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Quand le cerveau succombe aux addictions: Facteurs de vulnérabilité, implication des circuits cortico-striataux et développement d'une technique de neuromodulation pour favoriser le sevrage

Vincent van Waes

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Université de Franche-Comté
UFR Sciences et Techniques
Année 2016

Habilitation à diriger des recherches

Présentée et soutenue publiquement

Le 24 juin 2016 par

Vincent Van Waes

Maître de Conférences
en Neurosciences

Quand le cerveau succombe aux addictions:

Facteurs de vulnérabilité, implication des circuits cortico-striataux et développement d'une technique de neuromodulation pour favoriser le sevrage

Apports d'études précliniques

Composition du jury :

Dr. Christelle Baunez	Directeur de recherche CNRS, Marseille	(rapporteur)
Dr. Jean-Pol Tassin	Directeur de recherche INSERM, Paris	(rapporteur)
Dr. Emmanuel Valjent	Chargé de recherche INSERM, Montpellier	(rapporteur)
Pr. Muriel Darnaudéry	Professeur des Universités, Bordeaux	(examinateur)
Pr. Emmanuel Haffen	Professeur des Universités, Besançon	(examinateur)
Pr. Jean-Louis Millot	Professeur des Universités, Besançon	(Président)

Remerciements :

*Je tiens à remercier chaleureusement les Docteurs **Christelle Baunez**, **Jean-Pol Tassin** et **Emmanuel Valjent** (rapporteurs) ainsi que les Professeurs **Muriel Darnaudéry**, **Emmanuel Haffen et Jean-Louis Millot** (examinateurs) qui m'ont fait l'honneur de me consacrer de leur temps pour prendre connaissance et évaluer ce travail.*

Universités impliquées dans ce travail :

Université Lille1 Sciences et Technologies

Rosalind Franklin University of Medicine and Science

Université Lille 2 Droit et Santé

Sapienza Università di Roma

UFC Université de Franche-Comté

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Résumé

L'étude des phénomènes liés à l'addiction constitue le fil conducteur de ce travail. Pour ce faire, j'ai utilisé des modèles animaux (rats, souris) et mon approche était intégrative (du comportement à la biologie cellulaire et moléculaire) et, lorsque cela pouvait s'appliquer, translationnelle (études cliniques et précliniques menées en parallèle). Après une description de mes principales réalisations portant sur les deux versants de mon travail : enseignement et recherche, j'expose une rétrospective de mes recherches. Dans une troisième partie se trouve une sélection représentative de mes principales publications.

Mes premiers travaux portaient sur la différence de vulnérabilité aux drogues entre les individus et aux facteurs qui la contrôlent. Je me suis intéressé à un facteur environnemental, le stress, en particulier lorsqu'il est appliqué de manière précoce au cours du développement de l'individu. Ainsi, j'ai évalué les conséquences d'un stress *in utero* chez le rat sur la sensibilité aux effets de l'alcool et la propension à consommer à l'âge adulte. Ces études sont décrites dans le ***Chapitre 1 (thématique de la thèse, Université de Lille1/France, Université de Rome/Italie)***. Lors de mon post-doctorat, mon travail a porté sur l'implication des circuits cortico-striataux dans l'addiction. J'ai évalué chez le rat les conséquences de l'usage combiné de deux molécules : le méthylphénidate (Ritaline) - un psychostimulant utilisé pour traiter l'hyperactivité - et la fluoxétine (Prozac) - une molécule prescrite en première intention pour traiter la dépression -. Il ressort de cette étude que l'usage concomitant de ces deux traitements provoque des effets moléculaires et comportementaux comparables à ceux de la cocaïne. Mes données révèlent un potentiel effet addictogène de la combinaison de ces deux substances (pourtant largement co-prescrites), et soulèvent un éventuel problème de santé publique. Ces résultats sont exposés dans le ***Chapitre 2 (thématique principale du post-doctorat, Chicago Medical School/USA)***. J'ai par ailleurs lors de mon post-doctorat quantifié l'évolution en fonction de l'âge (pré-pubère, adolescent, adulte) de l'expression de récepteurs impliqués dans les processus addictifs (récepteur CB1) ou dans la modulation de la fonction dopaminergique (récepteur orphelin GPR88) ***(Chapitre 3, thématique secondaire du post-doctorat, Chicago Medical School/USA)***. Pour finir, je développe actuellement, dans le cadre d'études translationnelles, un modèle de stimulation transcrânienne par courant continu (tDCS) chez la souris. Cet outil clinique de neuromodulation innovant est à l'origine de résultats préliminaires enthousiasmants chez l'Homme pour le traitement de divers troubles psychiatriques (ex : dépression, troubles cognitifs, addiction). Cependant, ses mécanismes d'action restent peu connus, nécessitant la mise en place d'études comportementales et neurobiologiques chez l'animal. Ces travaux sont développés dans le ***Chapitre 4 (thématique actuelle, Maître de Conférences, Université de Franche Comté/France)***.

Mots clés:
Addiction, dépression, stress, neurostimulation non invasive, modèles animaux, comportement, expression génique.

PARTIE I : CURRICULUM VITAE

A) Présentation du candidat

Vincent Van Waes
Né le 21/07/1980 à Lille (59), France
1 enfant

Adresse :
Laboratoire de Neurosciences Intégratives et Cliniques EA481
Université de Franche-Comté - UFR Sciences et Techniques
2 Place Leclerc bureau 209 - 25030 Besançon Cedex - France
Tel: (+33)3.81.66.57.26
Fax: (+33)3.81.66.57.46

email :
vincent.van_waes@univ-fcomte.fr

Situation administrative :
Enseignant chercheur : Maître de Conférences en Neurosciences
Section 69 du Conseil National des Universités

Mots clés:
Addiction, dépression, stress, neurostimulation non invasive, modèles animaux, comportement, expression génique.

Formation universitaire

- **2008:** Doctorat en Neurosciences (Label Européen : Lille 1/Lille 2 et Universita di Roma “La Sapienza”, Rome)
- **2004:** DEA Biologie et Santé parcours Neurosciences (Lille 1/Lille 2)
- **2003 :** Master Sciences Cognitives (Lille 1/Lille 3)
- **2002:** Licence Sciences Cognitives (Lille 1/Lille 3)
- **2001:** DEUG Sciences de la Vie (Lille 1)

Expérience professionnelle

- **Depuis 2010:** **Maître de Conférences (69ème section)**
  Laboratoire de Neurosciences Intégratives et Cliniques EA481
  Besançon, France
  Thématique : Modélisation de la stimulation transcrânienne par courant continu chez la souris (études translationnelles)
  Référents : Pr Emmanuel Haffen, Pr Jean-Louis Millot

- **2011-2014** **Chercheur invité (Etés 2011-14; 6 mois au total)**
  Department of Cellular and Molecular Pharmacology
  The Chicago Medical School / RFUMS, North Chicago, IL, USA
  Thématique : Stimulation transcrâniennes par courant continu et addiction
  Référents : Pr Kuei Tseng, Pr Heinz Steiner

- **2008-2010:** **Chercheur Postdoctoral (USA)**
  Department of Cellular and Molecular Pharmacology
  The Chicago Medical School / RFUMS, North Chicago, IL, USA
  Thématique : Régulation des interactions cortico-striatales par les psychostimulants et les antidépresseurs.
  Référent : Pr Heinz Steiner

- **2005-2008:** **Doctorat en Neurosciences (Label Européen)**
  Laboratoire de Stress Périnatal, Université de Lille 1, France
  & Department of Human Physiology and Pharmacology, Universita di Roma "La Sapienza", Rome, Italie
  Vulnérabilité à l'alcool chez le rat adolescent et adulte : impact du stress prénatal.
  Référents : Pr Muriel Darnaudéry, Pr Stefania Maccari

Thèse soutenue le 04 février 2008 devant un jury composé de :

Pr. Stéfania Maccari	Université des Sciences et Technologies de Lille	Présidente
Pr. Philippe De Witte	Université Catholique de Louvain, Belgique	Rapporteur
Dr. Anna Moles	Istituto di Neuroscienze del CNR, Roma, Italie	Rapporteur
Dr. Gérard Barbanel	CNRS, Université Montpellier 2, France	Rapporteur
Dr. Muriel Darnaudéry	Université des Sciences et Technologies de Lille	Directeur
Pr. Michel Lhermitte	Université Droit et Santé de Lille	Directeur

Autres diplômes

- **2012:** **Initiation à la chirurgie expérimentale**
  VetAgro Sup, Lyon, Agrément n°R-69ENVL-CHIR-05

- **2007:** **Formation à l'expérimentation animale niveau 1**
  "De la modification génétique à l'exploration fonctionnelle et comportementale"
  Institut Pasteur, Lille, Agrément I-59LILLE-F1-04

Expertise technique

- **Pharmacologie:** Injections chez le rongeur (i.p., i.m., s.c., i.v.), microinjections de drogues dans le cerveau de rats libres de se mouvoir, prises de sang, perfusions intracardiaques, dissections d'organes, dissections du système nerveux central.

- **Endocrinologie:** Dosages RIA et ELISA.

- **Biologie moléculaire:** Hybridation *in situ* (avec isotopes ou fluorescence), Western blot, évaluation du stress oxydatif, marquage de la cytochrome oxydase, immunohistochimie.

- **Etudes comportementales chez le rongeur:** Protocole de stress prénatal, stimulation transcrânienne par courant continu chez la souris, détermination du cycle ovarien, évaluation de la consommation orale d'alcool, auto-administration de cocaïne, paradigme de préférence de place conditionnée, évaluation de l'activité locomotrice, des comportements associés à l'anxiété (labyrinthe en croix surélevé, champ ouvert, boîte noire et blanche), évaluation de la mémoire et de l'apprentissage (piscine de Morris, reconnaissance spatiale, labyrinthe en Y, reconnaissance d'objets), et des comportements associés à la dépression (test de la nage forcée, test de suspension par la queue).

Enseignements

- **Cours magistraux, travaux dirigés et travaux pratiques de neurosciences intégratives à l'Université de Franche Comté (~220 h/ans, depuis 2010):**
  - Licence Sciences de la Vie (1ère et 2ème année)
  - Licence de Psychologie (3ème année)
  - Médecine (3ème année)
  - Master 1 et Master 2 recherche « Physiologie, Neurosciences et Comportement »
  - Master BIOPS (Biologie et Produits de Santé)

- **Responsabilité d'unité :**

    - Neurobiologie et neurophysiologie cellulaire (60h)
      Master 1 Physiologie, Neurosciences et Comportement (Besançon)

- **Participation régulière à des jurys:**

  - 2010-2015 :
    - Jury Master 2 Recherche « Physiologie, Neurosciences et Comportement », Besançon (2 par an)
    - Jury Master 1 Biologie-Santé parcours « Physiologie, Neurosciences et Comportement », Besançon (1 par an)

  - 2015 :
    - Jury Master BIOPS (Biologie et Produits de Santé)

Encadrement : Master et Doctorat

- **Thèse de Neurosciences**

    - **Solène Pedron (2013-2015) 75%**

Titre :
« Utilisation de la stimulation transcrânienne par courant continu comme aide au sevrage (alcool, tabac, cocaïne): études comportementales et neurobiologiques chez la souris»

Situation actuelle de l'étudiante :
3ème année de thèse

Direction de thèse :
1) Pr Daniel Sechter
2) Pr Emmanuel Haffen
3) Dr Vincent Van Waes

Informations relatives à la thèse :

- Obtention d'un financement de 3 ans pour la thèse (Ville de Besançon, salaire Solène Pedron). Porteur du projet : Vincent Van Waes
- Solène Pedron a remporté le prix de thèse A'Doc en juin 2014 lui donnant l'opportunité de présenter ses résultats lors d'une conférence et de les publier sous forme d'article dans les Presses Universitaires de Franche-Comté.
- Obtention de financements pour que Solène Pedron effectue un stage à l'INSERM ERI 24 (Amiens, France) à la Chicago Medical School (Chicago, USA). Ces 2 stages d'une durée d'un mois chacun ont été effectués en 2014. Pour chaque stage, un article est en cours de rédaction (Amiens) ou soumis (Chicago Medical School, Addiction Biology).

- **M2 Recherche Physiologie, Neurosciences et Comportement, Besançon, stages d'un an (N=4)**

  - **Lilia Laribi (2016) 50% avec le Dr Yvan Peterschmitt**

Titre :
« Impact de la stimulation transcrânienne par courant continu (tDCS) sur la neurogenèse dans l'hippocampe chez la souris »

  - **Aurélie Salvadori (2015) 50% avec le Dr Yvan Peterschmitt**

Titre :
« Impact de la stimulation transcrânienne par courant continu sur la prolifération cellulaire dans l'hippocampe»
Situation actuelle de l'étudiant :
1ère année de thèse à Strasbourg

  - **Romain Monier (2014) 100%**

Titre :
« Impact de la stimulation transcrânienne par courant continu sur l'effet récompensant de la cocaïne chez la souris»
Situation actuelle de l'étudiant :
1ère année de thèse à Bordeaux

  - **Solène Pedron (2013) 100%**

Titre :
« Effets de la stimulation transcrânienne par courant continu chez la souris : Etudes comportementales »
Situation actuelle de l'étudiant :
3ème année de thèse à Besançon

- **M1 Biologie et Santé, Besançon, stage de 6 semaines (N=13)**

  - **Solène Pedron (2011) 100%**
  - **Mehdi Warid (2012) 100%**
  - **Lila Sid (2012) 100%**
  - **Collin Niarfeix (2013) 100%**
  - **Guillaume Bergot (2013) 100%**
  - **Quentin Chisin (2013) 100%**
  - **Alex Guerillot (2014) 100%**
  - **Amamata Ba (2014) 100%**
  - **Yassine Bougamale (2015) 100%**
  - **Viridiana Brenot (2015) 100%**
  - **Emel Laghouati (2016) 100%**
  - **Nagham Badreddine (2016) 100%**
  - **Maxime Aubry (2016) 100%**

- **M1 Sciences et Technologie, Mention Biologie Intégrative et Physiologie de l'Université Pierre et Marie Curie (Paris), stage de 6 mois (N=1)**

    - **Lilit Tonoyant (2014) 100%**

- **Etudiants dans d'autres cursus:**

  - 3ème année de pharmacie (Besançon)

    - **Mathilde Pitoy (2016), 6 semaines (100%)**

  - L3 Biologie parcours Biologie, Ecologie (Besançon)

    - **Virginie Guichon (2012), 3 semaines (100%)**

  - L2 Biologie parcours Biochimie, Biologie Cellulaire et Physiologie (Besançon)

    - **Marie-Claire Un (2012), 1 mois (50%)**
    - **Maeva Moyne (2012), 1 mois (50%)**

  - Ecole Polytechnique universitaire de Nice-Sophia

    - **Laibe Johanna (2013), 1 mois (50%)**

Collaborations

- **<u>Locale :</u>**

✓ **Dr Pierre-Yves Risold, CR INSERM**
EA3922 Laboratoire d'Histologie, Université de Franche Comté, Besançon.

Objet de la collaboration :
Noyaux para-sous thalamique et système de récompense : implication dans l'addiction ?

Publications communes: 1 + 1 en préparation

- **Nationale :**

✓ **Pr Mickael Naassila, Directeur de l'INSERM ERI 24**
Groupe de recherche sur l'alcool et les pharmacodépendances GRAP, Amiens, France

Objet de la collaboration :
Impact de la stimulation transcrânienne par courant continu sur la vulnérabilité à l'alcool ?

Projets communs financés par l'IREB en 2014 et 2015 (Institut de REcherche Scientifique sur les Boissons). Porteur de projet : Vincent Van Waes. http://www.ireb.fr/
Demande de financement Fondation pour la Recherche en Alcoologie 2016. Porteur de projet : Vincent Van Waes.

Publications communes: 1 en préparation

- **Internationales :**

✓ **Pr Heinz Steiner, Directeur du Département de Pharmacologie Cellulaire et Moléculaire, The Chicago Medical School, North Chicago, IL, USA**

Objet de la collaboration :
1) Interactions entre psychostimulants et antidépresseurs : potentiels effets addictogènes ?
2) Impact de la stimulation transcrânienne par courant continu sur la vulnérabilité à la cocaïne

Publications communes: 8 + 1 en révision + 1 chapitre de livre

✓ **Pr Kuei Tseng, Professeur au Département de Pharmacologie Cellulaire et Moléculaire, The Chicago Medical School, North Chicago, IL, USA**

Objet de la collaboration :
Application de la stimulation transcrânienne par courant continu chez l'adolescent : modification de la trajectoire développementale?

Publications communes: 2

✓ **Dr Dimitri De Bundel, Center for Neurosciences, Département de Chimie Pharmaceutique et de Toxicologie, Université de Bruxelles, Belgique**

Objet de la collaboration :
Stimulation transcrânienne par courant continu et extinction de la peur.

Demande commune pour obtenir une bourse doctorale FWO (2016), Porteur du projet : Dimitri De Bundel

- **Chercheur invité :**

Chicago Medical School (Pr Heinz Steiner)
6 mois au total depuis 2010 (juin à août 2011 et juillet-août 2012, 2013 et 2014)

Formation à de nouvelles techniques pour transfert au laboratoire de Besançon (biologie tissulaire et moléculaire; bases d'électrophysiologie), développement de collaborations pour la thèse de Solène Pedron, rédaction de publications.

- **Industrie : Dixi Medical**

Dixi Medical, Jean-Pierre Darnis, entreprise de biotechnologie, Besançon

Objet de la collaboration : Construction d'électrode pour la stimulation transcrânienne par courant continu chez la souris (NB : vente en 2015 de ces électrodes à plusieurs laboratoires américains). Construction d'un générateur pour les stimulations chez l'animal en collaboration avec notre ingénieur (Dr Patrice Andrieu, EA481).

Diffusion des résultats et vulgarisation

Depuis 2010, je participe régulièrement à la diffusion des résultats issus de mes recherches en donnant des conférences orales sur invitation et en participant régulièrement à des congrès nationaux et internationaux (voir liste des communications orales et écrites dans la section suivante). J'ai notamment eu l'opportunité d'organiser un symposium au IIIrd International Congress on Dual Disorders qui se tenait à Barcelone du 23 au 26 octobre 2013.

- **Organisation d'un Symposium au Congrès International Dual Disorders 2013 (Barcelona):**

- Adolescence and addictive disorders: insights from preclinical studies and animal models
  III International Congress on Dual Disorders
  October 23-26, 2013, Barcelona, Spain.

  Organisateur: Vincent Van Waes, EA481 Laboratory of Integrative and Clinical Neuroscience / SFR FED 4234 IBCT, Besançon, France
  Co-organisateur: Kuei Y Tseng, Chicago Medical School, North Chicago, IL USA

  Speakers:

  1. Kuei-Yuan Tseng, Chicago Medical School, North Chicago, USA

  Impact of periadolescent exposure to cocaine, MK-801, and the CB1 agonist WIN on prefrontal cortical maturation and function in adulthood.

2. Mickael Naassila, Inserm ERI-24 GRAP, Amiens, France

Alcohol intoxications during adolescence increase motivation for alcohol in adult rats: impact on gene expression in the nucleus accumbens and in a neurodevelopmental rat model of schizophrenia.

3. Vincent Van Waes, EA481 Laboratory of Integrative and Clinical Neuroscience, Besançon, France

SSRI antidepressants potentiate methylphenidate (Ritalin)-induced gene regulation in the adolescent striatum: concerns for addiction liability of methylphenidate?

4. Francesc Artigas, IIBB-CSIC, Barcelona, Spain

Disruption of prefrontal cortex function by psychotomimetic agents: Reversal by antipsychotic drugs

https://www.researchgate.net/profile/Vincent_Van_Waes/publication/257931151_ADOLESCENCE_AND_ADDICTIVE_DISORDERS_INSIGHTS_FROM_PRECLINICAL_STUDIES_AND_ANIMAL_MODELS/links/004635271475b7a678000000.pdf?origin=publication_detail

- **Vulgarisation scientifique**

- Participation à des émissions de radio :

  France Bleu, C'est bon à savoir, 21/09/2015 (tDCS)

- Actualité de l'Université de Franche-Comté (web):

  Toxicomanie : la stimulation transcrânienne favorise le sevrage des souris (03/09/2015)

- Participation à ***l'opération une classe/un chercheur*** :
  Présentation des thématiques de recherche du laboratoire à des lycéens et collégiens, encadrement des projets scientifiques élaborés par les élèves, visite du laboratoire.

  - 2016 : 3ème Collège Diderot à Besançon (Marion Griffoulière)
  - 2015 : 1ère S Lycée Paul Emile Victor à Champagnole (Catherine Bugada)
  - 2014 : 1ère S Lycée Gustave Courbet à Belfort (Catherine Do)
    1ère S Lycée Victor Hugo à Besançon (Magalie Quinanzoni)
  - 2013 : Ter S Lycée Victor Hugo à Besançon (Emmanuelle François)
  - 2012 : 1ère S Lycée Paul Emile Victor à Champagnole (Catherine Bugada)

- Participation à la ***semaine du cerveau* 2016** (15 mars) :
  Présentation orale à destination du grand public
  « Comment le cerveau succombe aux addictions ».

- **Sociétés savantes :**

- Membre de la “Society for Neuroscience” (depuis 2009).
- Membre de la “Société des Neurosciences Française” (depuis 2004).

- **Reviewer régulier pour :**

- Alcohol and Alcoholism
- Behavioural Brain Research
- Brain Stimulation
- European Neuropsychopharmacology
- European Journal of Neuroscience
- Neuropharmacology
- Neuroscience Letter
- Psychoneuroendocrinology
- Synapse
- The Journal of Child Psychology and Psychiatry

Responsabilités scientifiques

- **Co-responsable de l’axe de recherche «Récompense et Prise de Décision» à partir du 1er janvier 2017**

  Notre équipe EA481 comprend une trentaine de chercheurs titulaires et est divisée en 2 axes de recherches (axe 1 : Récompense et Prise de Décision; axe 2 : Perception et Hédonie). Je serai à partir du 1er janvier 2017 co-responsable, avec le Pr. Pierre Vandel, de l’axe 1.
  http://neurosciences.univ-fcomte.fr/pages/fr/menu4762/axe1-17456.html

- **Obtention de financements**

  Les expériences dont j’ai la responsabilité sont auto-financées. J’ai participé à diverses demandes de bourses (bourse de thèse ville de Besançon, ARN : Association pour la Recherche sur les Nicotianées, IREB : Institut de REcherche scientifique sur les Boissons, BQR université de Franche-Comté, BQR jeune chercheur, BQR PRES Bourgogne-Franche Comté, Fondation pour la Recherche en Alcoologie) en tant que porteur de projet ainsi qu’à une demande d’ANR SAMENTA en 2013 (en tant que task leader). J’ai obtenu un BQR de l’UFC en 2010 et un BQR jeune chercheur en 2012 ainsi que 3 financements consécutifs (2012, 2014 et 2015) de l’IREB. Par ailleurs, d’autres projets complémentaires (non détaillés dans le cadre de cette demande d’Habilitation à Diriger les Recherches) m’ont permis d’obtenir un financement BQR PRES en 2012 en collaboration avec le Professeur Naim Khan de l’université de Bourgogne («Impact de l’invalidation du gène d’ERK1 sur la signalisation cellulaire et sur l’activation de l’aire tegmentale ventrale (ATV) lors de la perception gustative lipidique chez la souris»).

Bourses depuis 2011 (porteur de projet):

- ✓ IREB (Institut de REcherche Scientifique sur les Boissons) n° 2015/29; **7k€** (2015)
- ✓ IREB n° 2014/20; **7.5k€** (2014)
- ✓ UFC international mobility, Solène Pedron **2k€** (2013)
- ✓ UFC BQR Jeune Chercheur; **4k€** (2012)
- ✓ BQR PRES Bourgogne/Franche Comté avec Naim Khan; **10k€** (2012)
- ✓ Bourse de thèse de 3 ans, Ville de Besançon; **86k€** (2012)
- ✓ IREB n° 2012/30; **7k€** (2012)

- **Conseils scientifiques :**

- Membre du **Conseil Scientifique de la SFR FED 4234**: Ingénierie et biologie cellulaire et tissulaire (depuis 2012)
- Membre du **Comité de bien être animal**, Animalerie Place Leclerc, Besançon (depuis 2013)

Distinctions

- **Prime d'Encadrement Doctoral et de Recherche** (2016)

- **Prix de thèse A'Doc** pour la thèse de Solène Pedron (2014)

  «Utilisation de la stimulation transcrânienne par courant continu comme aide au sevrage (alcool, tabac, cocaïne): études comportementales et neurobiologiques chez la souris»

- **Prix de thèse Gérard Vachonfrance** de l'Institut de Recherche Scientifique sur les Boissons (2008)

  «Vulnérabilité à l'alcool chez le rat adolescent et adulte : impact du stress prénatal»

B) Production scientifique

Publications internationales avec comité de lecture

1) **Van Waes V.**, Ehrlich S., Beverley J., and Steiner H.
Fluoxetine potentiation of methylphenidate-induced gene regulation in striatal output pathways: Potential role for 5HT1B receptor
Neuropharmacology, Vol. 89C, p77-86, 2015

2) Chometton S., Pedron S., Peterschmitt Y., **Van Waes V.**, Fellmann D. and Risold P.Y.
A preliminary lateral hypothalamic nuclear complex responds to hedonic but not aversive tastes in male rat
Brain Structure and Function, in press, 2015

3) Beverley J., Piekarski C., **Van Waes V.**, and Steiner H.
Potentiated gene regulation by methylphenidate plus fluoxetine treatment: Long-term gene blunting (Zif268, Homer1a) and behavioral correlates
Basal Ganglia, Vol. 4, p109-116, 2014

4) Bennabi D.*, Pedron S.*, Haffen E., Monnin J., Peterschmitt Y., and **Van Waes V.**
Transcranial direct current stimulation for cognitive enhancement: from clinical research to animal models
Frontiers in Systems Neuroscience, Vol. 8, Art. 159, 2014

5) Pedron S., Monnin J., Haffen E., Sechter D., and **Van Waes V.**
Repeated transcranial direct current stimulation prevents abnormal behaviors associated with abstinence from chronic nicotine consumption
Neuropsychopharmacology Vol. 39 (4), p981-988, 2014

6) Steiner H., Warren B., **Van Waes V.**, and Bolaños-Guzmán C.
Life-long consequences of juvenile exposure to psychotropic drugs on brain and behavior
Progress in Brain Research Vol. 21, p13-30, 2014

7) **Van Waes V.**, Vandrevala B., Beverley J., and Steiner H.
SSRIs potentiate gene blunting induced by repeated methylphenidate treatment: Zif268 vs. Homer1a
Addiction Biology Vol. 19(6), p986-995, 2014

8) Steiner H. and **Van Waes V.**
Addiction-Related Gene Regulation: Risks of exposure to cognitive enhancers vs. other psychostimulants
Progress in Neurobiology Vol. 100, p60-80, 2013

9) **Van Waes V.**, Carr B., Beverley J., and Steiner H.
Fluoxetine potentiation of methylphenidate-induced neuropeptide expression in the striatum occurs selectively in the direct (striatonigral) neurons
Journal of Neurochemistry Vol. 122(5), p1054-64, 2012

10) **Van Waes V.**, Beverley J., Siman H., Tseng K.Y, and Steiner H.
CB1 cannabinoid receptor expression in the striatum: association with corticostriatal circuits and developmental regulation
Frontiers in Neuropharmacology Vol. 3, Art. 21, 2012

11) **Van Waes V.**, Tseng K.Y, and Steiner H.
GPR88: a putative signaling molecule predominantly expressed in the striatum; Cellular localization and developmental regulation
Basal Ganglia Vol. 1(2), p83-89, 2011

12) **Van Waes V.**, Darnaudéry M., Marrocco J., Gruber S., Talavera E., Mairesse J., Van Camp G., Casolla B., Nicoletti F., Mathe A., Maccari S., and Morley-Fletcher S.
Impact of early life stress on alcohol consumption and on the short- and long-term responses to alcohol in adolescent female rats
Behavioural Brain Research Vol. 221(1), p43-49, 2011

13) **Van Waes V.**, Enache M., Berton O., Vinner E., Lhermitte M., Maccari S., and Darnaudéry M.
Effect of prenatal stress on alcohol preference and sensitivity to chronic alcohol exposure in male rats
Psychopharmacology Vol. 214(1), p197-208, 2011.

14) **Van Waes V.**, Beverley J., Marinelli M., and Steiner H.
SSRI antidepressants potentiate methylphenidate (Ritalin)-induced gene regulation in the adolescent striatum
European Journal of Neuroscience Vol. 32, p435-447, 2010.

15) Steiner H., **Van Waes V.,** and Marinelli M.
Fluoxetine potentiates methylphenidate-induced gene regulation in addiction related brain regions: concerns for use of cognitive enhancers?
Biological Psychiatry Vol. 67(6), p592-594, 2010.

16) **Van Waes V.**, Enache M., Zuenna A.R., Mairesse J., Nicoletti F., Vinner E., Lhermitte M., Maccari S., and Darnaudéry M.
Ethanol attenuates spatial memory deficits and increases mGlu1a receptor expression in the hippocampus of rats exposed to prenatal stress
Alcoholism: Clinical and experimental research Vol. 33(8), p1346-1354, 2009.

17) **Van Waes V.***, Enache M.*, Vinner E., Lhermitte M., Maccari S., and Darnaudéry M.
Impact of an acute exposure to ethanol on the oxidative stress status in the hippocampus of prenatally stressed adolescent male rats
Brain Research Vol. 1191C, p55-62, 2007. * co-first authorship.

18) **Van Waes V.**, Enache M., Dutriez I., Lesage J., Morley-Fletcher S., Vinner E., Lhermitte M., Vieau D., Maccari S., and Darnaudéry M.
Hyporesponse of the hypothalamic-pituitary-adrenocortical axis after an ethanol challenge in prenatally stressed adolescent male rats
European Journal of Neuroscience Vol. 24(4), p1193-1200, 2006.

En révision:

1) Pedron S., Beverley J., Haffen E., Andrieu P., Steiner H, and **Van Waes V.**
Transcranial direct current stimulation in mice produces long lasting attenuation of cocaine-induced gene regulation in striatum and cortex and reduces behavioral responses to cocaine
Addiction Biology, 2016, Révision

En préparation:

1) Coune F.*, Pedron S.*, Haffen E., Andrieu P., Sechter D., Naassila M., **Van Waes V.**[&,°]**,** and Gonzalez-Marin M.C[&].
Repeated anodal transcranial direct current stimulation decreases alcohol self-administration but not alcohol sensitization in mice
* co-first authorship, [&] co-last authorship, ° corresponding author

Publications nationales avec comité de lecture

1) Pedron S., Coune F., Haffen E., Andrieu P., Sechter D., Naassila M., Gonzalez-Marin M.C., and **Van Waes V.**
Effets de la stimulation transcrânienne par courant continu sur l'auto-administration d'alcool chez la souris.
Cahier de l'IREB n°22, in press, 2015.

2) Pedron S. and **Van Waes V.**
Effets de la stimulation transcrânienne par courant continu chez la souris: études comportementales
PUFC Presses Universitaires de Franche-Comté, 2014

3) Pedron S., Monnin J., Andrieu P., Nicolier M., Millot J., Sechter D., Haffen E., and **Van Waes V.** Effets de la stimulation transcrânienne par courant continu chez la souris: études comportementales.
Cahier de l'IREB n°21, p69-74, 2013.

4) Darnaudéry M., **Van Waes V.**, Enache M., Zuena A.R., Mairesse J., Nicoletti F., Vinner E., Lhermitte M., and Maccari S.
Stress prénatal et mémoire: effets paradoxaux de la consommation chronique d'alcool chez le rat
Cahier de l'IREB n°19, p63-69, 2009.

5) **Van Waes V.**, Enache M., Vinner E., Lhermitte M., Maccari S., and Darnaudéry M.
Impact du stress prénatal sur la vulnérabilité à l'éthanol chez le rat
Cahier de l'IREB n°18, p15-21, 2007.

6) Darnaudéry M., **Van Waes V.**, Enache M., Morley Fletcher S., Dutriez-Casteloot I., Lesage J., Vinner E., Lhermitte M., and Maccari S.
Conséquences d'un stress prénatal sur l'anxiété et l'activité de l'axe corticotrope après administration aiguë d'alcool chez le rat adolescent
Cahier de l'IREB n°17, p35-41, 2005.

Ouvrages et chapitres de livres

1) Etievant A., Monin J., and **Van Waes V.**
Bases neurobiologiques de la tDCS : apports des modèles animaux.
Editions Solal, Book Chapter, In Press, 2015

2) **Van Waes V.** and Steiner H.
SSRI antidepressants potentiate addiction-related gene regulation by psychostimulant medications (Book Chapter) in Fluoxetine: Pharmacology, Mechanisms of Action and Potential Side Effects.
Nova Biomedical, Book Chapter, p207-226, 2015

3) **Van Waes V.**
Vulnérabilité à l'alcool chez le rat: Impact du stress prénatal. Comment un stress précoce peut avoir des répercussions tout au long de la vie.
Éditions Universitaires Européennes, 2011

C) Participation à des congrès

Communications orales (invitations)

1) Mechanisms underlying individual variations in nicotine seeking: implications for addiction (Symposium).
P. Faure (Paris, France); P. Robledo (Barcelona, Spain); V. Van Waes (Besançon, France); V. Deroche-Gamonet (Bordeaux, France)
NeuroFrance 2017
May 17-19, 2017, Bordeaux, France.

2) Comment le cerveau succombe-t-il à l'addictions?
La semaine du cerveau
March 15, 2016, Besançon, France.

3) Modélisation de la tDCS chez la souris: effets comportementaux et neurobiologiques.
Formation Stimulation Transcrânienne En Psychiatrie
AFPBN, Association Française de la Psychiatrie Biologique et Neuropharmacologie
September 29-October 2, 2015, Bron, France.

4) La stimulation transcrânienne en courant continu comme aide au sevrage à la cocaine.
Solène Pedron. *Best Oral Communication Award.*
20ème Forum des Jeunes Chercheurs
June 23-24, 2014, Besançon, France.

5) Adolescence and addictive disorders: insights from preclinical studies and animal models.
Symposium chairman; invited: K. Tseng (Chicago, USA), M. Naassila (Amiens France), F Artigas (Barcelona, Spain)
III International Congress on Dual Disorders
October 23-26, 2013, Barcelona, Spain.

6) Effects of repeated transcranial direct current stimulation (tDCS) on depression and addiction-related behaviors in mice.
Solène Pedron/Vincent Van Waes
Cambridge & Luton International Conference on Mental Health 2013
September 5, 2013, Cambridge, UK.

7) SSRIs potentiate methylphenidate (Ritaline)-induced gene regulation in addiction related brain regions: risk for enhanced addiction liability?
Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC)
November 20, 2012, Strasbourg, France.

8) Effects of repeated transcranial direct current stimulation (tDCS) on addiction-related behaviors in mice: a preliminary study.
Chicago Medical School, July 24, 2012, North Chicago, Illinois, USA.

9) SSRI antidepressants potentiate psychostimulant (Ritalin)-induced gene regulation: risk for enhanced addiction liability?
American Society for Pharmacology and Experimental Therapeutics
Great Lake Chapter Annual Meeting, June 10, 2011, Chicago, Illinois, USA.

10) Ethanol vulnerability in adolescent and adult rats: Impact of prenatal stress.
Chicago Medical School, April 17, 2009, Zion, Illinois, USA.

11) Impact du stress prénatal sur la vulnérabilité à l'éthanol chez le rat.
18ème colloque de l'Institut de Recherche Scientifique sur les Boissons (IREB)
December 6-7, 2006, Fécamp, France.

Communications affichées

2015

1) Pedron S., Coune F., Andrieu P., Haffen E., Naassila M., Gonzalez-Marin M.C. and **Van Waes V.** Impact of transcranial direct current stimulation on alcohol self-administration in mice. *22ème Colloque scientifique de l'IREB, March 17-18 2015, Paris, France.*

2014

2) **Van Waes V.**, Ehrlich S., Beverley J., and Steiner H. Fluoxetine potentiates methylphenidate-induced gene regulation in the striatum: Role of 5-HT1B serotonin receptor. *SFN 2014, November 15-19 2014, Washington, DC, USA.*

3) Pedron S., Beverley J., Haffen E., Andrieu P., Monnin J., Monnier R., Sechter D., Steiner H. and **Van Waes V.** Repeated transcranial direct current stimulation in mice reduces the rewarding effect of cocaine and blunts cocaine-induced zif268 in the striatum. *9th FENS Forum of Neuroscience, July 5-9 2014, Milan, Italy.*

2013

4) Pedron S., Monnin J., Andrieu P., Nicolier M., Millot J., Sechter D., Haffen E. and **Van Waes V.** Utilisation de la stimulation transcrânienne en courant continu (tDCS) comme aide au sevrage (alcool, nicotine): Etude comportementale chez la souris. Forum des Jeunes Chercheurs 2013, *June 13-14 2013, Dijon, France.*

5) Pedron S., Monnin J., Andrieu P., Nicolier M., Millot J., Sechter D., Haffen E. and **Van Waes V.** Transcranial direct current stimulation (tDCS) and addiction-related behaviors in mice: a preliminary study. *21st European Congress of Psychiatry, April 6-9 2013, Nice, France. Selected for oral presentation (e-poster).*

6) Pedron S., Monnin J., Andrieu P., Nicolier M., Millot J., Sechter D., Haffen E. and **Van Waes V.** Effects of repeated transcranial direct current stimulation (tDCS) on addiction-related behaviors in mice: a behavioral study. *21ème Colloque scientifique de l'IREB, March 13-14 2013, Paris, France.*

2012

7) Pedron S., Monnin J., Andrieu P., Nicolier M., Jacquot L., Millot J., Sechter D., Risold P., Haffen E. and **Van Waes V.** Effects of repeated transcranial direct current stimulation (tDCS) on addiction-related behaviors in mice: a preliminary study. *SFN 2012, October 13-17, New Orleans, LA, USA.*

8) Jacquot L., **Van Waes V.**, Millot JL. and Paillard A. Olfactory perception and motion sickness: a psychophysical and psychophysiological approach. *SFN 2012, October 13-17, New Orleans, LA, USA.*

9) **Van Waes V.**, Vandrevala M., Beverley J. and Steiner H. SSRIs potentiate methylphenidate-induced blunting expression in the adolescent striatum. *SFN 2012, October 13-17, New Orleans, LA, USA.*

2011

10) **Van Waes V.**, Carr B., Beverley J. and Steiner H. SSRIs potentiate methylphenidate-induced gene regulation in the striatum: transcription factors (zif 268, c-fos) vs. neuropeptides (substance P, dynorphin). *SFN 2011, November 12-16, Washington, DC, USA.*

11) **Van Waes V.**, Carr B., Beverley J. and Steiner H. SSRIs potentiate methylphenidate-induced gene regulation in the striatum: transcription factors vs. neuropeptides. *ASPET Great Lake Chapter Annual Meeting, 2011, June 10, Chicago, Illinois, USA.*

12) Maccari S., **Van Waes V.**, Darnaudéry M., Marrocco, J., Gruber S.H., Talavera E., Mairesse, J., Van Camp G., Casolla B., Nicoletti F., Mathé, A.A. and Morley-Fletcher, S. Impact of early life stress on alcohol consumption and on the short- and long-term responses to alcohol in adolescent female rats. 10*ème* *Colloque de la Société des Neurosciences, May 24-27 2011, Marseille, France.*

2010

13) **Van Waes V.**, Tseng K.Y. and Steiner H. GPR88 - a putative G protein-coupled receptor highly expressed in the striatum: Cellular localization and developmental regulation. *SFN 2010, November 13-17, San Diego, California, USA.*

14) **Van Waes V.**, Tseng K.Y. and Steiner H. GPR88 - a putative G protein-coupled receptor selectively expressed in the striatum: Cellular localization and developmental regulation. *IBAGS Tenth Triennial Meeting, 2010, June 20-24, Long Branch, New Jersey, USA.*

15) **Van Waes V.**, Beverley J., Marinelli M. and Steiner H. SSRI antidepressants potentiate Ritalin-induced gene regulation in the striatum: Consequences for addiction liability? *ASPET Great Lake Chapter Annual Meeting, 2010, June 18, Chicago, Illinois, USA.*

16) **Van Waes V.**, Tseng K.Y. and Steiner H. Differential developmental trajectories for GPR88 expression in the caudate-putamen and nucleus accumbens. *SFN Chicago Chapter 2010, March 25th, Chicago, Illinois, USA.*

17) **Van Waes V.**, Beverley J., Marinelli M. and Steiner H. Fluoxetine (Prozac) potentiates methylphenidate (Ritalin)-induced gene regulation in addicted-related brain regions. *43rd winter Conference on Brain Research, 2010, January 23-29, Breckenridge, Colorado, USA.*

2009

18) **Van Waes V.**, Beverley J., Marinelli M. and Steiner H. Fluoxetine (Prozac) potentiates methylphenidate (Ritalin)-induced gene regulation in the striatum. *SFN Neuroscience 2009, October 17-21, Chicago, Illinois, USA.*

19) Darnaudery M., **Van Waes V.**, Enache M., Berton O., Vinner E., Lhermitte M. and Maccari S. Prenatal stress does not affect spontaneous ethanol preference but increases DeltaFosB levels in the nucleus accumbens after chronic ethanol consumption. *SFN 2009, October 17-21, Chicago, Illinois, USA.*

20) Van Camp G., Mairesse J., Giovine A., Siletti V., **Van Waes V.**, Navara P., Van Reeth O. and Maccari M. Prenatal stress alters sleep structure and circadian rhythms of locomotor activity: Relation with hypothalamic CRH levels. *36eme Colloque de la Société de Neuroendocrinologie, 2009, September 15-18, Nice, France*.

21) **Van Waes V.**, Marinelli M., Steiner H. Fluoxetine potentiates methylphenidate-induced gene regulation in the striatum: consequences for addiction liability? *SFN Chicago Chapter 2009, March 26th, Chicago, Illinois, USA.*

2008

22) **Van Waes V.**, Talavera E., Maccari S. and Darnaudery M. Influence of social housing condition on the ethanol preference and on emotional reactivity in prenatally stressed female rats. *6th Forum of European Neuroscience, July 12-16 2008, Geneva, Switzerland.*

23) Giovine A., Mairesse J., Zuena A., **Van Waes V.,** Giuli C., Cinque C., Catalani A., Mennuni G. , Van Reeth O. , Garcia C., Bergonzelli G. and Maccari S. **Postnatal** developmental profile of hippocampal metabotropic receptors expression of perinatal restraint stress rats. *6th Forum of European Neuroscience, July 12-16 2008, Geneva, Switzerland.*

2007

24) Tallavera E., **Van Waes V.,** Maccari S. and Darnaudéry M. Influence of social housing on the ethanol preference and on locomotor activity in female rats exposed to prenatal stress. Programme European Neuroscience Schools (PENS), Advanced Courses in Neuroplasticity*, September 5-11 2007, Roma, Italy.*

25) **Van Waes V.**, Enache M., Maccari S. and Darnaudéry M. Ethanol preference and induction of deltaFosB in the nucleus accumbens after a chronic ethanol treatment in prenatally stressed male rats. Programme European Neuroscience Schools (PENS), Advanced Courses in Neuroplasticity*, September 5-11 2007, Roma, Italy.*

26) **Van Waes V.**, Enache M., Maccari S. and Darnaudéry M. Ethanol preference and induction of deltaFosB in the nucleus accumbens after a chronic ethanol treatment in prenatally stressed male rats. *World Conference of Stress, August 23-26 2007, Budapest, Hungary.*

27) **Van Waes V.**, Enache M., Maccari S. and Darnaudéry M. Ethanol preference and induction of deltaFosB in the nucleus accumbens after a chronic ethanol treatment in prenatally stressed male rats. *8ème Colloque de la Société des Neurosciences, May 12-25 2007, Montpellier, France*. (Bourse de Congrès IREB)

28) Darnaudéry M., **Van Waes V.**, Enache M., Zuena A.R. and Maccari S. Impact of chronic ethanol consumption on spatial memory and hippocampal metabotropic glutamate receptors in prenatally-stressed rats. *8ème Colloque de la Société des Neurosciences, May 12-25 2007, Montpellier, France*.

2006

29) **Van Waes V.**, Enache M., Zuena A.R., Maccari S. and Darnaudéry M. Un traitement chronique à l'éthanol atténue le déficit mnésique observé chez le rat stressé prénatalement: rôle possible des récepteurs métabotropiques au glutamate du type I. *10éme Journée Scientifique du réseau LARC-Neurosciences, November 10th 2006, Lille, France.*

30) **Van Waes V.**, Enache M., Dutriez I., Lesage J., Vinner E., Morley-Fletcher S., Lhermitte M., Vieau D., Maccari S. and Darnaudéry M. Impact d'un stress prénatal sur l'activation de l'axe corticotrope en réponse à un traitement à l'éthanol et sur la consommation spontanée d'éthanol chez le rat adolescent. 6ème *Journée André Verbert, September 27th 2006, Villeneuve d'Ascq, France.*

31) **Van Waes V.**, Enache M., Dutriez I., Lesage J., Vinner E., Morley-Fletcher S., Lhermitte M., Vieau D., Maccari S. and Darnaudéry M. Impact of a prenatal stress on the HPA axis responsiveness to an ethanol challenge and on the ethanol preference in adolescent male rats. *Lille Summerschool in Neurosciences : Brain Plasticity in Life Span., September 2-7 2006 , Villeneuve d'Ascq, France.*

32) Enache M., **Van Waes V.**, Vinner E., Lhermitte M., Dutriez-Casteloot I., Vieau D., Morley Fletcher S.**,** Maccari S. and Darnaudéry M. Brain oxidative stress status in prenatally stressed adolescent rats after an acute alcohol administration. *Lille Summerschool in Neurosciences: Brain Plasticity in Life Span., September 2-7 2006 , Villeneuve d'Ascq, France.*

33) Enache M., **Van Waes V.**, Vinner E., Lhermitte M., Dutriez-Casteloot I., Vieau D., Maccari S. et Darnaudéry M. Impact of prenatal stress in brain damage and behavioural response to drugs in rats. *9éme Journée Scientifique du réseau LARC-Neurosciences, October 14th 2005, Amiens, France.*

2005

34) **Van Waes V.**, Enache M., Dutriez-Casteloot I., Maccari S. and Darnaudéry M. Prenatal stress and alcohol preference in male rats. *7ème Colloque de la Société des Neurosciences, May 17-20 2005, Lille, France*.

35) Enache M., **Van Waes V.**, Morley-Fletcher S., Vinner E., Lhermitte M., Dutriez-Casteloot I., Lesage J., Vieau D., Maccari S. and Darnaudéry M. Effects of an acute alcohol administration on HPA axis and brain oxidative stress status in prenatally stressed adolescent rats. *7ème Colloque de la Société des Neurosciences, May 17-20 2005, Lille, France*.

2004

36) **Van Waes V.**, Enache M., Morley-Fletcher S., Dutriez-Casteloot I., Lesage J., Vinner E., Lhermitte M., Vieau D.,Maccari S. et Darnaudéry M. Conséquences d'un stress prénatal sur l'anxiété et l'activité de l'axe corticotrope en réponse à une administration aiguë d'alcool chez le rat adolescent. *8éme Journée Scientifique du réseau LARC-Neurosciences, October 15th 2004, Paris, France.*

37) Enache M., **Van Waes V**., Morley-Fletcher S., Magni P., Lhermitte M,. Vinner E., Humbert L., Dutriez-Casteloot I., Vieau D., Maccari S. and Darnaudéry M. Effect of prenatal stress on endocrine and behavioural response to alcohol in adolescent male rats. *4th FENS Forum of Neuroscience, July 10-14 2004, Lisbon, Portugal.*

PARTIE II : ACTIVITE SCIENTIFIQUE

A) PREAMBULE

L'étude des phénomènes liés à l'addiction constitue le fil conducteur de ce travail. Pour ce faire, j'ai utilisé des modèles animaux (rats, souris) et mon approche était intégrative (du comportement à la biologie cellulaire et moléculaire) et, lorsque cela était possible, translationnelle (études cliniques et précliniques menées en parallèle). Les modèles animaux constituent un outil complémentaire et indispensable permettant des investigations qu'il est impossible d'envisager chez l'Homme pour des raisons d'ordres pratique et/ou éthique. L'ensemble des travaux présentés dans ce document a été accompli dans un cadre éthique strict sous le contrôle des instances *ad hoc* (CEBEA : Comité d'Ethique Bisontin pour l'Expérimentation Animale, n° d'agrément C-25-056-2 ; Cellule de bien être animal de l'EA481 ; Ministère de l'Agriculture). Ils suivent la réglementation en vigueur en France (articles R214-87 à R214-137 du code rural) mise à jour par le décret 2013-118 relatif à la protection des animaux utilisés à des fins scientifiques et cinq arrêtés interministériels publiés le 7 février 2013, en application de la directive 2010/63/UE.

Après une brève introduction générale sur l'addiction qui rappellera de manière non exhaustive les notions théoriques utiles pour aborder mes travaux de recherche, je décrirai mes principales activités scientifiques de manière chronologique selon un découpage en quatre chapitres.

Mes premières travaux portaient sur la différence de vulnérabilité aux drogues entre individus, phénomène largement décrit dans la littérature (Swendsen *et al*, 2011), et aux facteurs qui la contrôlent. La question centrale de cette thématique est de déterminer la raison pour laquelle, à exposition égale, un individu deviendra dépendant alors qu'un autre sujet en restera à un stade de consommations récréatives. De façon frappante, les différences interindividuelles de vulnérabilité aux drogues sont retrouvées chez l'animal de laboratoire (Deroche-Gamonet *et al*, 2004), validant l'utilité des modèles animaux pour l'étude des phénomènes liés à l'addiction. Dans ce cadre, je me suis intéressé à un facteur environnemental, le stress, en particulier lorsqu'il est appliqué lors de périodes précoces du développement. J'ai ainsi évalué les conséquences d'un stress *in utero* sur la sensibilité aux effets de

l'alcool et la propension à consommer des rats devenus adultes. Ces travaux sont décrits dans le ***Chapitre 1 (thématique de la thèse)***. Je me suis ensuite, lors de mon post-doctorat, focalisé sur le rôle des circuits cortico-striataux dans l'addiction. Mes recherches portaient sur les conséquences moléculaires et comportementales de l'usage combiné de deux molécules : le méthylphénidate - qui est un psychostimulant soulageant les troubles de l'attention associés à hyperactivité (Ritaline®) - et la fluoxétine - qui est une molécule prescrite en première intention pour traiter la dépression (Prozac®) -. L'interaction entre ces médicaments n'avait jamais été étudiée bien que ces produits soient souvent utilisés conjointement, pour diverses raisons qui seront détaillées dans le chapitre correspondant. Il ressort de ces études que la combinaison de ces deux traitements provoque des effets neurobiologiques et comportementaux comparables à ceux de la cocaïne, mettant perspective un potentiel effet addictogène de ces médicaments lorsqu'ils sont utilisés conjointement. Ces résultats sont décrits dans le ***Chapitre 2 (thématique principale du post-doctorat)***. J'ai par ailleurs lors de mon post-doctorat quantifié l'évolution à différents âges (pré-pubère, adolescent, adulte) de l'expression de récepteurs impliqués dans l'addiction ou la modulation de la fonction dopaminergique (*e.g. :* le récepteur CB1, le récepteur orphelin GPR88 ; ***Chapitre 3, thématique secondaire du post-doctorat)***. Je développe actuellement, dans le cadre d'études translationnelles, un modèle de stimulation transcrânienne par courant continu (tDCS) chez la souris. Cet outil clinique innovant est à l'origine de résultats préliminaires enthousiasmants chez l'Homme pour le traitement de divers troubles psychiatriques (*e.g. :* dépression, troubles cognitifs, addiction). Cependant, ses mécanismes d'action restent peu connus, nécessitant la mise en place d'études comportementales et neurobiologiques chez l'animal ***(Chapitre 4, thématique actuelle)***.

Si ce préambule n'est pas consacré aux remerciements - que j'exprimerai directement aux personnes concernées (Collègues, Amis, Famille) -, il me parait néanmoins indispensable de rendre hommage aux personnes qui ont permis/construit ce travail avec moi. Les résultats que je vais présenter sont issus de travaux façonnés par une succession de belles rencontres. Mon intérêt pour la thématique de l'addiction (ainsi que les troubles de l'humeur qui lui sont associés)

s'est révélé de lui même au début de ma carrière, mais les « frictions » avec mes Mentors m'ont permis de sélectionner les angles spécifiques avec lesquels je pouvais l'aborder. En premier lieu, j'ai rencontré le Professeur Muriel Darnaudéry qui m'a accepté en DEA puis en thèse à l'Université de Lille 1 sur la thématique du stress prénatal. Maintenant que je suis passé de l'autre côté de la frontière doctorant/encadrant, j'apprécie d'autant plus sa patience et j'utilise aujourd'hui encore, chaque jour, les méthodes et conseils qu'elle m'a prodigués. Le Professeur Stéfania Maccari qui dirigeait l'équipe lilloise m'a accueilli a bras ouvert, à Lille comme à Rome, et a toujours veillé à l'épanouissement professionnel et personnel des membres de son équipe. Je lui en suis reconnaissant. Muriel et Stéfania ont dirigé les recherches que je présenterai portant sur l'impact du stress prénatal sur la vulnérabilité à l'alcool. Je souhaiterais exprimer toute ma gratitude au Professeur Heinz Steiner qui m'a initié aux circuits cortico-striataux à la Chicago Medical School entre 2008 et 2010. Ce fut un stage postdoctoral sans concession et extrêmement enrichissant. J'ai beaucoup d'admiration pour sa rigueur et son honnêteté. C'est aussi grâce à son efficacité que j'ai eu l'opportunité d'obtenir par la suite le poste de Maître de Conférences que j'occupe aujourd'hui. Que dire du Professeur Kuei Tseng, qui illustre un fait contre-intuitif : le talent scientifique d'une personne peut être corrélé positivement à son accessibilité ! J'ai rarement rencontré une personne aussi brillante et passionnée qui, dans le même temps, peut être si proche de son équipe et des jeunes scientifiques en devenir. Dès mon premier jour à Chicago, Kuei m'a emmené dans son univers scientifique flamboyant (et boire un whisky) et m'a donné des conseils d'une valeur inestimable. Un merci chaleureux est également destiné à sa compagne, le Docteur Adriana Caballero qui m'a beaucoup aidé, en particulier pour la relecture critique de mes articles (forme et fond). Je salue également le Professeur Michaela Marinelli, avec qui je n'ai collaboré qu'indirectement mais qui m'a marqué par sa personnalité colorée. Ces personnes, en priorité le Professeur Heinz Steiner, ont porté les études sur les interactions entre le méthylphénidate et les antidépresseurs. Enfin, je suis redevable envers le Professeur Jean-Louis Millot qui m'a accueilli avec bienveillance au sein de son laboratoire de recherche à l'Université de Franche Comté en 2010. Ses encouragements quotidiens m'ont permis de trouver ma place facilement au sein de son équipe. Merci également au

Professeur Emmanuel Haffen, avec qui je collabore sur les projets portant sur les techniques de neuromodulation, pour sa confiance et son dynamisme, et de permettre à la recherche translationnelle de continuer à se développer au sein de notre laboratoire. Enfin, je félicite tout particulièrement Solène Pedron, ma première doctorante, qui a fourni un travail remarquable au cours de sa thèse et salue chaleureusement mes collègues du laboratoire bisontin directement impliqués dans ce projet (les Docteurs Patrice Andrieu, Adeline Etiévant, Julie Monnin, Yvan Peterschmitt et Pierre-Yves Risold et le Professeur Daniel Sechter), ou qui ne le sont pas mais avec qui j'interagis avec plaisir chaque jour au laboratoire. Ces rencontres me font penser que même si l'adversité est parfois présente… ce métier est formidable !

B) INTRODUCTION

B-1) La voie dopaminergique mésocorticolimbique

a) Découverte du système de récompense : Au commencement furent Old et Milner (1954)

Certains comportements sont bénéfiques pour la survie de l'espèce d'un point de vue évolutif : se nourrir, se reproduire, le comportement maternel, le comportement social. Il existe des régions cérébrales dont le rôle est de « récompenser » l'exécution de ces fonctions par une sensation agréable. Ces régions ont été découvertes par James Olds et Peter Milner en 1954 dans une désormais célèbre expérience d'auto-stimulation cérébrale (Olds and Milner, 1954). Milner étais un chercheur réputé pour ses études d'exploration des fonctions cérébrales. Sa principale méthode consistait à implanter des électrodes dans le cerveau de rats, d'y envoyer des courants de diverses intensités et d'en étudier les effets. La recherche de James Olds, son étudiant en thèse, portait plus précisément sur la stimulation d'un centre supposé de la vigilance, situé caudalement dans l'hypothalamus, afin de vérifier si l'on pouvait amener les rats à éviter certaines parties de leur cage en activant ces zones (sensations désagréables). L'expérience se déroulait à merveille : l'ensemble des rats stimulés avaient tendance à éviter les endroits «trop stimulants». Tous, sauf un. Contrairement aux autres, un rat «réfractaire» revenait systématiquement vers les endroits où les chocs électriques étaient administrés (préférence de place conditionnée induite par le courant électrique). Plus étonnant : plus l'intensité des chocs électriques était intense, plus le rat se dirigeait vers les zones où ils étaient administrés (effet dose-réponse sur l'induction de la préférence de place). Face à ce comportement singulier, Olds entreprit de disséquer l'animal. Il découvrit que suite à une erreur, l'électrode n'avait pas été implantée dans l'hypothalamus, mais dans une zone très proche, le septum. A partir de cette expérience princeps, bel exemple de sérendipité, une cartographie des zones induisant un comportement d'«auto-administration cérébrale» fut réalisée

par diverses équipes (Rolls, 1975). Les différentes régions associées à l'auto-stimulation se situent essentiellement autour du faisceau médian du télencéphale: l'aire septale, l'hypothalamus latéral, l'aire tegmentale ventrale et la région dorsale du pont. Ce protocole fut reproduit avec les mêmes effets chez la plupart des vertébrés (poissons, oiseaux, mammifères). Chez l'Homme, ce type de stimulation effectuée à des fins thérapeutiques suscite des impressions d'aise ou de joie qui poussent le sujet à demander sa répétition (Heath, 1972). Plus tard, des données pharmacologiques montreront le rapport entre l'auto-stimulation cérébrale et un neurotransmetteur: la dopamine. En effet, l'administration chez le rat d'un antagoniste des récepteurs de la dopamine - par exemple l'halopéridol, antagoniste des récepteurs D1 et D2 - réduit les comportements d'auto-stimulation suggérant ainsi l'implication de la dopamine dans ce phénomène (Wise, 1978, 2004).

b) Le système dopaminergique : la voie mésocorticolimbique et le plaisir

Le système dopaminergique est composé de trois voies principales : la voie nigrostriée, la voie mésocorticolimbique et la voie tubero-infendibulaire. De façon extrêmement simplifiée, la voie nigrostriée débute au niveau de la substance noire *pars compacta,* projette vers le striatum dorsal et est principalement impliquée dans l'initiation des mouvements volontaires. Cette voie est affectée, notamment, chez les patients Parkinsoniens chez qui 80% environ de la substance noire *pars compacta* est détruite, entrainant un déficit en dopamine au niveau du striatum dorsal à l'origine des symptômes moteurs observés chez les sujets atteints par la maladie. Elle pourrait également être impliquée dans les phénomènes addictifs ; ce sujet sera abordé plus loin dans ce document (Everitt and Robbins, 2005; Ilango *et al*, 2014; Wise, 2009). La voie mésocorticolimbique est la voie qui va principalement nous intéresser dans un premier temps car elle est activée par la majorité des substances « addictogènes » (Di Chiara and Imperato, 1988). Elle débute au niveau de l'aire tegmentale ventrale et projette vers le système limbique (en particulier le noyau accumbens, l'amygdale et l'hippocampe ; voie mésolimbique) et le cortex préfrontal médian (voie mésocorticale).

- La dopamine et le plaisir

La dopamine aurait en réalité un rôle plus complexe que celui de simple messager du plaisir. Les neurones dopaminergiques engagés dans la voie mésolimbique signaleraient la récompense potentielle (c'est-à-dire l'anticipation du plaisir) (Schultz, 2006; Schultz *et al*, 1997). La dopamine coderait en effet la différence entre ce que l'on a prédit et ce que l'on reçoit. La récompense est systématiquement comparée avec ce que l'on attend, et la dopamine n'est libérée que lorsque la récompense est plus importante que prévue.

Si par exemple un singe doit appuyer sur un levier pour obtenir une récompense : la première fois que l'animal fait un essai, il reçoit une récompense qu'il n'a pas anticipée ; la libération de dopamine fournit alors un signal renforçant qui indique la pertinence de ce comportement dans ce contexte précis. Le singe orientera par la suite sa décision grâce à la mémorisation d'une prédiction de la récompense et le contexte dans laquelle il peut l'obtenir. On parle alors de comportement motivé par le but. Lorsque le singe a appris qu'il devait appuyer sur le levier pour obtenir la récompense, les neurones dopaminergiques ont rempli leur rôle et ne s'activent plus. Pour plus d'informations sur l'anticipation du plaisir, se référer à l'ensemble des travaux de Schultz et collaborateurs (*e.g.* (Pessiglione *et al*, 2006; Schultz, 1998, 2006; Schultz *et al*, 1997; Schultz and Dickinson, 2000; Waelti *et al*, 2001))[1].

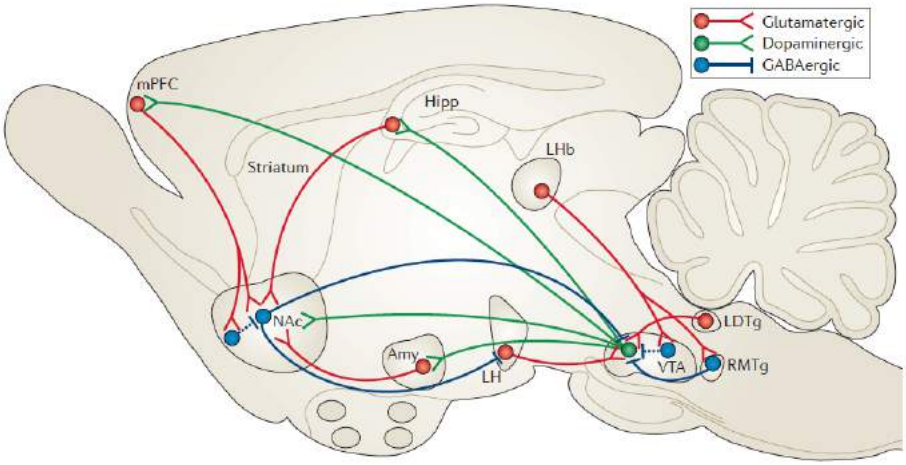


Figure 1 ***Schéma simplifié des principales connections dopaminergiques, glutamatergiques et GABAergiques (allant vers et provenant de) l'aire tegmentale ventrale et du noyau accumbens chez le rat (Russo and Nestler, 2013). VTA : aire tegmentale ventrale ; Amy : amygdale ; NAc : noyau accumbens ; mPFC : cortex préfrontal médian ; Hipp : hippocampe ; LH : hypothalamus latéral ; LHb : habenula latérale ; LDTg : noyau tegmental latéro-dorsal ; RMTg : noyau tegmental rostro-médian.***

[1] D'autres neurotransmetteurs, monoamines et opioïdes endogènes, interviennent également dans le circuit de la récompense, notamment par leurs actions sur les neurones dopaminergiques (*e.g.* (Tassin, 2008)).

- Quelles sont les principales afférences du noyau accumbens ?

Le système mésolimbique est constitué de neurones dopaminergique projetant vers le noyau accumbens, l'amygdale et l'hippocampe (Figure 1). L'amygdale et l'hippocampe envoient à leur tour des projections glutamatergiques en direction du noyau accumbens. Le système mésocortical projette vers le cortex préfrontal médian. Le noyau accumbens reçoit également des innervations denses de circuits glutamatergiques monosynaptiques du cortex préfrontal médian ainsi que d'autres régions (non détaillées dans la Figure 1).

- Quelles sont les principales afférences de l'aire tegmentale ventrale :

En retour, on trouve des projections GABAergiques du nucleus accumbens vers l'aire tegmentale ventrale; ces projections sont directes - *via* la « *voie directe* » - et font intervenir les neurones épineux moyens (Medium Spiny Neurons, MSNs) contenant principalement des récepteurs dopaminergiques de type D1.

Des projections GABAergiques indirectes - *via* la « voie indirecte » - (MSNs contenant principalement des récepteurs dopaminergiques de type D2) innervent l'aire tegmentale ventrale par l'intermédiaire des neurones GABAergiques du pallidum ventral (non représentées dans la Figure 1). Des données récentes indiquent toutefois que la description de cette ségrégation (voie directe : D1 / indirecte : D2) constitue un reflet simplifié de la réalité (Kupchik *et al*, 2015).

Outre ces voies de retour, l'aire tegmentale ventrale reçoit des afférences glutamatergiques du noyau tegmental latéro-dorsal, de l'habenula latérale et de l'hypothalamus latéral directement au niveau des neurones dopaminergiques ou via des interneurones GABAergiques (locaux). Ces afférences glutamatergiques variées contrôlent des aspects de la perception, de la récompense et de la mémoire. Finalement on relève une afférence GABAergique provenant du noyau tegmental rostro-médian.

c) Action des drogues sur la voie mésocorticolimbique

Malgré leur diversité, les drogues partagent toutes une caractéristique commune : elles activent le système de récompense et stimulent la libération de dopamine au niveau du noyau accumbens (Di Chiara *et al*, 1988). Plusieurs

catégorisations des drogues sont envisageables. On peut par exemple considérer qu'il existe 3 principales classes de drogues : les stimulants (ex : nicotine, cocaïne), les sédatifs (ex : héroïne) et les hallucinogènes (ex : cannabis). Remarquons que certaines drogues peuvent appartenir à plusieurs classes. C'est le cas par exemple de l'alcool qui peut être excitant à faible dose, et sédatif à forte dose. Le MDMA (3,4-méthylènedioxy-méthamphétamine ou ecstasy) est à la fois un stimulant et un hallucinogène. Bien qu'ayant des mécanismes d'actions et des cibles moléculaires distincts les unes des autres (Figure 2A), ces drogues ont pour effet commun d'activer, *in fine*, la voie mésolimbique en induisant une augmentation de la concentration de dopamine dans la fente synaptique du noyau accumbens à l'origine des sensations de plaisir (ou de l'anticipation du plaisir). Cet effet constitue le fondement neurobiologique de l'appétence pour les drogues.

Il existe trois mécanismes d'action principaux provoquant l'augmentation de la concentration de dopamine au niveau du noyau accumbens (Figure 2B) :

1. La facilitation de la libération de dopamine (cannabis, nicotine) au niveau de la terminaison présynaptique des neurones dopaminergiques.
2. L'inhibition de la recapture de la dopamine (cocaïne, amphétamines) au niveau de la terminaison présynaptique des neurones dopaminergiques.
3. La levée de l'inhibition des neurones dopaminergiques (opiacés, nicotine, alcool) au niveau des neurones GABAergiques inhibant l'aire tegmentale ventrale.

A

Drug of Abuse	Neurotransmitter	Sites
Cocaine and amphetamines	Dopamine Serotonin	Nucleus accumbens Amygdala
Opiates	Dopamine Opioid peptides	Ventral tegmental area Nucleus accumbens
Nicotine	Dopamine Opioid peptides	Ventral tegmental area Nucleus accumbens Amygdala
THC	Dopamine Opioid peptides	Ventral tegmental area
Alcohol	Dopamine Opioid peptides Serotonin GABA Glutamate	Ventral tegmental area Nucleus accumbens Amygdala

B

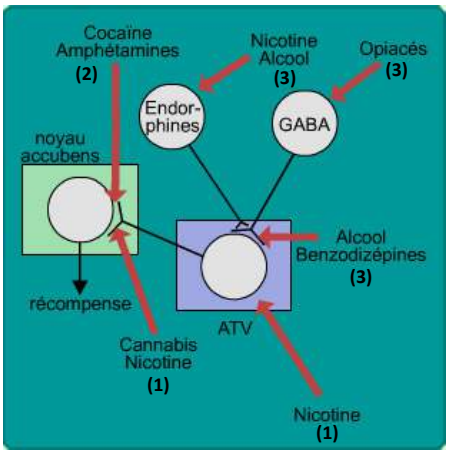
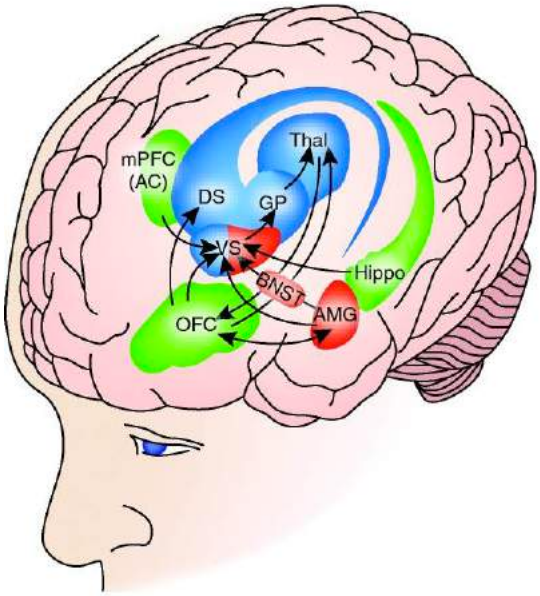


Figure 2 ***Activation par les drogues du système mésocorticolimbique. A) Table synthétisant les principaux substrats neurobiologiques de quelques drogues (Koob, 2013; Koob and Le Moal, 2001). B) Représentation schématique des principaux mécanismes par lesquelles les drogues augmentent la concentration de dopamine au niveau du noyau accumbens: 1) facilitation de la libération de dopamine, 2) inhibition de la recapture de dopamine ou 3) levée de l'inhibition des neurones dopaminergiques. D'après (Luscher and Ungless, 2006). Illustration de droite : http://lecerveau.mcgill.ca/index.php***

L'hyperactivation du système dopaminergique, en interaction avec d'autres systèmes de neurotransmission, va déclencher des modifications cérébrales durables par le truchement d'effets moléculaires détaillés dans la partie « B-3 Neurobiologie de l'addiction : effets moléculaires ».

d) Les 3 phases de l'addiction et les structures cérébrales impliquées

Le système mésocorticolimbique n'est pas le seul à être impliqué dans la dépendance aux drogues (Koob *et al*, 2010). L'addiction peut être divisée en 3 phases : l'intoxication, le sevrage et l'anticipation. Selon la phase concernée, différentes structures seront recrutées. Ces circuits cérébraux sont détaillés dans la Figure 3.



Intoxication

- Striatum ventral
(euphorie, récompense)
- Striatum dorsal
- Globus pallidus
- Thalamus
(habitudes, persévération)

Sevrage

- Amygdale étendue
Amygdale + noyau de la strie terminale
(malaise, dysphoria, état emotionel négatif)
- Striatum ventral
(diminution de la récompense)

Anticipation

- Cingulaire, préfrontal médian, orbitofrontal
(fonctions exécutives)
- Amygdale : noyau basolateral
(stimuli conditionnés)
- Hippocampe
(rôle du contexte)

Figure 3 ***Les 3 étapes de l'addiction : l'intoxication, le sevrage et l'anticipation et les circuits cérébraux recrutés (Koob and Volkow, 2010).***

B-2) L'addiction et les différences de vulnérabilité interindividuelles

a) Qu'est-ce que la dépendance à une substance ? Quelle est son origine ?

Le mot drogue désignait au XIVème un ingrédient servant à la teinture et aux préparations chimiques. Au XIXème siècle, il a pris son sens actuel et fait mention à une substance naturelle ou de synthèse, licite ou non, dont la consommation provoque un état modifié de la conscience. Les drogues peuvent être administrées par ingestion (ex : alcool), par injection (ex : héroïne), par inhalation (ex : nicotine, cannabis) ou par absorption par les membranes muqueuses (ex : cocaïne). La dépendance aux drogues est une pathologie chronique, hautement récidivante. Le taux de rechute est extrêmement important, même après plusieurs années d'abstinence.

L'addiction peut être définie comme une pathologie du choix et de l'apprentissage dans laquelle les patients apprennent en excès à reproduire, sans cesse, des choix mal adaptés et/ou biaisés. Selon la dernière édition du manuel de classification des troubles mentaux publiée par l'Association Américaine de Psychiatrie (Diagnostic and Statistical Manual of Mental Disorders - Edition 5, DSM-5; parue en 2013), les critères de la dépendance aux drogues sont au nombre de 11 (Tableau 1). Un sujet est considéré comme dépendant lorsqu'il présente ou a présenté, au cours des 12 derniers mois, au moins deux de ces onze critères. La sévérité des troubles est basée sur le nombre de critères rencontrés: 2-3 critères signent un trouble léger; 4-5 critères, un trouble modéré, et 6 ou plus, un trouble sévère. En outre, le DSM-5 décrit pour chaque substance des critères pour l'intoxication, le sevrage et les troubles induits par la substance.

La rémission récente est définie comme consistant en au moins 3 mois (et moins que 12) sans qu'aucun des critères ne soient applicables (sauf celui de l'envie de consommer, « craving ») alors que la rémission durable consiste en au moins 12 mois sans critère applicable (sauf, une fois encore, l'envie de consommer).

Deux phénomènes physiques sont associés à l'apparition d'une dépendance aux drogues : la <u>tolérance</u> (diminution de l'effet de la drogue au fur et à mesure des prises, ce qui implique comme corollaire la nécessité d'augmenter la consommation

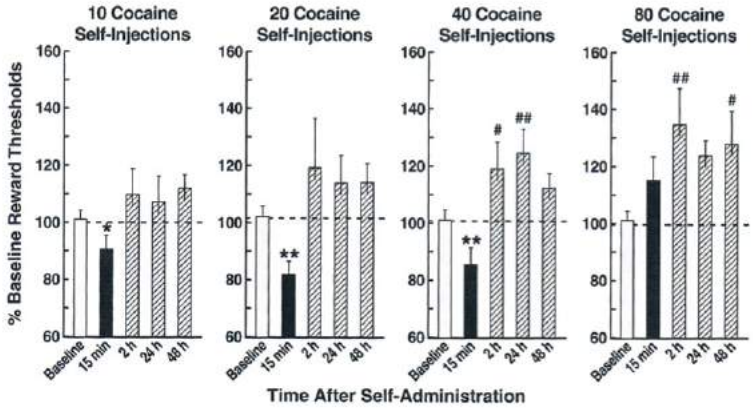
de la drogue pour maintenir des effets d'intensités similaires) et le sevrage (symptômes physiques et/ou psychologiques désagréables survenant lors de l'arrêt brutal de la consommation).

Tableau 1 ***Les 11 critères de dépendance selon le Diagnostic and Statistical Manual of Mental Disorders - Edition 5 (DSM-5) publié en 2013 par l'Association Américaine de Psychiatrie.***

Les 11 critères de la dépendance (DSM-5)
• Besoin impérieux et irrépressible de consommer la substance (**craving**)
• Perte de contrôle sur la quantité et le temps dédié à la prise de substance
• Beaucoup de temps consacré à la recherche de substances
• Augmentation de la **tolérance** au produit addictif
• Présence d'un **syndrome de sevrage**, c'est-à-dire de l'ensemble des symptômes provoqués par l'arrêt brutal de la consommation
• Incapacité de remplir des obligations importantes
• Usage même lorsqu'il y a un risque physique
• Problèmes personnels ou sociaux
• Désir ou efforts persistants pour diminuer les doses ou l'activité
• **Activités réduites** au profit de la consommation
• **Poursuite de la consommation malgré les dégâts physiques ou psychologiques**

Ces manifestations physiques sont à l'origine de la progression d'une consommation chronique vers un état de dépendance (Figure 4). En effet, une exposition répétée aux substances addictives engendre des changements adaptatifs au niveau cérébral qui vont s'opposer à l'effet des drogues et produire la tolérance. Suite à l'arrêt de la consommation, ces mêmes changements restent actifs un certain temps et occasionnent un effet opposé à celui de la prise de drogue : le sevrage (Figure 4B). Le syndrome de sevrages (dont la nature dépendra de la drogue considérée) est soulagé si le sujet consomme à nouveau. On passe alors d'un renforcement positif (consommation pour l'obtention d'une récompense = le plaisir induit par la drogue) à un renforcement négatif (consommation pour éviter le syndrome de sevrage). Cette transition d'un renforcement positif à un renforcement négatif pourrait signer le début de la dépendance (Figure 4C, D).

A



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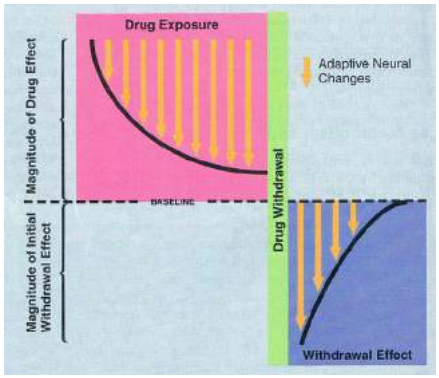

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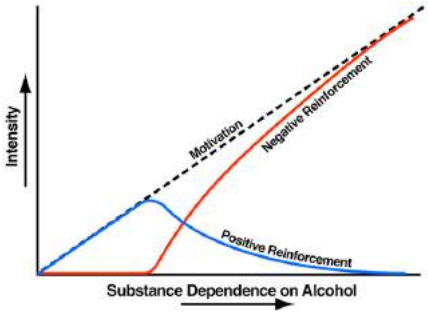
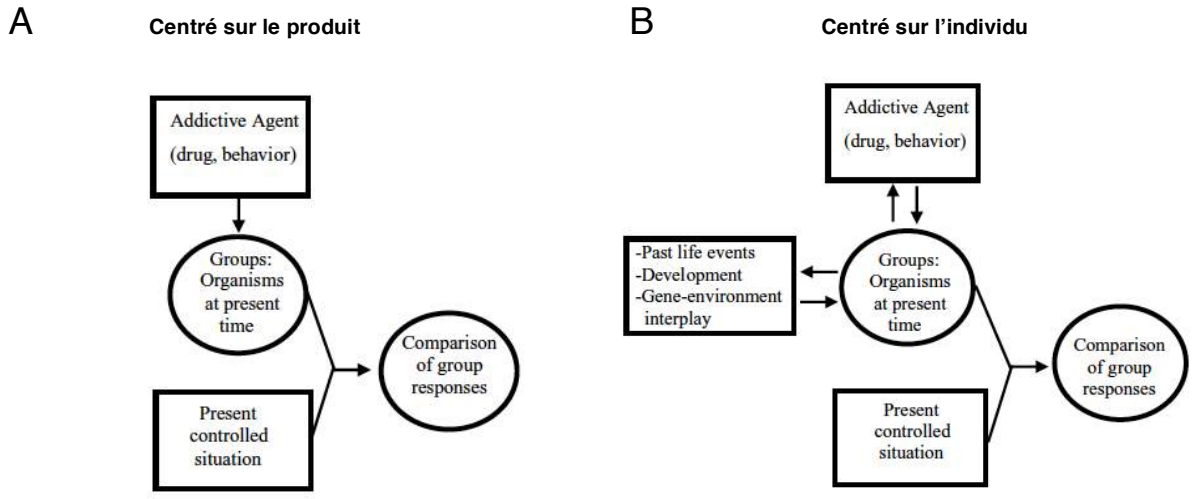


Figure 4 ***A) La diminution du seuil d'auto-stimulation électrique immédiatement après la consommation de drogue est suivie par une augmentation de celui-ci (cf. théorie des processus opposants) (Kenny et al, 2003). B) Des changements adaptatifs intervenant à la suite d'une prise répétée de drogues rendent compte à la fois du phénomène de tolérance (partie rose) et de sevrage (partie bleue). C) La transition d'un renforcement positif (prise 1 et 2) à un renforcement négatif (prise 3 et 4) expliquerait la progression d'un usage récréatif vers une addiction. D) Représentation théorique de la transition progressive d'un renforcement positif à un renforcement négatif.***
(Koob, 2013; Koob et al, 2001)

b) Approche centrée sur le produit versus approche centrée sur l'individu : la drogue n'est pas l'unique moteur de l'addiction

La toxicomanie est principalement décrite comme étant une dépendance à une substance chimique (nicotine, alcool, héroïne…). C'est une approche centrée sur le produit c'est à dire que l'on considère que la substance, et uniquement elle, va déclencher une dépendance (Figure 5A). Plus récemment a été introduit le terme anglo-saxon «addiction» englobant à la fois les dépendances à des substances chimiques mais également des dépendances sans drogue (addictions

comportementales). Parmi ces dernières, nous pouvons citer le jeu pathologique (souvent lié à des gains d'argent), les achats compulsifs, l'exercice physique, le sexe, les troubles alimentaires. Le terme « addiction » introduit également une approche centrée sur l'individu (Figure 5B). Nous ne sommes pas tous égaux face à l'addiction. Celle-ci est induite par une substance (ou un comportement), en interaction avec un individu plus ou moins vulnérable, qui a une histoire propre et qui évolue dans un environnement spécifique (ex : environnement stressant favorisant les dépendances). Cette conception plus intégrée permet de rendre compte des différences individuelles de vulnérabilité aux drogues. En effet, si une majorité de la population est confrontée à des substances addictogènes (ex : alcool), seule une partie restreinte des individus va développer une dépendance. Par exemple, concernant les utilisateurs réguliers de cocaïne, il est estimé que seulement 15 à 17% vont développer une dépendance. Qu'est ce qui différencie ces 15 à 17% de sujets des autres utilisateurs ? Quels sont les facteurs favorisant la transition d'un usage chronique à une dépendance ?



Référence clinique :
- Désordre iatrogénique
- Différences interindividuelles non considérées

Recherche et traitement :
- Comprendre les effets des substances d'abus sur le système nerveux central
- Tenter de contrecarrer ces effets par des moyens pharmacologiques

Politique sociale :
- Contrôler et réprimer la disponibilité et l'usage de drogue

Référence clinique :
- Phénotype vulnérable (génétique/environnement)
- Prédisposition, comorbidité
- Désordre 'bio-comportementale' (interface individu/société)

Recherche et traitement :
- Orienté vers la prédiction et la prévention
- Identification des individus vulnérables

Politique sociale :
- Prévention ; soins sociaux et médicaux

Figure 5 ***A) Modèle centré sur le produit : le produit et lui seul crée la dépendance. B) Modèle centré sur l'individu prenant en compte les différences interindividuelles de vulnérabilité aux drogues (Swendsen and Le Moal, 2011).***

c) Les facteurs influençant la vulnérabilité aux drogues

Nous pouvons diviser les facteurs influençant la vulnérabilité aux drogues en 4 grandes catégories : le produit, l'individu, le contexte social et l'environnement (Figure 6). Dans ce bref rappel, non exhaustif, nous ne traiterons pas du contexte social.

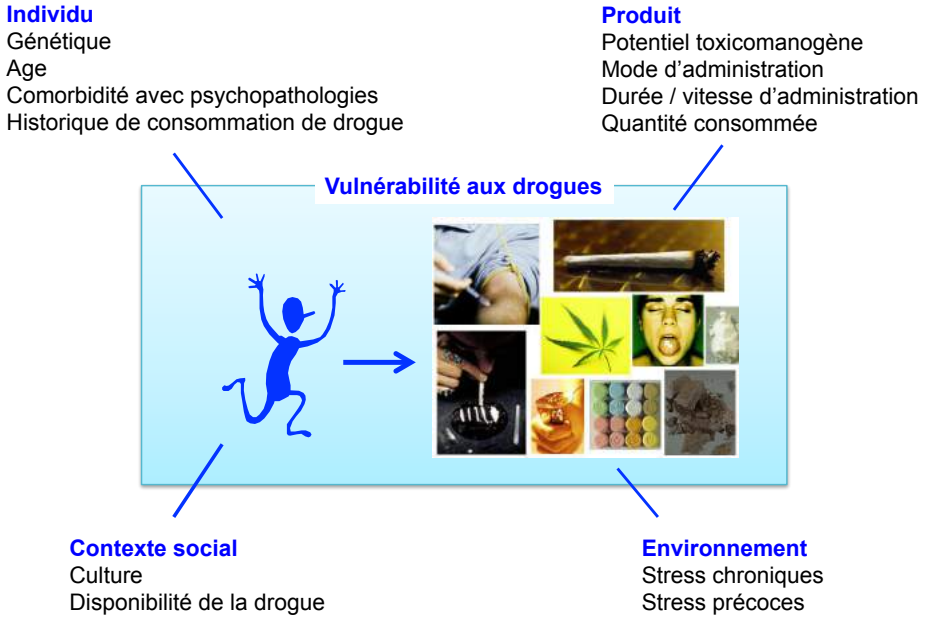


Figure 6 ***La vulnérabilité aux drogues dépend de facteurs génétiques et environnementaux.***

Le potentiel toxicomanogène du <u>produit</u> est indubitablement un critère primordial pour que la dépendance apparaisse. Certains produits sont par nature plus addictogènes que d'autres (Figure 7) (Anthony *et al*, 1994). De même, la quantité consommée est proportionnelle au taux d'apparition de la dépendance. D'autres paramètres tel que le mode d'administration (ex : nicotine : patch [-] *versus* inhalation [+++]; methylphénidate : voie orale [-] *versus* absorption par les muqueuses nasales lors d'un usage récréatif [+++]; alcool : consommation étalée dans le temps [++] *versus* ivresse expresse, «binge drinking» [+++]) influence pour une substance donnée la propension à développer une dépendance. De manière générale, plus le produit est absorbé rapidement, plus les processus de plasticité mis en jeu au niveau cérébral pour s'opposer aux effets de la drogue seront robustes.

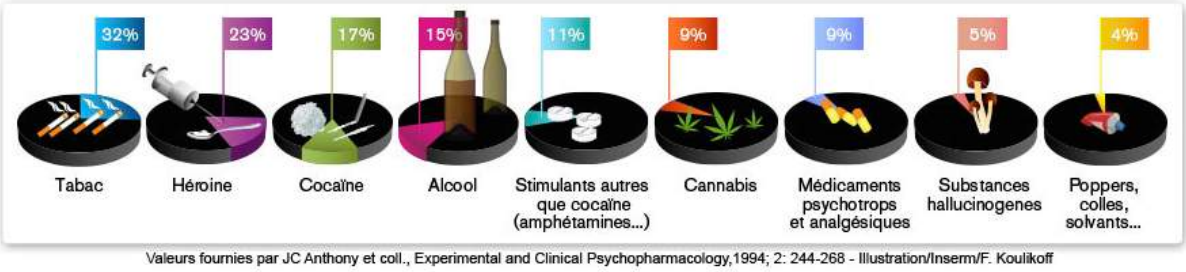


Figure 7 ***Part des usagers développant une dépendance à la substance (Anthony et al, 1994).***

Concernant l'individu, son bagage génétique va influencer le risque de développer une dépendance. Des travaux indiquent que la prévalence de l'addiction est comparable chez les jumeaux homozygotes (vrais jumeaux). En revanche, une telle similitude des risques n'est pas retrouvée chez les jumeaux hétérozygotes (faux jumeaux) (Goldman *et al*, 2005). Il n'existe pas un gène spécifique à la dépendance, mais il est probable que plusieurs gènes influencent les risques, notamment ceux qui sont impliqués dans le mode d'action des substances psychoactives (récepteurs, transporteurs) mais également dans leur pharmacodynamique et leur métabolisme (Tableau 2) (Bierut, 2011; Gorwood *et al*, 2007).

Tableau 2 ***Gènes associés à l'addiction (Bierut, 2011).***

Nicotine Dependence
CHRNA5-CHRNA3-CHRNB4
CHRNA6-CHRNB3 Region
Cyp2a6 Region
Alcohol Dependence
ADH1B
ALDH2
Cocaine Dependence
CHRNA5-CHRNA3-CHRNB4

L'âge du sujet est aussi à prendre en considération. L'adolescence est en effet une période critique pour le développement des conduites addictives, et ce, à double titre. D'une part, c'est souvent lors de l'adolescence que sont initiées les conduites d'abus vis-à-vis des substances addictives (Spear, 2000). L'adolescence est de plus une période sensible du développement lors de laquelle le cerveau n'est pas encore complètement mature. Le cerveau adolescent est donc plus vulnérable aux effets à long terme induit par les drogues (cf. (Iniguez *et al*, 2009)). La Figure 8 représente la

maturation du cerveau (élagage des connexions synaptiques qui ne sont pas nécessaires, couleurs chaudes) entre 5 et 20 ans. L'exposition à des drogues lors de cette fenêtre temporelle peut affecter ce mécanisme et ainsi induire des effets permanents sur cet organe.

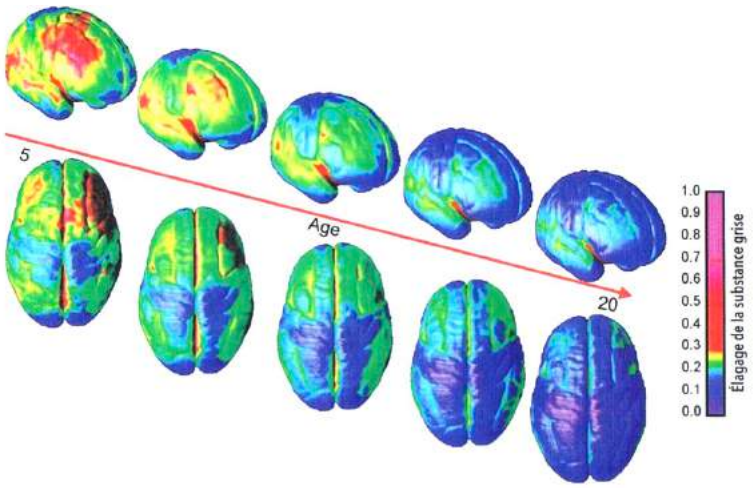


Figure 8 ***La maturation du cerveau (figurée en bleu entre 5 et 20 ans) se produit de l'arrière vers l'avant. Elle correspond à une perte de synapses qui ne sont pas utiles au développement. Cet élagage se prolonge au-delà de 20 ans. La consommation de drogue lors de l'adolescence perturbe ce processus, entrainant des anomalies de connexion entre les neurones (Gogtay et al, 2004).***

Il existe par ailleurs une forte comorbidité entre la dépendance à des substances et les troubles de l'humeur (Merikangas *et al*, 1998). Ainsi, l'anxiété et la dépression sont fréquemment associés à l'alcoolo-dépendance, sans que l'on puisse conclure de façon certaine sur le paramètre qui était présent au départ et qui entraine l'autre. Gardons à l'esprit qu'un rapport de causalité entre ces différents troubles n'est pas démontré ; il est possible que cette association soit uniquement due à des facteurs confondants. L'impulsivité pourrait constituer un autre facteur prédictif d'un risque élevé de développer une addiction. Kreek et collaborateurs (Kreek *et al*, 2005) ont reporté une corrélation positive entre l'augmentation de la dopamine dans le striatum ventral lors de la prise de substances et un niveau d'impulsivité élevé. De plus, certains polymorphismes génétiques des récepteurs ou des transporteurs de la dopamine sont associés à la fois à une forte impulsivité et à un risque plus élevé de développer un comportement addictif (Kreek *et al*, 2005).

L'environnement est aussi un facteur déterminant entrant dans l'équation. L'exposition au stress en particulier est communément associée à une prévalence

accrue d'addictions. Elle constitue un facteur aggravant les risques de rechute. Chez les rongeurs, le stress augmente les propriétés hédoniques des substances addictives (Koob, 2008). Sur le plan cellulaire, le stress se traduit par une activation de l'axe hypothalamo-hypophyso-surrénalien (axe corticotrope, cf. Chapitre 1). De nombreuses études soulignent la convergence de ces mécanismes avec ceux mis en jeu dans l'addiction. Ainsi, un stress aigu déclenche une plasticité synaptique au niveau de l'aire tegmentale ventrale similaire à celle obtenue après une exposition à des substances addictives (Saal *et al*, 2003). D'autres expériences ont mis en exergue le fait que des stress précoces, lors de l'enfance, peuvent augmenter durablement (tout au long de la vie) la propension à consommer des substances addictives (Huot *et al*, 2001; Vazquez *et al*, 2006). Dans le Chapitre 1, j'exposerai mes travaux de thèses qui montrent qu'un stress plus précoce encore (subi *in utero*) influence à long terme la sensibilité aux effets neurobiologiques de l'alcool.

B-3) Neurobiologie de l'addiction : effets moléculaires

La prise répétée de drogues modifie la plasticité des synapses en influençant divers processus tels que : 1) l'expression génique, 2) le traitement post-traductionnel des protéines; 3) l'excitabilité des membranes; 4) l'architecture des neurones.

a) Modulation de l'expression génique

La dopamine qui s'accumule au niveau de la fente synaptique du noyau accumbens se lie à deux familles de récepteurs métabotropiques: les "D1-like" et "D2-like". Les D1-like (D1 et D5) sont couplés *via* une protéine G stimulatrice (Gs) à l'adénylate cyclase (AC) et permettent la production d'adénosine monophosphate cyclique (AMPc). L'AMPc déclenche de nombreuses réponses métaboliques dépendantes de la Protéine Kinase A (PKA) en activant cette dernière (Figure 9). Citons par exemple la phosphorylation des canaux ioniques facilitant les courants Na^+ et inhibant les courants K^+, phénomène à l'origine d'une plus forte excitation neuronale.

Les D2-like (D2, D3, D4), quant à eux, sont couplés à une protéine inhibitrice (Gi) et inhibent la synthèse d'AMPc. Ceci facilite l'ouverture de canaux K^+ hyperpolarisant et inhibe les neurones. Ainsi, selon la nature du récepteur auquel elle se lie, la dopamine stimulera (D1-like) ou inhibera (D2-like) les neurones.

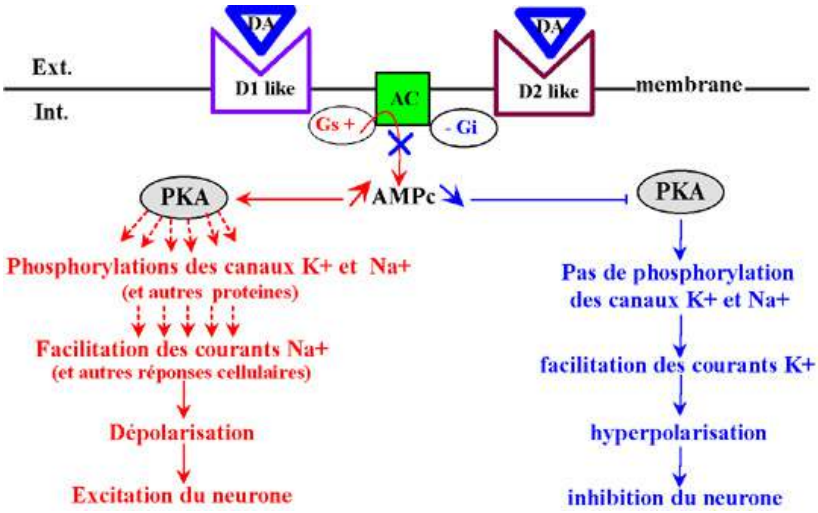


Figure 9 ***Effets de l'activation des récepteurs dopaminergiques du type D1 ou D2.***

La dopamine joue un rôle critique dans la régulation de l'expression génique induite par les substances addictogènes (*via* l'activation des récepteurs D1 et D2). Cette régulation génique est responsable des changements adaptatifs à long terme observés dans le circuit de la récompense suite à une consommation répétée.

Les récepteurs D1 sont indispensables à l'augmentation de l'expression génique dans les neurones du striatum. Preuve en est, l'induction de gènes précoces (immediate-early genes, IEGs) par l'amphétamine et la cocaïne est totalement inhibée en cas d'administration systémique ou intra-striatale d'un antagoniste spécifique des récepteurs D1 (Cole *et al*, 1992; Graybiel *et al*, 1990; Moratalla *et al*, 1992; Steiner and Gerfen, 1995; Young *et al*, 1991) ou en cas de délétion ciblée des récepteurs D1 (souris «knockout» pour les récepteurs D1) (Drago *et al*, 1996; Moratalla *et al*, 1996b; Zhang *et al*, 2004). En adéquation avec l'effet opposé des récepteurs D1 et D2 sur l'adénylate cyclase, les récepteurs D2, eux, inhibent l'expression génique dans les neurones striataux. En revanche, la stimulation concomitante des récepteurs D1 + D2 potentialise l'expression génique induite par les récepteurs D1 (Gerfen *et al*, 1995; LaHoste *et al*, 1993; Paul *et al*, 1992). Les

autres neurotransmetteurs tels que la noradrénaline, la sérotonine et le glutamate ont un rôle modulateur sur l'induction de la régulation génique par la dopamine (Figure 10).

- <u>Exemple de la dynorphine :</u>

L'activation des récepteurs dopaminergiques D1 provoque une cascade de réactions aboutissant à la phosphorylation du facteur de transcription CREB (cAMP Response Element Binding Protein) (Figure 10B). Ceci conduit à une élévation de la synthèse de dynorphine au niveau des MSNs projetant vers les neurones dopaminergiques du système mésolimbique (Figure 10C). La dynorphine est alors libérée (au niveau de l'aire tegmentale ventrale et de la terminaison synaptique des neurones dopaminergiques) et inhibe la production/libération de dopamine dans le noyau accumbens. La dynorphine a donc un rôle de rétrocontrôle négatif.

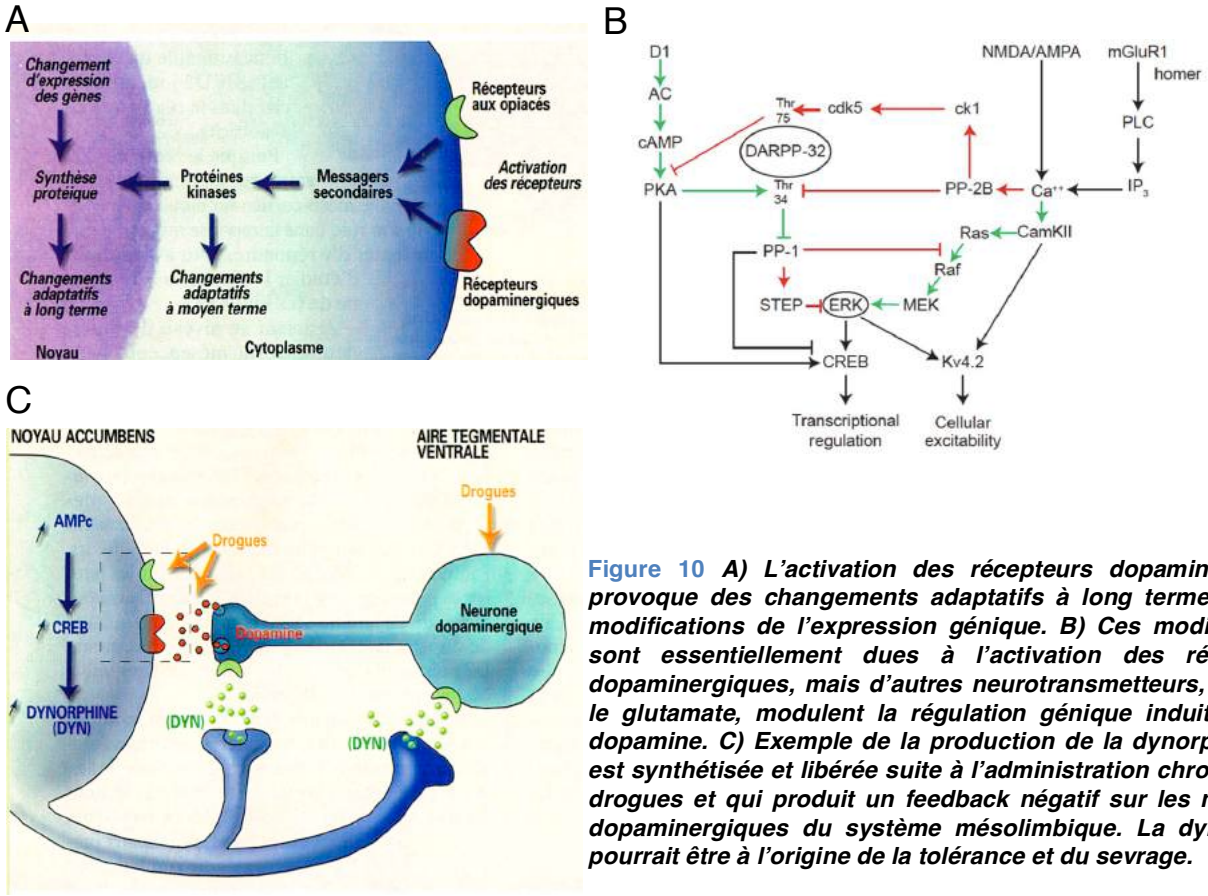


Figure 10 ***A) L'activation des récepteurs dopaminergiques provoque des changements adaptatifs à long terme via des modifications de l'expression génique. B) Ces modifications sont essentiellement dues à l'activation des récepteurs dopaminergiques, mais d'autres neurotransmetteurs, tels que le glutamate, modulent la régulation génique induite par la dopamine. C) Exemple de la production de la dynorphine qui est synthétisée et libérée suite à l'administration chronique de drogues et qui produit un feedback négatif sur les neurones dopaminergiques du système mésolimbique. La dynorphine pourrait être à l'origine de la tolérance et du sevrage.***

La dynorphine pourrait expliquer le phénomène de tolérance (diminution de l'effet de la drogue au fur et à mesure des prises puisque la dynorphine s'oppose à

l'effet de la drogue), mais également le syndrome de sevrage puisque son augmentation est maintenue après l'arrêt de la consommation, provoquant une « hypodopaminergie » transitoire.

- Exemple du Delta-FosB

Suite à un usage répété, le système mésolimbique peut devenir, ultérieurement, plus sensible aux effets de la drogue. Ce phénomène serait dû à une augmentation graduelle de la protéine Delta-FosB dans le noyau accumbens, un facteur de transcription faiblement activé par les drogues après un usage unique mais qui a tendance à s'accumuler en réponse à des stimulations chroniques (dû à l'extrême stabilité du Delta-FosB, forme tronquée du FosB). Cette accumulation est maintenue pendant plusieurs semaines voire plusieurs mois (Figure 11A).

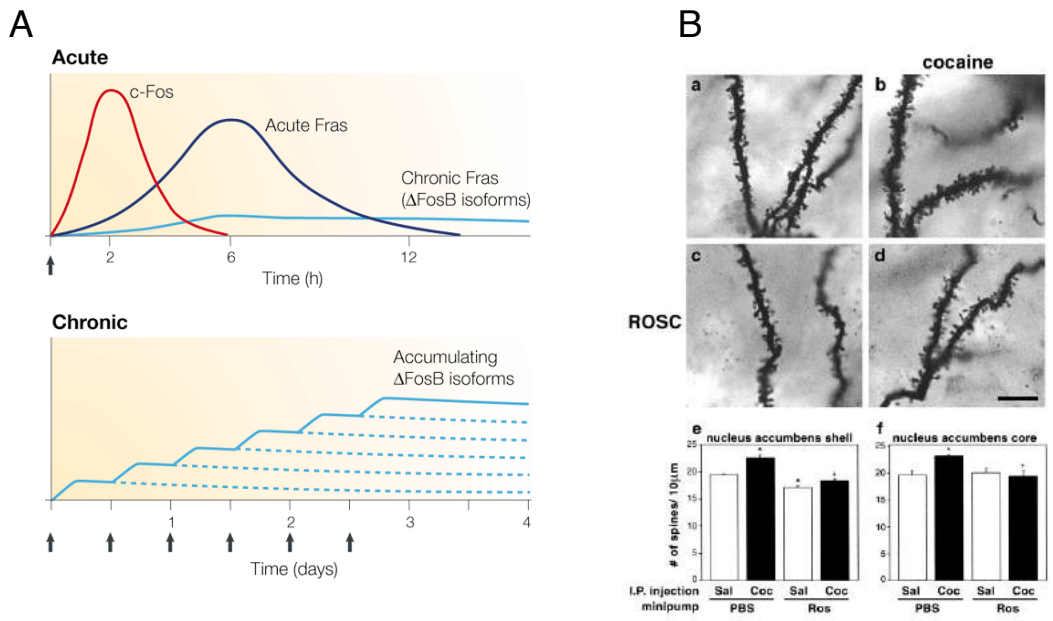


Figure 11 A) Accumulation de Delta-FosB suite à la prise répétée de drogue (Renthal and Nestler, 2008). L'une des cibles du facteur de transcription Delta-FosB est la CDK5. B) L'inhibition de la CDK55 bloque la production d'épines dendritiques par la cocaïne. a, b, c, d sont des illustrations de dendrites au niveau du noyau accumbens de rats dans diverses conditions expérimentales. a: témoins non traités, b : cocaïne seule, c : roscovitine (inhibiteur de la CDK5) seule, d : cocaïne + roscovitine. e et f : quantifications du nombre d'épines comptées le long de dendrite distale dans deux régions du noyau accumbens, l'enveloppe (shell) et le cœur (core). Ces quantifications montrent que, par rapport à la condition témoin (Sal & PBS) la cocaïne (Coc & PBS) accroît la densité épineuse, et que la roscovitine s'oppose à cet effet (Coc + Ros). PBS : phosphate-buffered saline (Norrholm *et al*, 2003).

Ce facteur de transcription pourrait de ce fait jouer le rôle d'un interrupteur moléculaire. Il augmente par exemple l'expression de la sous-unité $GluR_2$ des

récepteurs AMPA et du Brain-Derived Neurotrophic Factor (BDNF) qui stimule la croissance des dendrites. Une autre cible du Delta-FosB serait le gène codant pour l'enzyme Cell Division Protein Kinase 5 (CDK5), responsable de l'augmentation du nombre d'épines dendritiques au niveau des neurones du noyau accumbens après un traitement chronique à la cocaïne (Figure 11B). Comme indiqué dans le Tableau 3, la surexpression de Delta-FosB induite expérimentalement dans les MSNs de la voie directe engendre une augmentation de la sensibilité aux drogues et à la consommation de nourriture.

Tableau 3 ***Conséquences comportementales de la surexpression de Delta-FosB à l'âge adulte (système de régulation des gènes par la tétracycline) dans les neurones Dynorphin + /Substance P+ du noyau accumbens et du striatum dorsal (voie directe). D'après (McClung et al, 2004).***

Stimulus	Phénotype	
Cocaïne	↑ réponse locomotrice après administration aiguë	(Kelz et coll., 1999)
	↑ sensibilisation locomotrice suite à une administration répétée	(Kelz et coll., 1999)
	↑ place préférence	(Kelz et coll., 1999)
	↑ acquisition de l'auto-administration de cocaïne pour des doses faibles	(Colby et coll., 2003)
	↑ motivation pour la drogue dans un protocole de ratio progressif	(Colby et coll., 2003)
Morphine	↑ place préférence pour des doses faibles	(Zachariou et coll., 2006)
	↑ syndrome de sevrage	(Zachariou et coll., 2006)
	↑ tolérance, ↓ de l'effet analgésique	(Zachariou et coll., 2006)
Alcool	↑ de l'effet anxiolytique	(McClung et coll., 2004)
Nourriture	↑ motivation pour la nourriture dans un protocole de ratio progressif	(Olausson et coll., 2006)

Dans le Chapitre 1, je montrerai que l'induction de Delta-FosB par l'alcool est exacerbée chez les rats adultes qui ont subi un stress prénatal comparés à des rats adultes témoins. Dans le Chapitre 2, j'exposerai des résultats portant sur les effets moléculaires (modulation de l'expression génique) du méthylphénidate (utilisé pour le traitement de l'hyperactivité avec trouble de l'attention) seul ou en co-administration avec la fluoxétine (inhibiteur sélectif de la recapture de la sérotonine, utilisé comme antidépresseur). Je m'efforcerai de démontrer que la fluoxétine potentialise l'expression génique induite par le méthylphénidate et pourrait en ce sens augmenter son potentiel addictif.

b) Mécanismes épigénétiques

L'épigénétique désigne l'étude des influences de l'environnement sur l'expression de nos gènes. Pour utiliser une métaphore, la génétique renvoie à l'écriture des gènes, l'épigénétique à leur lecture : un même gène pourra être lu différemment selon certaines circonstances. Le terme épigénétique définit donc les modifications transmissibles et réversibles de l'expression des gènes ne s'accompagnant pas de changements de séquences nucléotidiques au niveau de l'ADN.

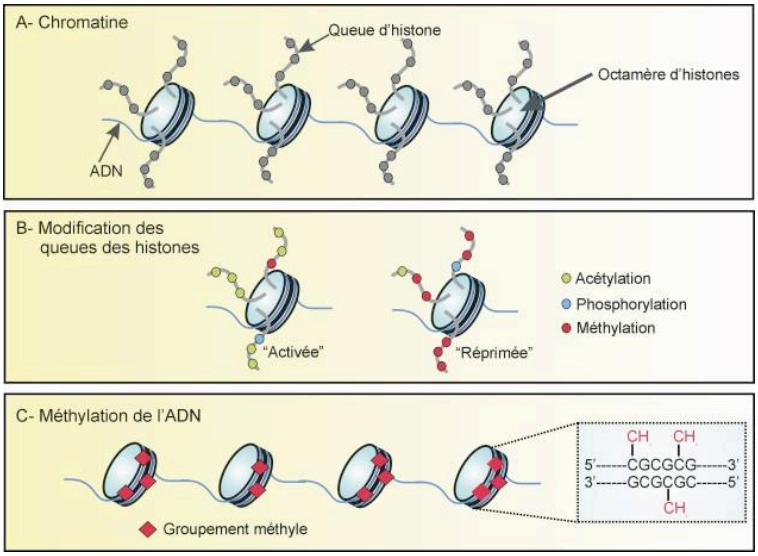


Figure 12 L'état compacté ou décompacté de la chromatine est contrôlé par la modifications des queues des histones (acétylation, phosphorylation, méthylation ou ubiquitinylation) et la méthylation de l'ADN (Jiang *et al*, 2008).

L'expression d'un gène peut être régulée par l'état compacté ou décompacté de la chromatine. Ces états sont contrôlés par :

1) les modifications des histones liées à l'ADN (acétylation, méthylation, phosphorylation ou ubiquitinylation) (Figure 12). Ces modifications post-traductionnelles nécessitent l'action d'enzymes spécifiques modifiant l'extrémité N-terminale des histones. Par exemple, l'acétylation de l'histone par l'Histone acétyltransférase (HAT) entraîne une ouverture de la chromatine, facilitant ainsi la transcription (Figure 13).
2) la méthylation de l'ADN au niveau des résidus cytosine des îlots CpG qui se trouvent essentiellement dans les régions proximales des promoteurs

des gènes (Figure 12 et Figure 13). Une faible méthylation se traduit par une forte expression du gène, alors qu'un haut niveau de méthylation inactive le gène. Cette méthylation s'effectue par les enzymes spécifiques appelées ADN méthyltransférases. Le dérèglement de la méthylation des îlots CpG est associé à diverses pathologies.

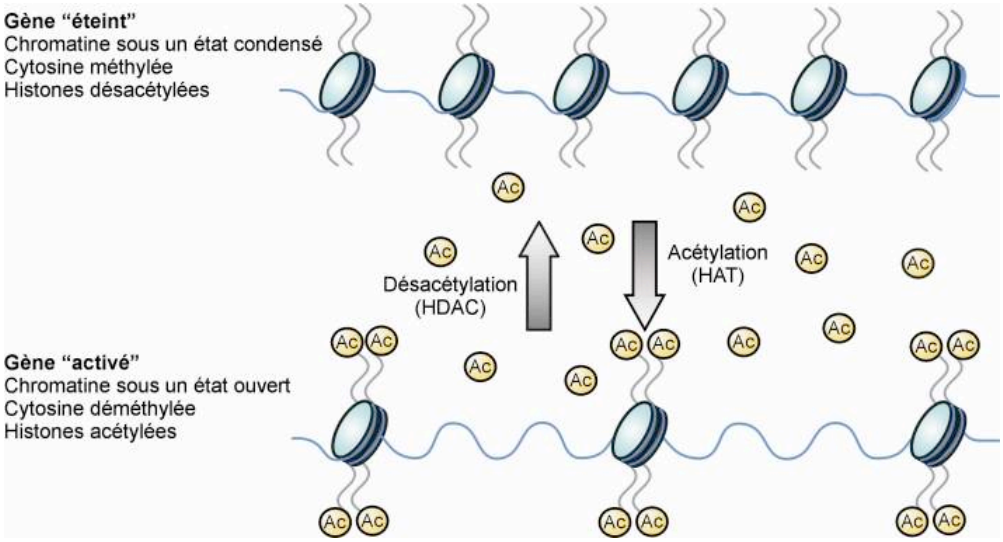


Figure 13 Régulation épigénétique de la transcription des gènes (Pons *et al*, 2009). HDAC : Histone désacétylase ; HAT : Histone acétyltransférase

Des hypothèses émergentes suggèrent que ces altérations épigénétiques sont des mécanismes importants sous-tendant la réponse neurobiologique aux substances addictives. Ainsi, la cocaïne augmente l'acétylation de l'histone H3 au niveau des promoteurs de certains gènes comme celui du BDNF (Renthal *et al*, 2008). Néanmoins, elle induit aussi des modifications épigénétiques particulières qui dépendent de la fréquence d'administration de la drogue. Aussi, après une administration unique à dose élevée, les histones H4 sont acétylées, alors qu'à la suite d'une administration chronique, ce sont les histones H3 qui sont acétylées (Tsankova *et al*, 2007). Ces divers mécanismes pourraient être ciblés pour développer de nouveaux traitements contre la plasticité cérébrale induite par les drogues.

c) Implication des circuits cortico-striataux dans l'addiction et comportements automatisés

- Implication exclusive du striatum ventral ?

La dichotomie fonctionnelle entre le système dopaminergique mésocorticolimbique (~ motivation) et le système nigrostrié (~ moteur) est de plus en plus remise en question. Un nombre croissant d'indices montre que ces deux voies participent aux processus de récompense. Par exemple, la stimulation spécifique de la voie nigrostriée peu induire un phénomène de préférence de place similaire à celui produit par l'activation de la voie mésolimbique (Ilango *et al*, 2014). Le blocage des entrées glutamatergiques et cholinergiques dans la VTA, mais aussi dans la substance noire, atténuent la compulsion pour l'auto-administration de cocaïne (Wise, 2009). La dopamine dans la voie mésocorticolimbique, comme dans la voie nigrostriée, participe au renforcement de la consolidation de la mémoire des récompenses (Wise, 2009).

- Quelle est alors l'implication du striatum dorsal dans le développement d'une dépendance ?

Lors de la perte de contrôle du comportement (dépendance), les décisions prises par le sujet ne sont plus guidées par la volonté de consommer la substance, mais dépendent d'une automatisation de son comportement (qui est sensible aux indices contextuels). La transition d'un comportement planifié et motivé à un comportement automatique et compulsif serait sous tendu par le recrutement progressif du striatum dorsal (caudate-putamen) (Everitt and Robbins, 2013).

Le système de prise de décision planifiée implique une boucle neuronale striato-pallido-thalamo-corticale, médio-ventrale (gris clair, Figure 14A), régulée par les projections dopaminergiques issues de l'aire tegmentale ventrale. Les projections dopaminergiques de la substance noire modulent quant à elles une boucle striato-pallido-thalamo-corticale située latéro-dorsalement par rapport à la précédente et qui gère la prise de décision automatique (gris foncé, Figure 14A). Chez le sujet sain, les transitions d'un mode de décision planifiée à un mode de décision automatique sont courantes et nécessaires pour libérer des ressources cognitives intrinsèquement limitées. Une transition progressive en faveur du mode automatique pourrait être

également à l'origine du développement de comportements compulsifs retrouvés dans l'addiction (Everitt *et al*, 2008).

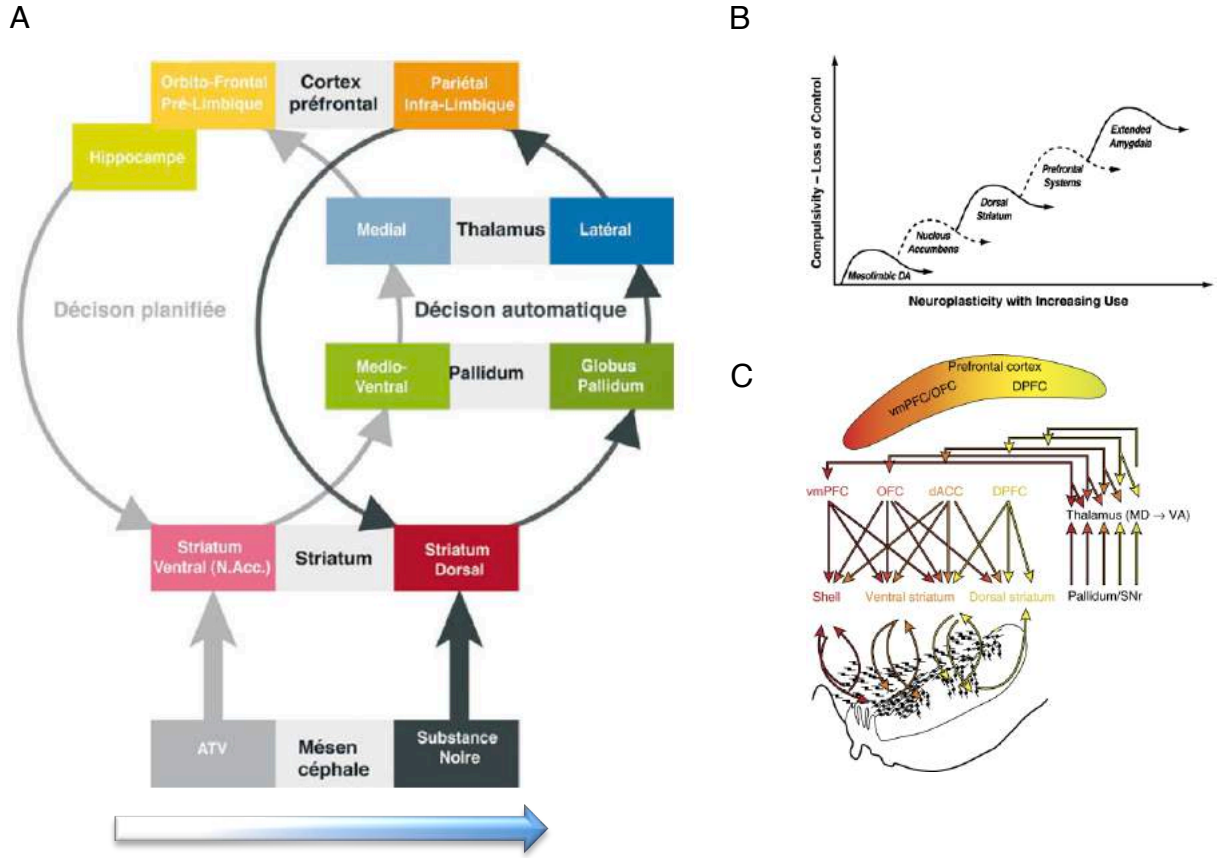


Figure 14 A) Représentation schématique des boucles neuronales sous-tendant les mécanismes de prise de décision planifiée et automatique (Redish *et al*, 2008). B) Modèle théorique de la séquence des structures impliquées (neuroplasticité) au cours de l'apparition d'une dépendance, en particulier une transition d'une implication du noyau accumbens à une implication du striatum dorsal (Koob *et al*, 2010). Le striatum dorsal serait impliqué dans l'automatisation, les associations stimuli-actions et donc dans la compulsion. C) Schéma anatomo-fonctionnel illustrant le recrutement progressif des circuits recevant des afférences dopaminergiques de la substance noire impliquant le striatum dorsal et le cortex préfrontal dorsal *via* des boucles ouvertes non réciproques (Haber and Knutson, 2010).

Dans un premier temps, une plasticité synaptique précoce est observée au niveau de l'aire tegmentale ventrale et des noyaux accumbens initiant une cascade de changements moléculaires à la base d'un remodelage fonctionnel de certains réseaux (Carr and Kalivas, 2008; Engblom *et al*, 2008; Hyman *et al*, 2006) (Figure 14B). L'activation soutenue et persistante de l'aire tegmentale ventrale associée à la libération excessive de dopamine vers le cortex orbitofrontal, l'hippocampe et le noyau accumbens (Figure 1) pourrait recruter progressivement les afférences dopaminergiques modulatrices de la substance noire, qui projettent vers le cortex infralimbique et le striatum dorsal (Figure 14B et C). Une démonstration explicite

étayant cette hypothèse est que, chez des rats entraînés à s'auto-injecter de la cocaïne, la déconnexion chirurgicale du striatum ventral et dorsal interrompt le développement des processus addictifs (Belin and Everitt, 2008; Luscher and Bellone, 2008; Murray *et al*, 2015)[2].

B-4) Thérapeutiques ?

a) Traitements médicamenteux

L'addiction est un trouble psychiatrique incurable à ce jour. Les rechutes, même après de longues périodes de sevrage, constituent un défi majeur du traitement de cette pathologie. Il existe cependant un certain nombre de thérapeutiques qui visent à accompagner le patient lors de l'arrêt de la consommation. L'utilisation d'agents pharmacologiques est une approche standard dans la prise en charge des addictions, en combinaison de traitements psycho-comportementaux et d'une aide social.

Concernant les traitements médicamenteux, différentes stratégies sont possibles (Tableau 4). Une première option consiste à administrer une substance antagoniste qui bloque les récepteurs ciblés par la drogue et par conséquent inhibe son effet euphorisant. La naltrexone est un exemple d'antagoniste compétitif spécifique des opiacés utilisé dans le traitement de l'alcoolisme et de la dépendance aux opiacés. Bien que cette stratégie puisse dans une certaine mesure diminuer la consommation de drogue, le taux de rechute est significatif.

Une seconde option consiste à maintenir une activité minimale du système de récompense lors de l'arrêt de la consommation en utilisant des produits de substitution. La molécule utilisée agit de la même façon que le produit substitué. Cependant, ses effets comportementaux ou sa toxicité sont plus faibles. Cette approche permet au patient de diminuer sa consommation en douceur et au praticien

[2] Il faut garder à l'esprit que d'autres structures des ganglions de la base sont impliquées dans les processus de récompense. C'est le cas notamment du noyau sous-thalamique (Baunez *et al*, 2005; Breysse *et al*, 2015; Espinosa-Parrilla *et al*, 2015; Hachem-Delaunay *et al*, 2015; Lardeux *et al*, 2013; Pelloux and Baunez, 2013; Pelloux *et al*, 2014; Rouaud *et al*, 2010). Cet aspect ne sera pas développé dans ce document.

de contrôler le mode d'administration et le dosage du médicament utilisé. En usage depuis plus de 40 ans pour le traitement de la dépendance aux opiacés (ex : héroïne), la méthadone est un agoniste des récepteurs opioïdes de type mu. L'usage du patch nicotinique ou de la cigarette électronique entre également dans cette catégorie.

Tableau 4 Quelques exemples de traitements pharmacologiques de l'addiction.
(1) Ces produits n'ont pas l'autorisation de mise sur le marché pour cette indication

Mode d'action	Effets pharmacologiques	Produit/ *Nom pharmaceutique*	Indication
Blocage du système de récompense	Antagoniste opiacé	**Naltrexone** *Revia ®*	Alcool
		Naltrexone *Nalorex ®*	Opiacés
	Agoniste GABA/ Antagoniste NMDA	**Acamprosate** *Aotal ®*	Alcool
Maintien d'une activité minimale du système de récompense	Agoniste opiacé	**Méthadone**	Opiacés
		Buprénorphine *Sulbutex®*	Opiacés
	Agoniste dopaminergique	**Bromocriptine** *Parlodel ®*	Alcool (1)
		Bupropion *Zyban ®*	Tabac
	Agoniste sérotoninergique	**Antidépresseurs ISRS**	Alcool (1)
		Lithium	Alcool (1)
	Agoniste nicotinique	**Nicotine**	Tabac

Une troisième option thérapeutique consiste en une stratégie dite «aversive» qui décourage l'individu dépendant de consommer. C'est le cas notamment pour le disulfirame (Antabuse®, inhibiteur de l'aldéhyde déshydrogénase). Son utilisation concomitante avec l'alcool entraîne la survenue de symptômes désagréables causés par l'accumulation d'acétaldéhyde : flush, tachycardie, céphalée, nausées.

Récemment, de nouvelles molécules ont fait leur apparition. Le baclofène a reçu une recommandation de mise sur le marché temporaire de la part des autorités de santé en France (Agence Nationale de Sécurité du Médicament et des produits de santé, ANSM, 2014) pour le traitement des patients alcoolo-dépendantes. Ce traitement prometteur, à l'origine utilisé comme myorelaxant, est un agoniste du récepteur $GABA_B$. Il est indiqué à la fois pour réduire drastiquement la consommation d'alcool, et pour l'aide au maintien de l'abstinence après sevrage. La varénicline (Champix®), qui est un agoniste partiel des récepteurs nicotiniques (en particulier les

α4β2), est utilisée pour faciliter le sevrage tabagique mais reste controversée due à sa mauvaise tolérance (précipitation de troubles psychiatriques ?).

D'autres stratégies ont également vu le jour tel le «vaccin anti-cocaïne » (Hicks *et al*, 2011). Celui-ci enseigne au système immunitaire à identifier la cocaïne comme un intrus. L'organisme développe alors une immunité naturelle contre la substance, les anticorps produits empêchant la cocaïne d'atteindre le cerveau. Il est à parier que ces vaccins feront partie de la gamme des réponses disponibles pour lutter contre les addictions. Cependant, les espoirs concernant cette approche sont limités étant donné sa faible «vertu thérapeutique» intrinsèque. Pour finir, de nouvelles molécules attrayantes sont à l'études chez l'animal incluant des modulateurs des mécanismes épigénétiques (ex : inhibiteurs des HDAC1, cf. Figure 13) (Kennedy and Harvey, 2015) ou de la fonction glutamatergique (N-acétylcystéine) (Ducret *et al*, 2015; Murray *et al*, 2012).

Si ces traitements sont utiles chez certains sujets, aucun d'entre eux ne peut s'enorgueillir à ce jour d'éliminer complètement les rechutes. Ceci souligne l'importance d'explorer de nouvelles options thérapeutiques pouvant renforcer l'arsenal existant. Les techniques de stimulation cérébrales non invasives sont en ce sens être une piste intéressante à développer (Feil and Zangen, 2010).

b) Les techniques de stimulation cérébrale non invasives : de nouveaux outils thérapeutiques ?

Ces techniques peuvent être utilisées à des fins de recherches fondamentales ou thérapeutiques. La stimulation non invasive de régions cérébrales peut être obtenue par:

- La stimulation magnétique transcrânienne répétée (rTMS) qui induit des stimulations/interruptions transitoires de l'activité des neurones de régions corticales spécifiques.

- La stimulation transcrânienne par courant continu (tDCS) qui permet de moduler le potentiel de repos de la membrane des neurones et d'altérer ainsi leur excitabilité (Stagg *et al*, 2011).

Bien que des études récentes et enthousiasmantes révèlent que la tDCS diminue l'envie impérieuse de consommer de l'alcool et du tabac (den Uyl *et al*,

2015; Feil *et al*, 2010), son mode d'action sur les comportements associés à l'addiction reste incompris. Je traiterai dans le Chapitre 4 de la validation d'un modèle animal de tDCS qui nous permettra d'investiguer ses mécanismes d'actions à l'origine de son impact bénéfique sur les comportements associés à l'addiction.

C) CHAPITRE 1:

Vulnérabilité à l'alcool chez le rat adolescent et adulte : Impact du stress prénatal (Thématique de la thèse)

C-1) Principaux résultats obtenus

a) Résumé des résultats

Identifier les facteurs pouvant prédire et/ou influencer la consommation excessive d'alcool constitue une étape importante dans la compréhension des origines de l'alcoolo-dépendance. Parmi ces facteurs, l'exposition au stress augmente la consommation d'alcool et favorise la rechute chez le sujet sevré. L'objectif de ce travail de thèse était de déterminer si l'exposition à un stress lors d'une période très précoce du développement : la période intra-utérine, peut moduler durablement (à l'âge adulte) la vulnérabilité à l'alcool. Pour ce faire, nous avons utilisé le modèle du stress prénatal chez le rat (stress de contention de la mère gestante lors des 10 derniers jours de la gestation). Deux aspects furent étudiés chez les rats stressés *in utero*: 1) la sensibilité de ces animaux aux effets hormonaux, neurobiologiques et comportementaux induits par une administration d'alcool ; 2) leur propension à consommer la substance.

1) Quel est l'impact du stress prénatal sur la sensibilité aux effets de l'éthanol à l'âge adulte?

Nous avons démontré que les animaux stressés *in utero* sont moins sensibles aux effets d'une injection d'alcool lors de l'adolescence au niveau hormonal (hypo-activation de l'axe corticotrope) (Van Waes *et al*, 2006) et neurobiologique (hypo-activation des défenses anti-oxydantes dans l'hippocampe) (Enache *et al*, 2008). En étudiant les effets de l'alcool sur la mémoire, nous avons mis en évidence qu'une alcoolisation chronique (alcool 10% comme seul accès à la boisson, voie orale, 9

mois) engendrait des altérations partiels de la reconnaissance spatiale chez les rats témoins. Au contraire, de façon surprenante, ce même traitement abolissait les déficits mnésiques constitutifs des animaux stressés prénatalement. Les animaux, qu'ils soient stressés ou témoins, ne présentaient pas de dommage oxydatif dans l'hippocampe après l'alcoolisation chronique. En revanche, dans cette structure cérébrale, les récepteurs métabotropiques au glutamate du type I et II, impliqués dans les processus mnésiques, étaient affectés différemment par l'éthanol chez les rats exposés au stress prénatal (témoins : diminution des quantités de mGluR5 et mGlu2/3 par l'alcool; stressés : augmentation des mGlu1). Ces variations pourraient en partie expliquer les effets opposés de l'alcool sur la mémoire chez les animaux témoins et stressés prénatalement (Van Waes *et al*, 2009).

2) Quel est l'impact du stress prénatal sur l'appétence pour l'alcool ?

Nous avons évalué chez les rats stressés *in utero* la préférence pour l'alcool lors de l'adolescence et à l'âge adulte (libre choix entre de l'eau et différentes doses d'alcool). Nos résultats montrent que le stress prénatal n'altère pas la préférence pour l'alcool chez le rat mâle. Cependant, un traitement chronique à l'alcool augmente de manière dose dépendante les quantités de Delta-FosB (un facteur de transcription impliqué dans la vulnérabilité à la consommation de drogues) dans le noyau accumbens, sélectivement chez les rats stressés *in utero* (Van Waes *et al*, 2011b). Finalement, en faisant varier les conditions expérimentales, nous avons mis en évidence une augmentation de la préférence pour l'alcool chez les animaux stressés *in utero* lorsque ceux-ci étaient soumis de nouveau à un stress à l'âge adulte (choc électrique léger au niveau des pattes) (Darnaudery *et al*, 2007).

L'ensemble de ces résultats indique que le stress *in utero*, en interaction avec d'autres facteurs expérimentaux, modifie durablement chez le rat la sensibilité aux effets produits par l'alcool, ainsi que la propension à consommer cette substance. Nos données complètent les travaux antérieurs démontrant qu'une exposition à un stress prénatal augmente la vulnérabilité à certaines substances d'abus (*i.e.* psychostimulants et opiacés). De surcroît, elles soulignent l'importance de prendre en compte l'histoire de l'individu, même très précoce, pour appréhender la genèse des conduites addictives. *Thèse récompensée par le prix Gérard Vachonfrance de l'Institut de REcherche scientifique sur les Boissons (IREB), 2010. http://www.ireb.com/node/1061. Synthèse des résultats page 71.*

Synthèse : démarche expérimentale et principaux résultats

NEUROTOXICITE DE L'ETHANOL

Résultat principal
Résultats secondaires

ARTICLE 1

Stress *in utero* → Axe corticotrope *Adolescence* ← Ethanol

Observe-t-on une hyper-activation de l'axe corticotrope en réponse à une injection d'éthanol chez les rats stressés adolescents?

NON: Hypo-activation à tous les niveaux de l'axe

↓ constitutive des ARNm des MR et GR dans l'hippocampe des animaux stressés;
↓ ARNm des MR dans le gyrus dentatus après l'alcoolisation chez les animaux témoins;

ARTICLE 2

Quel est le statut oxydatif dans l'hippocampe des animaux adolescents en réponse à une injection d'éthanol?

↓ des réponses anti-oxydantes chez les rats stressés

↓ constitutive de l'activité de la catalase chez les rats stressés;
Pas de peroxydation lipidique;

ARTICLE 3

Quel est l'impact d'un traitement chronique de 7 mois sur les performances mnésiques des rats stressés mâles?

↓ performances chez témoins mais abolition des déficits mnésiques constitutifs des animaux stressés

Pas de peroxydation lipidique dans l'hippocampe;
↓ constitutive des récepteurs mGlu du type I et II dans l'hippocampe des animaux stressés;
Variation différentielle des récepteurs mGlu du type I et II dans l'hippocampe chez les rats témoins et stressés après alcoolisation;

ARTICLE 4

Le stress prénatal augmente-t-il la consommation d'éthanol lors de l'adolescence chez les rats mâles?

NON

Préférence modulée par la concentration d'éthanol;

Le stress prénatal augmente-t-il la consommation d'éthanol chez l'adulte chroniquement exposé à l'éthanol?

NON

Pas de dépendance à l'éthanol;
Augmentation dose dépendante du ΔFosB dans noyaux accumbens chez les rats stressés après alcoolisation;

ARTICLE 5

Impact de l'environnement social et du stress prénatal sur la consommation d'éthanol des rats femelles?

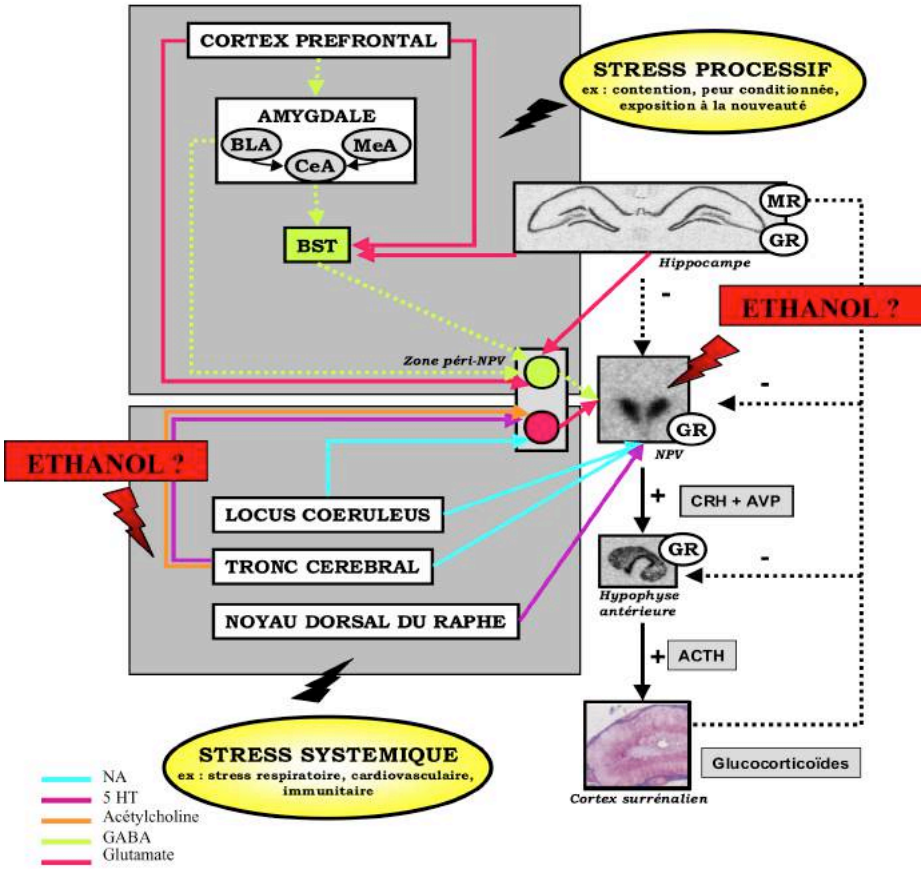
Interaction conditions d'élevage x SP

↑ préférence pour l'éthanol chez les rates adolescentes stressées élevées en groupe;
↑ réactivité locomotrice à la nouveauté chez les rates stressées isolées;

PROPENSION A CONSOMMER L'ETHANOL

b) Focus 1: L'activation de l'axe corticotrope par l'alcool lors de l'adolescence est plus faible chez les animaux stressés in utero

Une diminution de la réponse de l'axe corticotrope en réponse à l'alcool constitue un indice prédictif chez l'Homme d'un risque accru de développer des conduites d'abus (Schuckit *et al*, 1987; Schuckit *et al*, 1988; Schuckit and Smith, 2000). Puisque d'une part l'axe corticotrope est activé par l'alcool (Figure 15) (Rivier, 1996) et que d'autre part cet axe est altéré durablement par le stress *in utero* (Maccari *et al*, 2014), nous avons comparé l'activation de l'axe corticotrope en réponse à une administration d'alcool chez des d'animaux adolescents témoins et stressés prénatalement (stress de contention de la mère sous lumière vive 3 x 45 minutes par jours lors des 10 derniers jours de la gestation).



BLA : noyau basolatéral de l'amygdale, CeA : noyau central de l'amygdale, MeA : noyau médial de l'amygdale, BST : noyau du lit de la strie terminale, NPV : noyau paraventriculaire de l'hypothalamus, GR : récepteurs aux glucocorticoïdes, MR : récepteurs aux minéralocorticoïdes, CRH : corticolibérine, AVP : arginine vasopressine, ACTH : corticotropine.

Figure 15 Représentation schématique du contrôle de l'axe corticotrope par les structures limbiques et lieux putatifs de l'activation de l'axe par l'alcool. D'après (Herman and Cullinan, 1997; Rivier, 1996).

Nous avons injecté l'alcool par voie intrapéritonéale (1,5 g/kg corporel) ou un volume équivalent de liquide physiologique (NaCl 0,9 %) au début de l'adolescence (J28) (Spear, 2014). Nous avons alors examiné la cinétique des taux sanguins d'ACTH et de corticostérone par dosage radioimmunologique (0, 30, 60 et 240 minutes post-injection). L'impact de l'alcool sur l'expression génique (ARN messagers, ARNm) de plusieurs acteurs centraux de l'axe corticotope (récepteurs aux glucocorticoïdes [MR] et aux minéralocorticoïdes [GR] hippocampiques, la corticotropin-releasing hormone [CRH] hypothalamique, la pro-opiomélanocortine [POMC] hypophysaire) a été estimé par hybridation *in situ* (60 et 240 minutes post-injection). En parallèle, nous avons évalué la cinétique d'élimination des taux sanguins d'éthanol par chromatographie en phase gazeuse (0, 30, 60 et 240 minutes post-injection) (Figure 16).

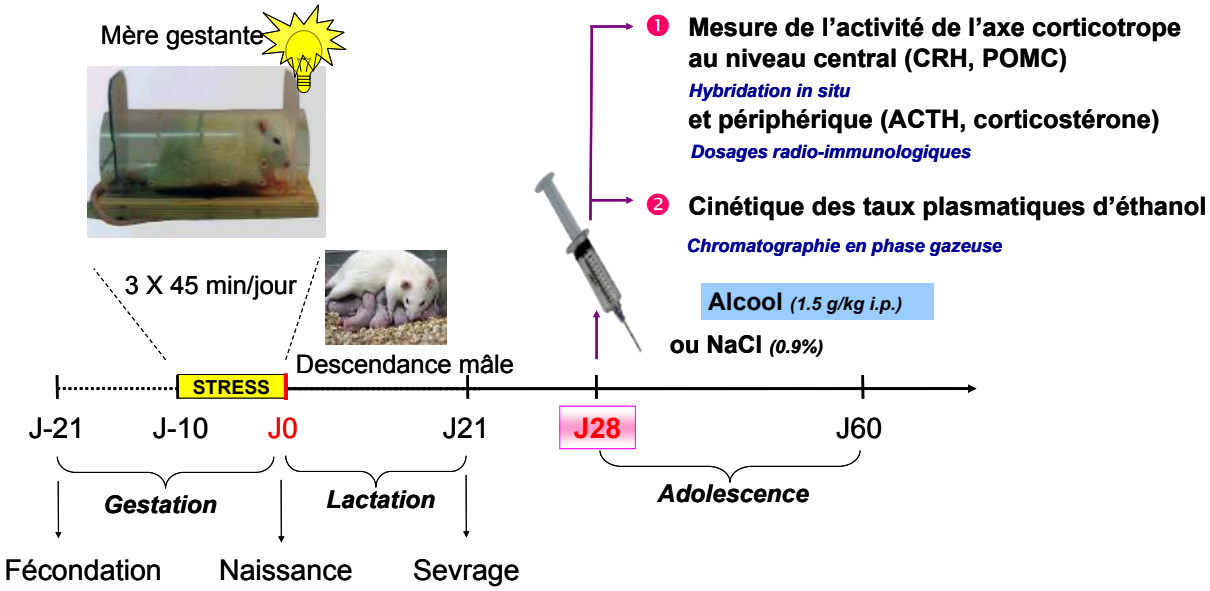


Figure 16 Protocole expérimental utilisé pour étudier l'activation de l'axe corticotrope par l'alcool.

L'alcool induisait une activation robuste de l'axe corticotrope chez tous les sujets. Cependant, comparés aux témoins, les animaux stressés lors de la phase précoce de leur développement présentaient une augmentation des taux de corticostérone et d'ACTH de moindre ampleur suite à l'injection d'alcool (Figure 17). De plus, l'alcool augmentait les taux d'ARNm codant la CRH (dans le noyau

paraventriculaire de l'hypothalamus) et la POMC (dans l'anté-hypophyse) chez les témoins alors que ces taux n'étaient pas affectés chez les animaux stressés.

Nous avons également montré que les animaux stressés n'étaient pas sensibles, contrairement aux animaux témoins, à la diminution par l'éthanol des ARNm codant les MR dans le gyrus denté.

L'ensemble de ces résultats suggère que le stress prénatal diminue la réponse endocrinienne à l'alcool lors de l'adolescence sans altérer sa cinétique d'élimination. Ces données contrastent avec l'hyperactivité de l'axe corticotrope classiquement reportée chez ces animaux lors d'une exposition à un stress. Ils indiquent donc que le stress prénatal peut induire des effets opposés sur l'activité de l'axe corticotropre selon le type de stimulation considérée (stress processif, ex : contention *versus* stress systémique, ex : alcool).

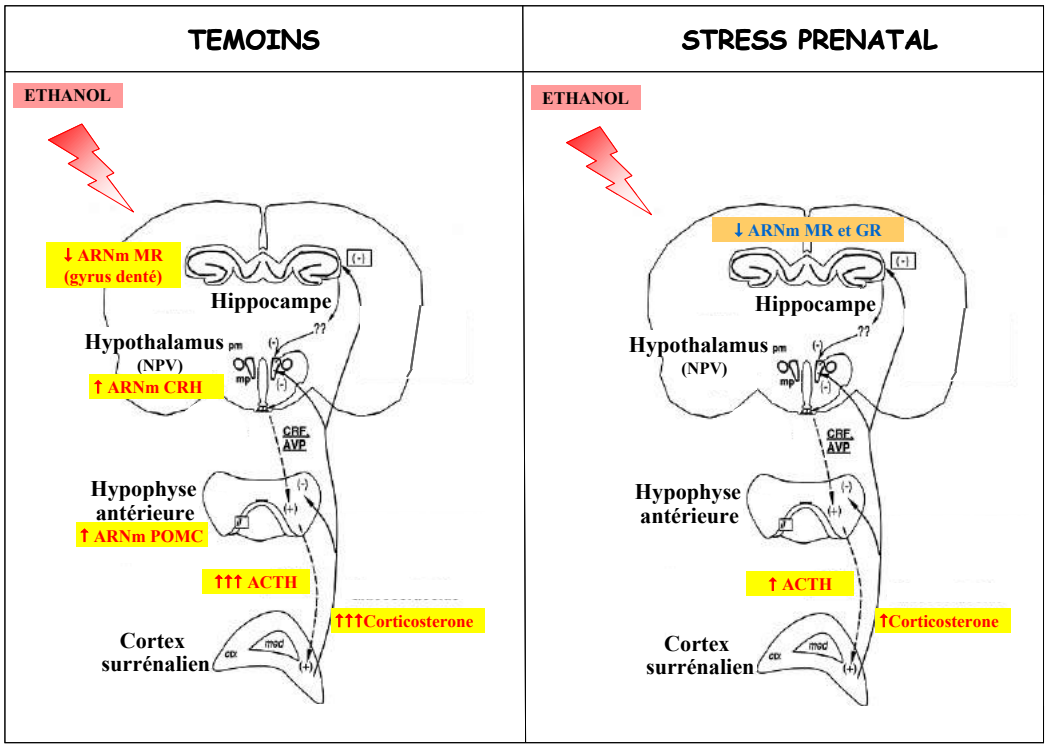


Figure 17 Activation de l'axe corticotrope suite à l'injection d'éthanol (1.5 g/kg i.p.) lors de l'adolescence (J28) chez les rats témoins ou stressés pendant la période prénatale. Les animaux stressés présentent à tous les niveaux de l'axe une activation endocrinienne de moindre ampleur comparée à celle des animaux témoins. La cinétique d'élimination de l'éthanol n'était pas altérée chez les animaux stressés.

Il restait à déterminer si cette réponse atténuée de l'axe est associée à une augmentation de la consommation spontanée d'alcool.

c) Focus 2: L'alcool augmente les concentrations de Delta-FosB dans le noyau accumbens sélectivement chez les animaux stressés in utero

Des études antérieures menées chez le rat ont montré que des stress précoces post-nataux peuvent moduler durablement l'appétence pour l'alcool (Fahlke *et al*, 1997; Huot *et al*, 2001; Lancaster, 1998). Par ailleurs, les animaux stressés *in utero* présentent des altérations du système dopaminergique mésolimbique (Adrover *et al*, 2007; Barros *et al*, 2004; Berger *et al*, 2002; Henry *et al*, 1995) et une augmentation de la consommation spontanée de certaines drogues, telles que l'amphétamine (Deminiere *et al*, 1992) ou la cocaïne (Kippin *et al*, 2015; Kippin *et al*, 2008). Ces données, ainsi que l'observation d'une hypo-réponse de l'axe corticotrope en réponse à l'alcool chez les animaux stressés, nous ont conduit à évaluer l'influence du stress prénatal sur l'appétence spontanée pour l'éthanol.

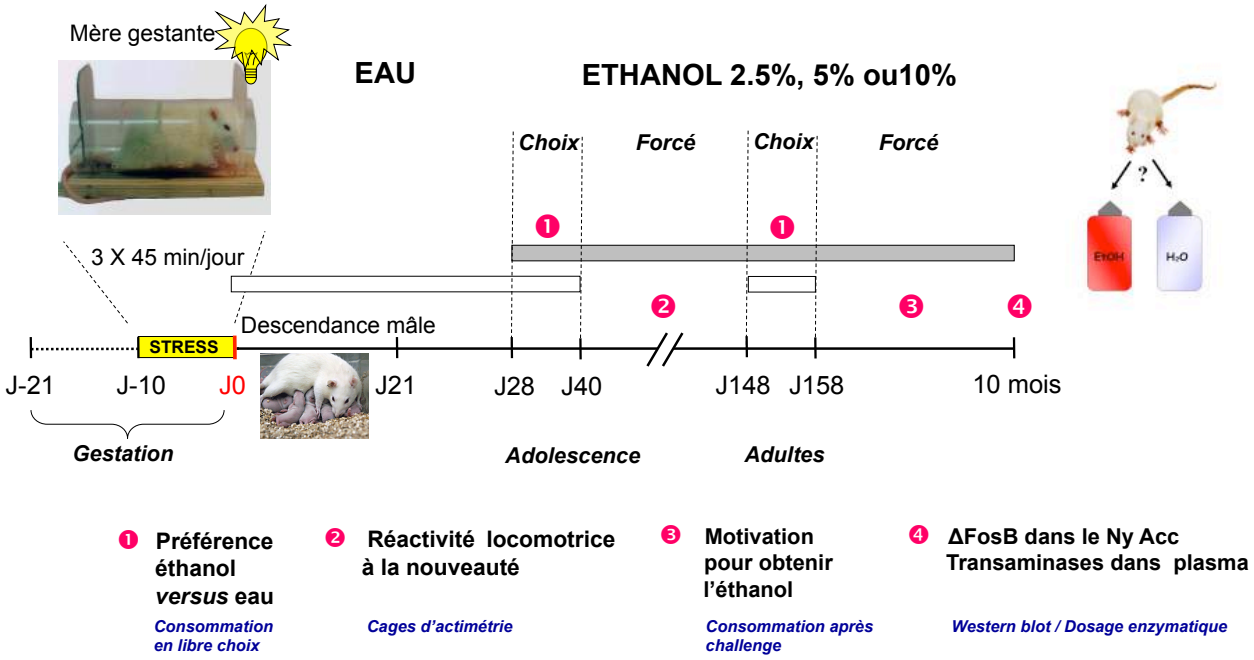


Figure 18 Protocole expérimental utilisé pour évaluer la consommation d'alcool.

Nous avons évalué la préférence pour une solution d'éthanol (2,5 ; 5 ou 10 %) dans une population d'animaux naïfs (*i.e.* jamais exposés à l'alcool) lors de l'adolescence (J28-J38), ainsi que chez des animaux adultes (J148-J158) préalablement exposés à l'alcool pendant plusieurs mois (de manière non contingente, Figure 18). La préférence pour l'éthanol était évaluée en condition de

libre choix où chaque rat avait la possibilité de choisir entre deux biberons. Le premier contenait de l'eau, le second de l'éthanol (2,5% ; 5% ou 10%).

De nombreuses données suggèrent que le Delta-FosB, forme tronquée du facteur de transcription FosB, contribue aux changements à long terme de la plasticité des systèmes dopaminergiques lors de la prise répétée de substances d'abus (Nestler *et al*, 2001) (cf. Introduction). L'administration répétée de drogues, telles que la cocaïne (Moratalla *et al*, 1996a), l'amphétamine (Ehrlich *et al*, 2002), la morphine (Nye and Nestler, 1996) ou la nicotine (Pich *et al*, 1997) induit l'accumulation durable de Delta-FosB dans le noyau accumbens (grande stabilité de ce dernier). L'existence d'une telle accumulation après une alcoolisation chronique restait en revanche peu documentée (McClung *et al*, 2004). Dans ce cadre, en parallèle des mesures de consommation d'éthanol, nous avons étudié l'effet de l'exposition chronique à l'alcool sur les taux de Delta-FosB dans le noyau accumbens des rats stressés et témoins.

Il ressort de cette étude que le stress prénatal ne modulait pas la préférence pour l'éthanol. Nonobstant l'absence de résultat concernant la consommation d'alcool, le facteur de transcription Delta-FosB était différemment modulé par l'éthanol chez les animaux témoins et stressés. En absence d'éthanol, les animaux stressés et témoins présentaient des quantités de Delta-FosB similaires. En revanche, le traitement chronique à l'éthanol (9 mois) augmentait de manière dose dépendante les quantités de Delta-FosB dans le noyau accumbens des animaux stressés alors même que ce traitement n'avait aucun effet significatif chez les animaux témoins (Figure 19). La dissociation entre nos résultats neurobiologiques (Delta-FosB) et comportementaux (consommation) suggère qu'il serait nécessaire d'évaluer plus finement l'appétence pour l'éthanol, en particulier, en modulant sa palatabilité ou en situation de stress.

A la suite de cette étude, nous avons mené des investigations complémentaires et avons démontré que les animaux stressés *in utero* consommaient plus d'alcool que des animaux témoins lorsqu'ils étaient soumis à nouveau à un stress à l'âge adulte (ex : choc électrique léger) (Darnaudery *et al*, 2007; Van Waes *et al*, 2011a).

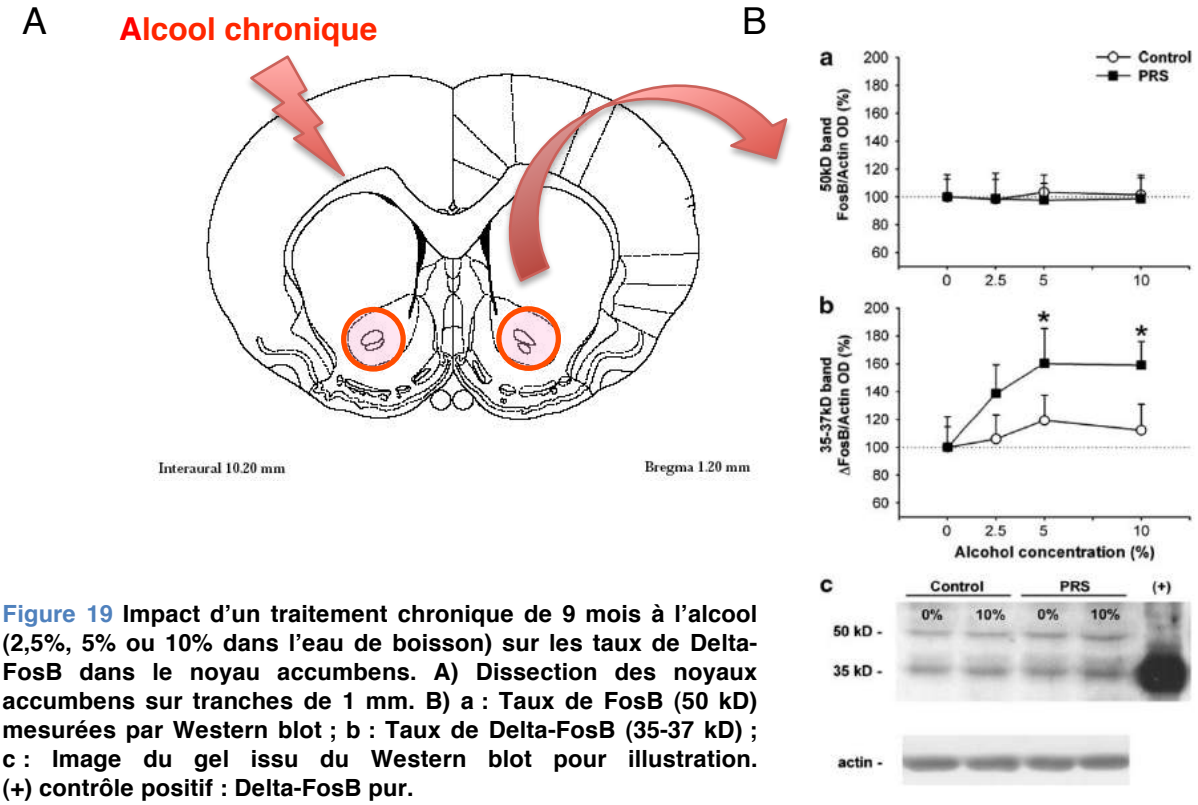


Figure 19 **Impact d'un traitement chronique de 9 mois à l'alcool (2,5%, 5% ou 10% dans l'eau de boisson) sur les taux de Delta-FosB dans le noyau accumbens. A) Dissection des noyaux accumbens sur tranches de 1 mm. B) a : Taux de FosB (50 kD) mesurées par Western blot ; b : Taux de Delta-FosB (35-37 kD) ; c : Image du gel issu du Western blot pour illustration. (+) contrôle positif : Delta-FosB pur.**

C-2) Publications résultant de ces travaux

- **Publications avec comité de lecture :**

1) **Van Waes V.**, Darnaudéry M., Marrocco J., Gruber S., Talavera E., Mairesse J., Van Camp G., Casolla B., Nicoletti F., Mathe A., Maccari S., and Morley-Fletcher S.
Impact of early life stress on alcohol consumption and on the short- and long-term responses to alcohol in adolescent female rats
Behavioural Brain Research Vol. 221 (1), p43-49, 2011

2) **Van Waes V.**, Enache M., Berton O., Vinner E., Lhermitte M., Maccari S., and Darnaudéry M.
Effect of prenatal stress on alcohol preference and sensitivity to chronic alcohol exposure in male rats
Psychopharmacology Vol. 214 (1), p197-208, 2011.

3) **Van Waes V.**, Enache M., Zuenna A.R., Mairesse J., Nicoletti F., Vinner E., Lhermitte M., Maccari S., and Darnaudéry M.
Ethanol attenuates spatial memory deficits and increases mGlu1a receptor expression in the hippocampus of rats exposed to prenatal stress
Alcoholism: Clinical and experimental research Vol. 33 (8), p1346-1354, 2009.

4) **Van Waes V.***, Enache M.*, Vinner E., Lhermitte M., Maccari S., and Darnaudéry M.
Impact of an acute exposure to ethanol on the oxidative stress status in the hippocampus of prenatally stressed adolescent male rats
Brain Research Vol. 1191C, p55-62, 2007. * co-first authorship.

5) **Van Waes V.**, Enache M., Dutriez I., Lesage J., Morley-Fletcher S., Vinner E., Lhermitte M., Vieau D., Maccari S., and Darnaudéry M.
Hyporesponse of the hypothalamic-pituitary-adrenocortical axis after an ethanol challenge in prenatally stressed adolescent male rats
European Journal of Neuroscience Vol. 24 (4), p1193-1200, 2006.

- **Ouvrage :**

1) **Van Waes V.**
Vulnérabilité à l'alcool chez le rat: Impact du stress prénatal. Comment un stress précoce peut avoir des répercussions tout au long de la vie.
Éditions Universitaires Européennes, 2011

- **Autres publications :**

1) Darnaudéry M., **Van Waes V.**, Enache M., Zuena A.R., Mairesse J., Nicoletti F., Vinner E., Lhermitte M., and Maccari S.
Stress prénatal et mémoire: effets paradoxaux de la consommation chronique d'alcool chez le rat
Cahier de l'IREB n°19, p63-69, 2009.

2) **Van Waes V.**, Enache M., Vinner E., Lhermitte M., Maccari S., and Darnaudéry M.
Impact du stress prénatal sur la vulnérabilité à l'éthanol chez le rat
Cahier de l'IREB n°18, p15-21, 2007.

3) Darnaudéry M., **Van Waes V.**, Enache M., Morley Fletcher S., Dutriez-Casteloot I., Lesage J., Vinner E., Lhermitte M., and Maccari S.
Conséquences d'un stress prénatal sur l'anxiété et l'activité de l'axe corticotrope après administration aiguë d'alcool chez le rat adolescent
Cahier de l'IREB n°17, p35-41, 2005.

D) CHAPITRE 2 :

Ritaline® + Prozac®: une combinaison addictogène ? (Thématique principale du Post-doctorat)

D-1) Principaux résultats obtenus

a) Résumé des résultats

Lors de mon post-doctorat (Chicago Medical School), je me suis intéressé aux effets comportementaux et moléculaires (modèle animal : rat) induits par la co-administration de méthylphénidate (Ritaline®, psychostimulant prescrit à grande échelle pour le traitement de l'hyperactivité avec trouble de l'attention) et la fluoxétine (Prozac®, antidépresseur utilisé en première intention, inhibiteur sélectif de la recapture de la sérotonine) en me focalisant sur leur impact sur les circuits cortico-striataux.

En quoi l'usage combiné de ces deux molécules pourrait-il constituer un danger ?

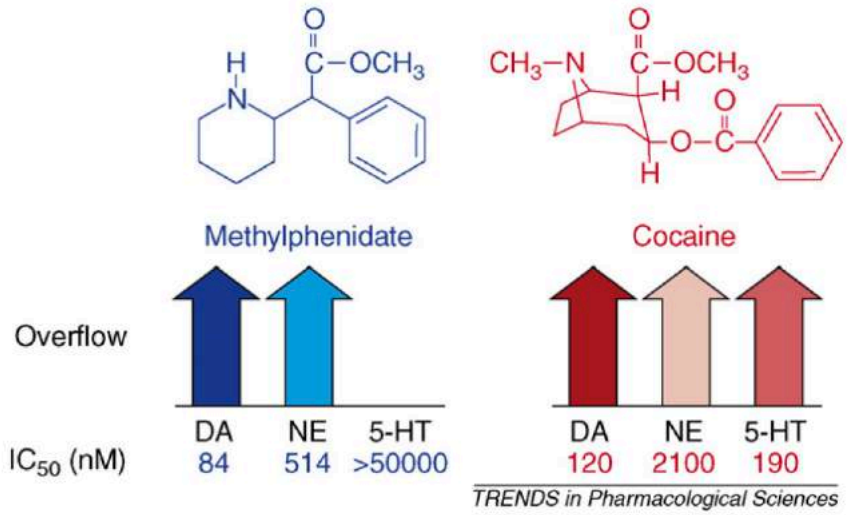
Le méthylphénidate est utilisé de façon croissante dans des populations infantiles et chez l'adolescent pour réduire les symptômes associés à l'hyperactivité. Environ 8% des enfants et adolescents et 4% des adultes sont diagnostiqués avec des troubles du déficit de l'attention associés à l'hyperactivité (Kollins, 2008). Trois millions d'enfants et adolescents, aux Etats-Unis uniquement, sont traités avec ce type de psychostimulants (Swanson and Volkow, 2008). Bien que se révélant très efficace contre les symptômes de la pathologie, son emploi reste encore aujourd'hui controversé et à l'origine de débats houleux. Des questions se posent notamment sur les éventuelles conséquences néfastes de l'exposition chronique aux psychostimulants lors de périodes critiques du développement cérébral (enfance, adolescence) (Krishnan *et al*, 2015; Steiner and Van Waes, 2013; Steiner *et al*, 2014). Par ailleurs, le méthylphénidate est de plus en plus utilisé de façon détournée comme améliorateur cognitif (Greely *et al*, 2008), ou sniffé/injecté lors d'usages récréatifs (White *et al*, 2006). Les doses administrées dans ce cas ne sont pas

contrôlées et peuvent donc induire des effets plus importants/différents de ceux obtenus avec des doses thérapeutiques.

Quel est le mode d'action du méthylphénidate ?

De façon similaire à la cocaïne, il inhibe la recapture de la dopamine (et de la noradrénaline, Figure 20A). Contrairement à la cocaïne, cependant, il ne bloque pas la recapture de la sérotonine (la cocaïne inhibe la recapture de la dopamine, de la noradrénaline ET de la sérotonine, Figure 20A). Cette différence (absence d'effet sur la fonction sérotoninergique) expliquerait en partie pourquoi le méthylphénidate mime certains - mais pas tous - les effets moléculaires et comportementaux de la cocaïne ((Yano *et al*, 2007), Figure 20B). Ce serait également la raison du plus faible (ou de l'absence de) potentiel addictogène du méthylphénidate comparé à la cocaïne (Steiner *et al*, 2013; Yano *et al*, 2007).

A



B

	Molecule[a]	Methylphenidate Basal/challenge[b]	Effect[c]	Refs	Cocaine Basal/challenge[b]	Effect[c]	Refs
Acute[d]	*c-fos*	Challenge	++++	[17,27,28,89]	Challenge	++++	[18,35,36,45]
	zif268	Challenge	++++	[16,27,38]	Challenge	++++	[27,45]
	homer 1a	Challenge	++++	[16,38]	Challenge	++++	[90],[f]
	Substance P	Challenge	++++	[17]	Challenge	++++	[27,36,41]
	Dynorphin	Challenge	(+)	[17]	Challenge	++	[18,36,41]
	Enkephalin	Challenge	0	[17]	Challenge	+	[36,41]
Repeated[e]	*c-fos*	Challenge	++	[27,28,89]	Challenge	++	[18,35,36,45]
	zif268	Challenge	++	[27,38]	Challenge	++	[45]
	homer 1a	Challenge	+++(+)	[38]	Challenge	++	[f]
	Substance P	Challenge	++	[27]	Challenge	++	[36]
	Dynorphin	Basal	0, +	[12,27]	Basal	++++	[18,36,40,45,46]
	Enkephalin	Basal	0	[27]	Basal	++	[36,46]

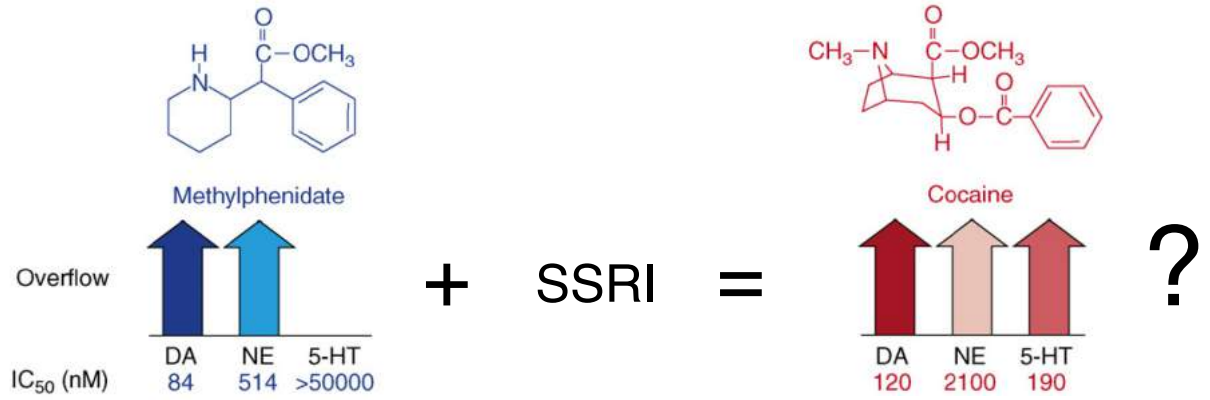
Figure 20 A) Méthylphénidate *versus* cocaïne : effets sur la transmission monoaminergique. Le méthylphénidate et la cocaïne augmentent les concentrations extracellulaires de dopamine (DA) et de noradrénaline (NE) mais, contrairement à la cocaïne, le méthylphénidate n'affecte pas les quantités extracellulaires de sérotonine (5-HT). B) Effets sur la régulation génique dans le striatum. Bien que similaire, les effets du méthylphénidate sur la régulation génique diffèrent de ceux de la cocaïne (ex : cadres en rouge, dynorphine et enképhaline) (Yano and Steiner, 2007).

Les effets combinés du méthylphénidate + fluoxétine aux niveaux moléculaire et comportemental n'ont jamais été évalués. Le méthylphénidate est pourtant souvent utilisé conjointement avec des inhibiteurs sélectifs de la recapture de la sérotonine (selective serotonin reuptake inhibitors, SSRIs) tels que la fluoxétine (Prozac®).

En effet :

1) les troubles de l'attention sont co-morbides avec d'autres psychopathologies tels que l'anxiété et la dépression (Bhatara *et al*, 2004; Kollins, 2008; Safer *et al*, 2003).
2) le méthylphénidate est combiné aux SSRIs pour accélérer les effets des antidépresseurs (Lavretsky *et al*, 2003) et pour soulager les dysfonctions sexuelles associées à l'utilisation de SSRIs (Csoka *et al*, 2008).
3) un usage du méthylphénidate ayant pour finalité d'améliorer les performances cognitives chez des patients déprimés déjà traités avec des SSRIs voit son nombre d'occurrences se décupler dû à l'intensification spectaculaire de la prise détournée de méthylphénidate comme améliorateur cognitif (Kollins, 2008; Svetlov *et al*, 2007; Swanson *et al*, 2008; Wilens *et al*, 2008).

Le but de ce travail était de caractériser l'effet combiné de ces deux drogues et d'évaluer l'innocuité ou la dangerosité de ce cocktail de molécules souvent prescrit durant l'enfance/l'adolescence. La question centrale est schématisée dans la Figure 21. Nous nous sommes intéressés aux effets comportementaux de ce traitement ainsi qu'à l'induction de gènes dans les circuits cortico-striataux.



Méthylphénidate (dopamine) + Fluoxétine (sérotonine) = Cocaïne (dopamine + sérotonine)?
- *Régulation génique dans les circuits cortico-striataux impliquées dans l'addiction*
- *Effets comportementaux*

Figure 21 Hypothèse de travail simplifiée.

L'ensemble de nos résultats révèle que l'usage de ces deux composés provoque des effets moléculaires similaires à ceux de la cocaïne. La fluoxétine exacerbait dans les circuits cortico-striataux l'induction par le méthylphénidate de facteurs de transcription impliqués dans la plasticité cérébrale (prise unique ; c-Fos, Zif268) (Steiner *et al*, 2010; Van Waes *et al*, 2010). Les effets d'un traitement chronique avec le méthylphénidate sur la régulation génique (Zif268, Homer1a) étaient également amplifiés par la fluoxétine (Van Waes *et al*, 2014). Cette potentialisation était par ailleurs observée en étudiant d'autres paramètres tels que l'induction de peptides opioïdes dans le striatum après administration aiguë (dynorphine, Dyn ; substance P, SP) (Van Waes *et al*, 2012b) ou répétée (Dyn ; enképhaline, Enk) de ces psychotropes (Beverley *et al*, 2014). Enfin, nous avons montré que cette potentialisation pourrait être sous-tendue, au moins en partie, par l'activation des récepteurs 5-HT1B (Van Waes *et al*, 2015).

Au niveau comportemental, des données non publiées indiquent que l'administration de méthylphénidate + fluoxétine entraîne un comportement de recherche de cocaïne dans un modèle de rechute (auto-administration de cocaïne suivi d'une période d'extinction et d'une réexposition à la drogue), soulevant un potentiel problème de santé publique (cf. Focus 2).

Je poursuis actuellement ce travail en collaboration avec le Pr Heinz Steiner, en me rendant chaque été dans son laboratoire (depuis 2011), comme peut l'attester la liste de publications issues de ce projet commun.

b) Focus 1: La fluoxétine exacerbe la régulation génique induite par le méthylphénidate dans le striatum

Nous avons traité des rats adolescents avec une injection intrapéritonéale de liquide physiologique (vehicle, V), méthylphénidate (2 ou 5 mg/kg, MP2 et MP5), fluoxétine (5 mg/kg, FLX) ou de la combinaison méthylphénidate + fluoxétine. Nous avons sacrifié les animaux 40 minutes après l'injection, récupéré les cerveaux et quantifié par hybridation *in situ* les niveaux d'ARNm codant pour c-Fos et Zif268 dans 22 régions corticales et 23 secteurs du striatum principalement définis par leurs afférences corticales (Willuhn *et al*, 2003) et ceci, sur 4 niveaux de coupes coronales

(frontal, approximativement +2,7 mm par rapport au bregma (Paxinos and Watson, 1998); rostral, +1,6 ; middle, +0,4 ; et caudal -0,8) (Figure 22).

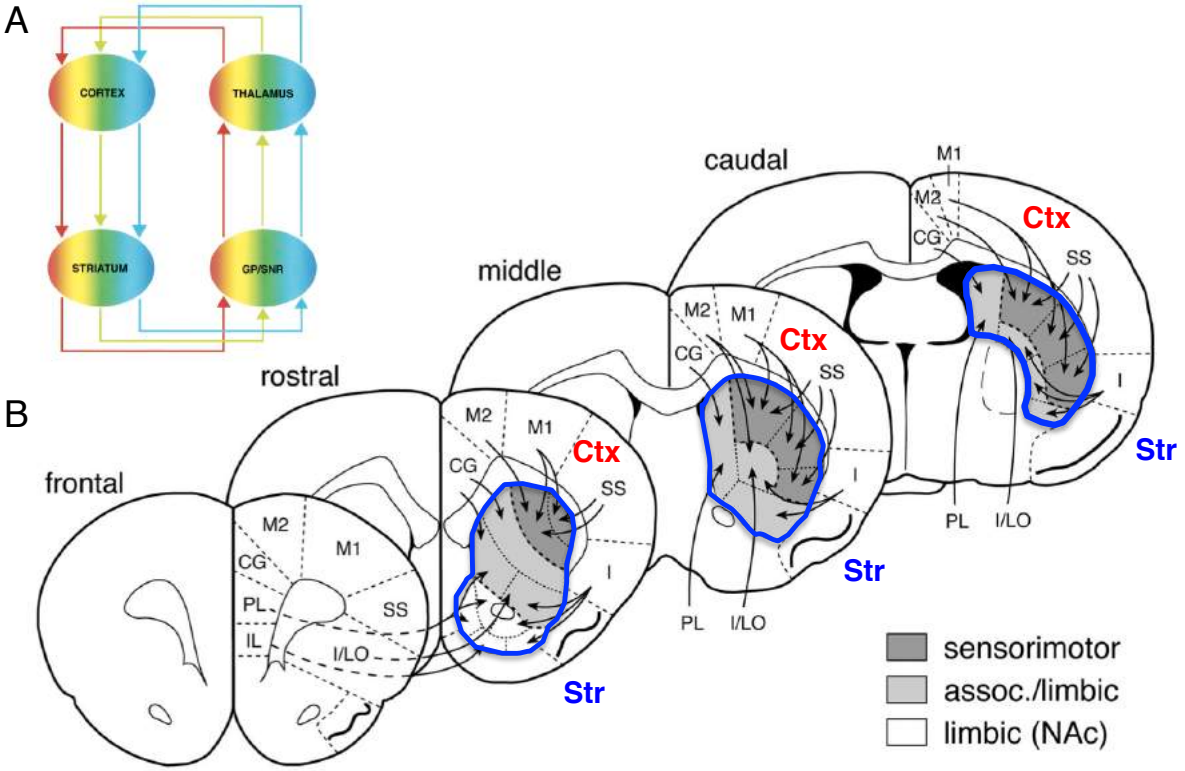


Figure 22 A) Schéma simplifié illustrant les boucles anatomiques parallèles (projections topographiques) des circuits cortico - ganglions de la base - thalamo - corticaux (Haber and McFarland, 2001). B) Les niveaux d'ARNm étaient mesurés dans 22 régions corticales : cingulate, medial agranular, motor, somatosensory and insular cortex sur les niveaux « frontal » à « caudal », et : infralimbic, prelimbic et insular/lateral orbital cortex sur le niveau « frontal ». L'expression génique dans le striatum était déterminée sur les niveaux « rostral » à « caudal » dans 23 secteurs du striatum définis par leurs principales afférences corticales (Willuhn *et al*, 2003). Dix-huit représentaient le caudate/putamen (medial, dorsomedial, dorsal, dorsolateral, ventrolateral, ventral, central, dorsal central, et ventral central) et 5 le noyau accumbens (medial core, lateral core, medial shell, ventral shell et lateral shell) (Van Waes *et al*, 2010).

Nos résultats indiquent que la fluoxétine exacerbe la régulation génique induite par le méthylphénidate dans le striatum (facteur de transcription Zif268, Figure 23). Cette potentialisation commençait à apparaître pour la dose de 2 mg/kg de méthylphénidate (données non montrées) et était robuste pour la dose 5 mg/kg (Figure 23). Les secteurs du striatum les plus touchés étaient les secteurs sensorimoteurs et les secteurs associatifs. Le noyau accumbens était également impacté, mais dans une moindre mesure (lateral shell uniquement). Par ailleurs, la distribution de cette potentialisation était similaire pour les gènes précoces Zif268 et c-Fos (Figure 23C).

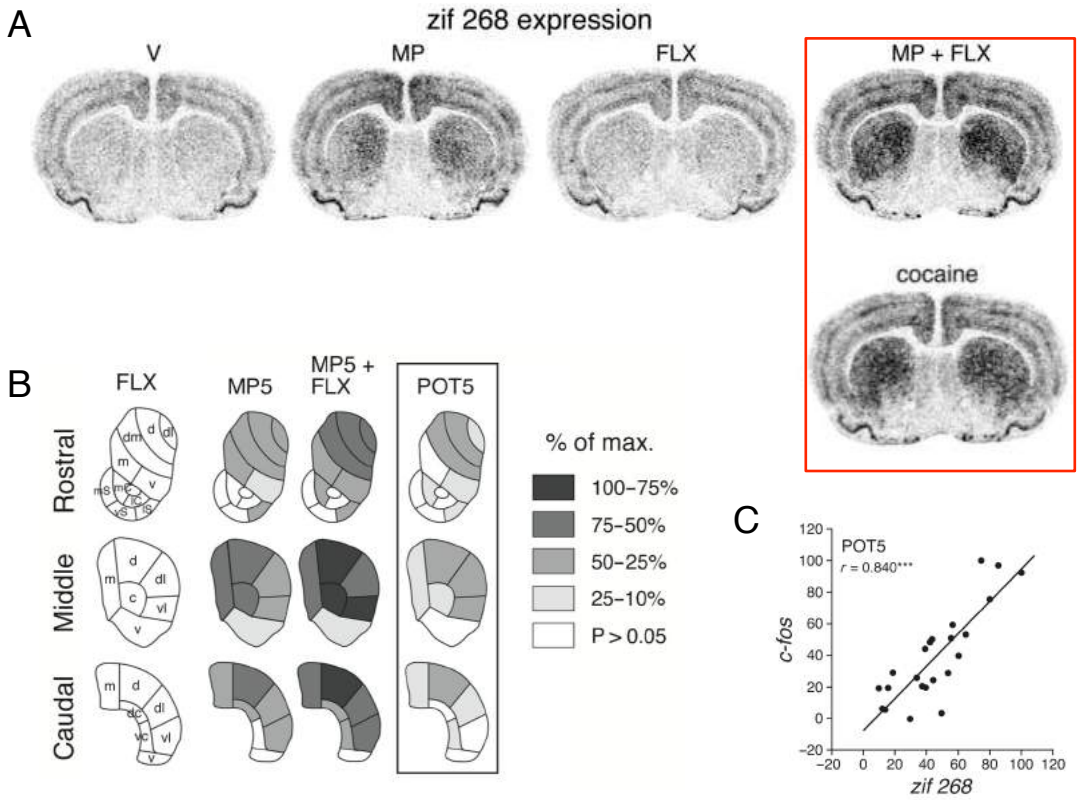
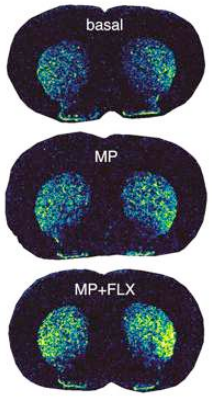


Figure 23 A) Autoradiogrammes (hybridation *in situ*) illustrant l'expression des ARNm codant pour le facteur de transcription Zif268 au niveau du striatum pour un rat ayant reçu une injection de liquide physiologique (V, i.p.), de méthylphénidate (MP, 5 mg/kg) de fluoxétine (FLX, 5mg/kg) ou de la combinaison méthylphénidate + fluoxétine (MP+FLX). La fluoxétine n'a pas d'effet *per se* sur l'expression de Zif268 mais elle potentialise l'effet du méthylphénidate. Pour comparaison, l'expression de Zif268 dans le striatum est montrée en réponse à une injection de cocaïne (25 mg/kg; i.p.). B) Induction de Zif268 dans les 23 secteurs du striatum exprimée en fonction de l'augmentation maximale reportée dans le striatum (100%). Les secteurs grisés sont codés comme indiqué. Les secteurs en blanc sont non significatifs (P>0.05). POT5 : potentialisation pour la dose de méthylphénidate de 5 mg/kg. Correspond à la différence entre le méthylphénidate + fluoxétine et le méthylphénidate seul. C) Corrélation entre la potentialisation observée pour le facteur de transcription Zif268 et celle observée pour c-Fos dans les 23 secteurs du striatum indiquant une régionalisation similaire pour les deux marqueurs d'activité utilisés.

Nous nous sommes également intéressés à l'induction de peptides opioïdes (SP/Dyn → voie directe ; Enk → voie indirecte) après administration aiguë ou chronique de méthylphénidate et fluoxétine. Les résultats obtenus sont synthétisés dans la Figure 24. Ils indiquent qu'en réponse à une administration aiguë, la potentialisation est restreinte à la voie directe (potentialisation de la substance P et de la dynorphine mais pas de l'enképhaline). En revanche, suite à une administration chronique, l'induction de l'enképhaline par le méthylphénidate était également potentialisée lorsque nous ajoutions la fluoxétine indiquant dans ce cas un effet sur les deux voies de sortie du striatum. Le fait que l'induction de la dynorphine soit potentialisée nous questionne sur l'éventuel effet toxicomanogène du mélange MP + FLX comparé au MP seul étant donné la probable implication de la dynorphine dans

le développement des addictions (cf. introduction « B-3) Neurobiologie de l'addiction : effets moléculaires » et Figure 10).

A



B

MP + FLX

Potentialisation?	SP	Dyn	ENK
aiguë	oui	oui	non
chronique	nt	oui	oui
	Voie directe		Voie indirecte

Figure 24 Potentialisation des effets du méthylphénidate par la fluoxétine (peptides opioïdes). A) Autoradiogrammes illustrant l'induction de substance P 90 minutes après une injection de liquide physiologique (basal), de méthylphénidate (MP, 5 mg/kg) ou de méthylphénidate + fluoxétine (MP+FLX, 5+5 mg/kg). B) Synthèse des résultats obtenus suite à une administration aiguë ou chronique de méthylphénidate et fluoxétine. nt : non testé.

c) Focus 2: La combinaison méthylphénidate + fluoxétine provoque la rechute du comportement de recherche de cocaïne

Des rats péri-adolescents étaient placés dans une cage d'auto-administration et recevaient une dose de cocaïne (600 μg / 100 μl/kg / infusion, i.v.) à chaque visite du trou actif (« nose poke », ratio fixe 1). Les sessions d'auto-administration étaient journalières et duraient deux heures. Après huit jours consécutifs d'auto-administration, la solution de cocaïne n'était plus distribuée et les rats subissaient une procédure d'extinction pendant 32 jours (Figure 25). La rechute du comportement de recherche de cocaïne était testée le jour 23 (suite à une injection de liquide physiologique), le jour 25 (suite à une injection de méthylphénidate + fluoxétine, 5 mg/kg chacun) puis le jour 32 (suite à une injection de cocaïne). Ces drogues étaient administrées par voie intrapéritonéale immédiatement avant la session d'auto-administration, durant laquelle le nombre de visites du trou actif était comptabilisé. L'injection de liquide physiologique (Vehicle, J23) n'avait pas d'effet sur le nombre de visites du trou actif au cours de la session (Figure 25). En revanche, l'injection du méthylphénidate + fluoxétine (J25) induisait un comportement actif de

recherche de la cocaïne (augmentation du nombre de visites du trou actif alors que la drogue n'est plus distribuée), tout comme le faisait une injection de cocaïne au jour 32. Ceci indique que la combinaison méthylphénidate + fluoxétine induit un comportement de rechute dans un modèle d'auto-administration de cocaïne. Cette combinaison pourrait donc favoriser l'émergence d'un désir impérieux de consommer de la cocaïne, en particulier chez des sujets dépendants sevrés. Des études complémentaires sont en cours pour mesurer le comportement d'auto-administration du mélange méthylphénidate + fluoxétine (nombre de visites du trou actif en utilisant successivement un ratio fixe puis un ratio progressif).

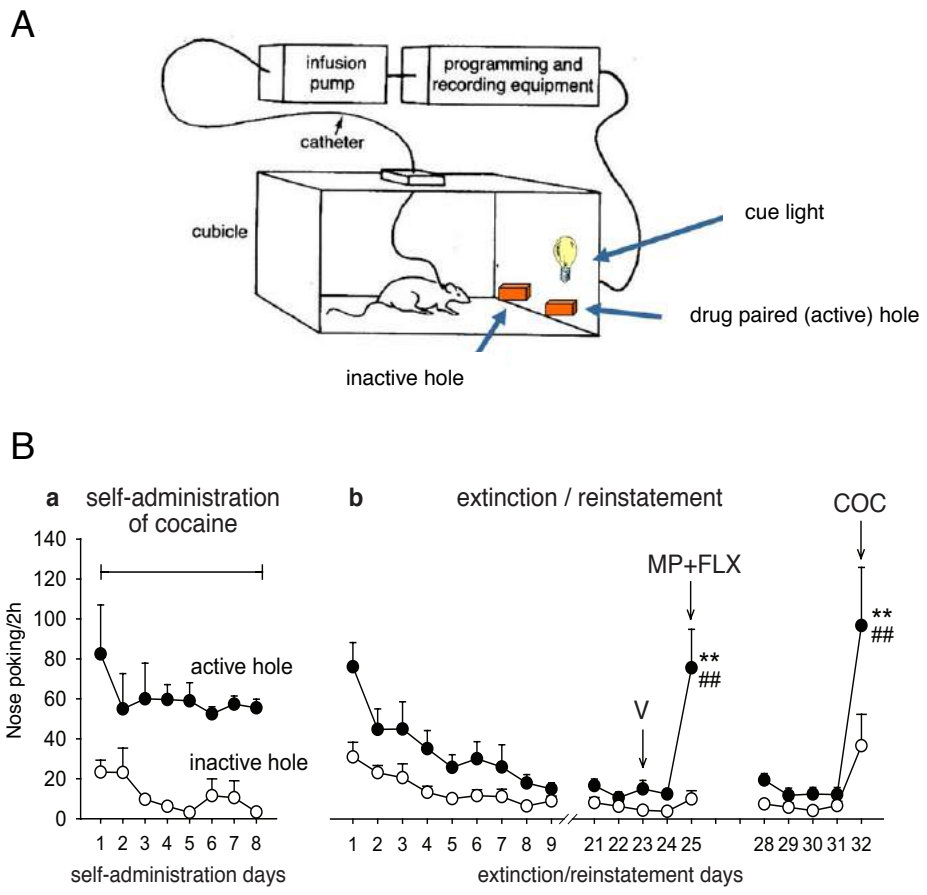


Figure 25 A) Cage utilisée pour l'auto-administration intraveineuse de cocaïne (conditionnement opérant). B) L'injection concomitante de méthylphénidate + fluoxétine (MP + FLX, 5+5 mg/kg, J25) induit un comportement de recherche de cocaïne dans un modèle classiquement utilisé pour évaluer la rechute chez le rat (auto-administration, données préliminaires non publiées, Dr Michaela Marinelli).

d) Focus 3: Les récepteurs 5-HT1B sont en partie responsables de la potentialisation de l'effet du méthylphénidate par la fluoxétine

Nous avons montré que le fait de combiner des SSRIs avec le méthylphénidate exacerbait l'induction de Zif268 par le méthylphénidate. La présente étude avait pour objectif de déterminer quel sous-type de récepteur sérotoninergique pourrait sous-tendre cet effet. Nos résultats montrent qu'un traitement répété de 5 jours avec le méthylphénidate et la fluoxétine (5+5 mg/kg) induit une augmentation de l'expression des récepteurs 5-HT1B, mais pas des 5-HT2C dans le striatum. Une seconde expérience montrait qu'un agoniste spécifique des récepteurs 5-HT1B : le CP94253 (3-10 mg/kg ; CP3, CP10 ; i.p.) mimait la potentialisation provoquée par l'ajout de fluoxétine au méthylphénidate (Figure 26). Ces données suggèrent un rôle des récepteurs 5-HT1B dans l'amplification de l'effet du méthylphénidate par la fluoxétine.

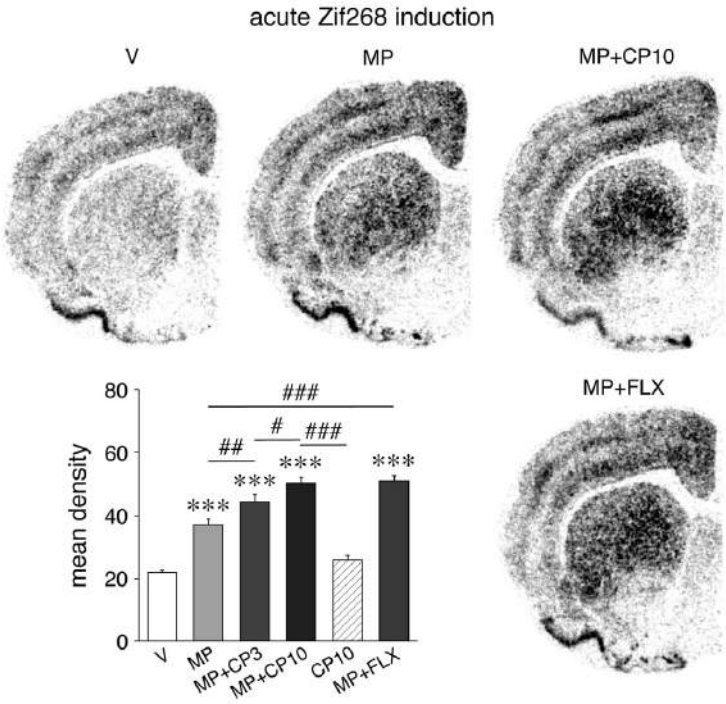


Figure 26 Implication des récepteurs 5-HT1B dans la potentialisation de l'effet du méthylphénidate par la fluoxétine.

D-2) Publications résultant de ces travaux

Peer-reviewed publications:

1) **Van Waes V.**, Ehrlich S., Beverley J., and Steiner H.
   Fluoxetine potentiation of methylphenidate-induced gene regulation in striatal output pathways: association with increased 5-HT1B receptor expression
   Neuropharmacology, Vol. 89C, p77-86, 2015

2) Steiner H., Warren B., **Van Waes V.**, and Bolaños-Guzmán C.
   Life-long consequences of juvenile exposure to psychotropic drugs on brain and behavior
   Progress in Brain Research Vol. 211, p13-30, 2014

3) **Van Waes V.**, Vandrevala B., Beverley J., and Steiner H.
   SSRIs potentiate gene blunting induced by repeated methylphenidate treatment: Zif268 vs. Homer1a
   Addiction Biology Vol. 19(6), p986-995, 2014

4) Beverley J., Piekarski C., **Van Waes V.**, and Steiner H.
   Potentiated gene regulation by methylphenidate plus fluoxetine treatment: Long-term gene blunting (Zif268, Homer1a) and behavioral correlates
   Basal Ganglia, Vol. 4, p109-116, 2014

5) Steiner H. and **Van Waes V.**
   Addiction-Related Gene Regulation: Risks of exposure to cognitive enhancers vs. other psychostimulants
   Progress in Neurobiology Vol. 100, p60-80, 2013

6) **Van Waes V.**, Carr B., Beverley J., and Steiner H.
   Fluoxetine potentiation of methylphenidate-induced neuropeptide expression in the striatum occurs selectively in the direct (striatonigral) neurons
   Journal of Neurochemistry Vol. 122 (5), p1054-64, 2012

7) **Van Waes V.**, Beverley J., Marinelli M., and Steiner H.
SSRI antidepressants potentiate methylphenidate (Ritalin)-induced gene regulation in the adolescent striatum
European Journal of Neuroscience Vol. 32, p435-447, 2010.

8) Steiner H., **Van Waes V.,** and Marinelli M.
Fluoxetine potentiates methylphenidate-induced gene regulation in addiction related brain regions: concerns for use of cognitive enhancers?
Biological Psychiatry Vol. 67 (6), p592-594, 2010.

Books and book chapters

1) **Van Waes V.** and Steiner H.
SSRI antidepressants potentiate addiction-related gene regulation by psychostimulant medications (Book Chapter) in Fluoxetine: Pharmacology, Mechanisms of Action and Potential Side Effects.
Nova Publisher, Book Chapter, p207-226, 2015.

E) CHAPITRE 3 :

Etude anatomique au cours du développement de récepteurs impliqués dans l'addiction / la fonction dopaminergique

(Thématique secondaire du Post-doctorat)

E-1) Principaux résultats obtenus

a) Résumé des résultats

J'ai étudié l'évolution au cours du développement de récepteurs dans des zones cérébrales associées à l'addiction (striatum dorsal et ventral): le récepteur orphelin GPR88 et le récepteur aux cannabinoïdes CB1. En effet, un second objectif de mon post-doctorat était de mettre en place une nouvelle technique au sein du laboratoire américain: l'hybridation *in situ* (fluorescence) combinée à l'immunohistochimie. Le développement de cet outil, en partant de zéro, m'a pris 6 mois et a été couronné de succès. Cette technique est maintenant mise à profit dans diverses expériences menées par plusieurs équipes de la Chicago Medical School. En particulier, nous avons étudié grâce à elle les niveaux d'expression au cours du développement d'un récepteur métabotropique orphelin exprimé de façon sélective dans le striatum, et dont le ligand endogène reste inconnu à ce jour : le GPR88 (Van Waes *et al*, 2011c) (Focus 1). Nous avons par ailleurs quantifié les niveaux d'expression du récepteur CB1 dans le striatum au cours du développement et nous les avons corrélés aux niveaux d'expression du récepteur dans les régions corticales fonctionnellement associées (Van Waes *et al*, 2012a) (cf. article en annexe).

b) Focus 1: GPR88

Le récepteur orphelin GPR88 est un récepteur métabotropique (supposé) exprimé majoritairement - mais pas exclusivement - dans le striatum (Ghate *et al*, 2007; Logue *et al*, 2009; Massart *et al*, 2009; Mizushima *et al*, 2000) et dont

l'expression est modulée par des interventions neuropharmacologiques (Befort *et al*, 2008; Bohm *et al*, 2006; Conti *et al*, 2007; Le Merrer *et al*, 2012). Sa localisation sélective dans le striatum en fait une cible de choix pour le développement de thérapeutiques visant à moduler les fonctions sous tendus par le striatum (motricité/Parkinson, compulsion, addiction…). Le gène GPR88 est distribué dans le striatum dorsal (caudate-putamen) et dans le striatum ventral (noyau accumbens). Il est détecté dans les neurones de projection (neurones épineux moyens MSNs, GABAergiques) appartenant aussi bien à la voie directe qu'à la voie indirecte (Massart *et al*, 2009). Malgré le nombre important de recherches qui lui est consacré, seul un agoniste synthétique de ce récepteur a été décrit à ce jour (Jin *et al*, 2014; Li *et al*, 2013) ; aucun agoniste endogène n'a été découvert. Les approches utilisées pour étudier sa fonction dans le système nerveux central ont donc, jusqu'à maintenant, essentiellement consistées en des études génétiques (Ingallinesi *et al*, 2015; Logue *et al*, 2009; Quintana *et al*, 2012). Les données obtenues à partir de souris pour lesquelles le gène GPR88 a été inactivé indiquent que ce dernier serait impliqué dans la modulation de la transmission dopaminergique. Les souris mutantes (*i.e.* gène GPR88 invalidé) présentent une diminution des taux de base de dopamine et des niveaux plus élevés de la protéine DARPP-32 sous sa forme phosphorylée (Thr-34) dans le striatum (protéine appartenant à une voie de transduction régulée par la dopamine). Ceci était accompagné d'une augmentation de l'activité locomotrice induite par l'apomorphine (agoniste compétitif de la dopamine) et l'amphétamine (Logue *et al*, 2009). Chez les souris mutantes, on note également une augmentation de l'excitabilité des neurones épineux moyens par le glutamate, une diminution de la coordination motrice et un déficit d'apprentissage associé au contexte (Quintana *et al*, 2012). Une autre étude récente révèle que ces souris mutantes présentent par ailleurs une amélioration de la mémoire spatiale et des niveaux d'anxiété atténués (Meirsman *et al*, 2015). Finalement, une inactivation sélective de GPR88 dans le noyau accumbens atténue les altérations comportementales observées dans un modèle neurodéveloppemental de schizophrénie chez le rat (hyperlocomotion induite par l'amphétamine, déficit de discrimination de la nouveauté dans un contexte sociale) (Ingallinesi *et al*, 2015).

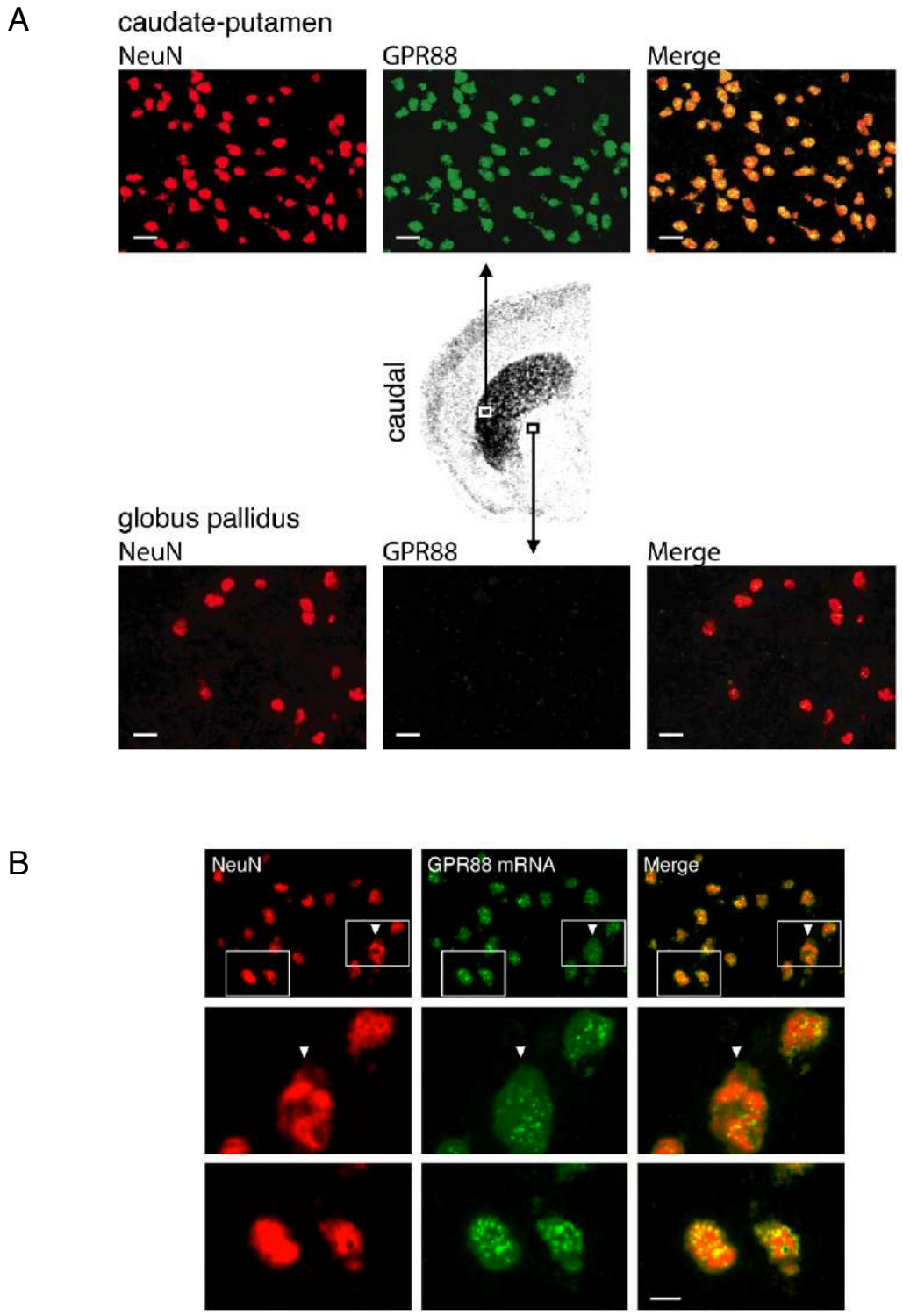


Figure 27 Illustration des résultats obtenus en utilisant la technique d'hybridation in situ (fluorescence) combinée à l'immunohistochimie pour caractériser l'expression du récepteur orphelin GPR88 dans le striatum, région impliquée entre autres dans les processus addictifs. A) Les ARNm codant le récepteur GPR88 (vert) étaient détectés dans toutes les cellules NeuN-positives (rouge) dans le striatum (caudate-putamen, en haut). En revanche, dans le globus pallidus, les cellules NeuN-positives n'exprimaient pas les ARNm codant le GPR88. Barre d'échelle = 25 microns. B) Localisation de GPR88 (vert) dans les neurones (rouge) de taille moyenne et dans ceux de grande taille dans le striatum (ligne du haut). Grossissement (ligne du milieu) montrant un exemple de neurone large (probablement un interneurone cholinergique, triangle blanc) exprimant faiblement GPR88. La ligne du bas montre une expression plus élevée du GPR88 dans les neurones de taille moyenne (MSNs gabergiques) comparés aux interneurones cholinergiques. Barre d'échelle = 10 microns.

Bien que la localisation du GPR88 soit maintenant bien établi, le décours de son expression au cours du développement n'a jamais été investigué.

Nous avons dans une étude descriptive, grâce à la technique d'hybridation *in situ* par fluorescence couplée à l'immunohistochimie, montré que le GPR88 était présent quasiment exclusivement dans le striatum, et ceci dans la totalité des neurones c'est à dire dans l'ensemble des neurones épineux moyens (MSNs) d'une part (voies directe et indirecte) mais également dans l'ensemble des interneurones du striatum (Figure 27A). L'expression de GPR88, bien que visible dans l'ensemble des neurones du striatum, présentait des différences de niveau d'expression marquées entre les cellules (Figure 27B).

Nous avons également montré que la trajectoire développementale des quantités d'ARNm codant pour ce récepteur est différente dans le striatum dorsal et dans le noyau accumbens (Figure 28). La diminution était continue dans le striatum dorsal (caudate-putamen) de l'âge de 25 jours (préadolescents, P25) à l'âge de 70 jours (jeunes adultes, P70, Figure 28B). En revanche, une diminution des taux d'ARNm codant pour ce récepteur était observée au niveau du noyau accumbens entre la pré-adolescence (P25) et l'adolescence (P40), mais pas de l'adolescence (P40) à l'âge adulte (P70). Des études complémentaires seront nécessaires pour déterminer le rôle fonctionnel de ce récepteur présumé et un effort particulier devra être entrepris pour découvrir un éventuel ligand endogène de ce récepteur.

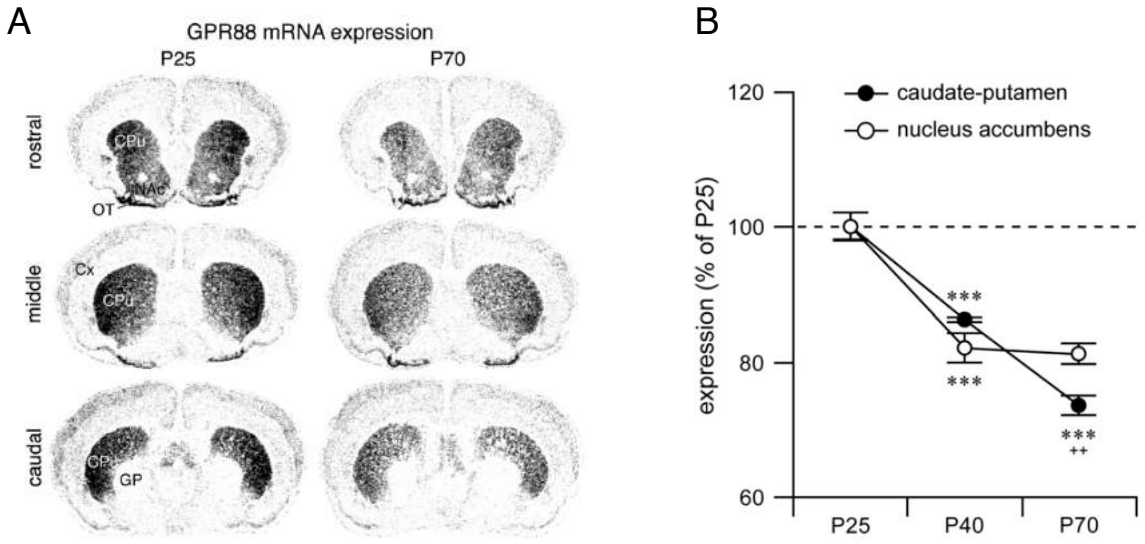


Figure 28 A) Autoradiogrammes illustrant la diminution de l'expression de GPR88 en fonction de l'âge dans le striatum dorsal (caudate-putamen, CPu) et dans le striatum ventral (nucleus accumbens, NAc). P25 : 25 jours, P70 : 70 jours. B) Quantification de l'expression de GPR88 dans le caudate-putamen et dans le noyau accumbens à 25, 40 et 70 jours.

E-2) Publications résultant de ces travaux

Peer-reviewed publications:

1) **Van Waes V.**, Tseng K.Y, and Steiner H.
GPR88: a putative signaling molecule predominantly expressed in the striatum; Cellular localization and developmental regulation
Basal Ganglia Vol. 1 (2), p 83-89, 2011

2) **Van Waes V.**, Beverley J., Siman H., Tseng K.Y, and Steiner H.
CB1 cannabinoid receptor expression in the striatum: association with corticostriatal circuits and developmental regulation
Frontiers in Neuropharmacology Vol. 3, Art. 21, 2012

F) CHAPITRE 4 :

Utilisation de la stimulation transcrânienne par courant continu (tDCS) comme aide au sevrage (Thématique actuelle)

F-1) Intégration dans l'équipe de recherche EA481

J'ai incorporé le Laboratoire de Neurosciences Intégratives et Cliniques (EA481) en 2010 ou j'ai commencé par encadrer des étudiants en Master 1 (« Physiologie, Neurosciences et Comportement », Besançon) puis des étudiants en Master 2 (du même Master) avant de co-diriger une étudiante en thèse (Solène Pedron) qui soutiendra en juin 2016 et est à ce jour co-signataire de 3 articles dans des journaux internationaux de rang A (Frontiers in Systems Neuroscience, Brain Structure and Function, Neuropsychopharmacology). Deux autres articles sont soumis (Addiction Biology, en révision, collaboration avec la Chicago Medical School) ou en préparation (rédaction en collaboration avec l'INSERM ERI24, Amiens). Enfin 3 articles dans des revues nationales ont également été publiés (deux dans les cahiers de l'IREB, un aux Presses Universitaires de Franche Comté).

Dans ce cadre, en sus de mon investissement dans un certain nombre de collaborations internes ou externes à l'équipe non détaillées dans ce document (ex : (Chometton *et al*, 2015)), j'ai débuté une collaboration avec le Professeur Emmanuel Haffen qui développe des outils thérapeutiques innovants pour la recherche en psychiatrie. J'ai pu apporter à l'équipe en place mon expertise dans le champ du comportement animal et de l'addiction et j'ai proposé de développer une thématique de recherche : « Stimulation transcrânienne et addiction : modèle animal ». J'ai en particulier rédigé un sujet de thèse en 2012 en collaboration avec les Professeurs Emmanuel Haffen et Daniel Sechter et obtenu un financement de 3 ans (2012-2015, Ville de Besançon) sur la thématique : « **Utilisation de la stimulation transcrânienne par courant continu comme aide au sevrage (alcool, tabac, cocaïne): études comportementale et neurobiologique chez la souris.** »

Mon étudiante en thèse, Solène Pedron, a remporté le Prix de thèse de l'association A'DOC en juin 2014 lui donnant l'opportunité de présenter ses résultats lors d'une communication orale et de les publier sous forme d'article dans les Presses Universitaires de Franche-Comté :

Effets de la stimulation transcrânienne par courant continu chez la souris: études comportementales

Solène Pedron

PUFC, 2014

Elle a également reçu le Prix de la meilleure présentation orale au forum des jeunes chercheurs en 2014 pour sa présentation intitulée :

La stimulation transcrânienne par courant continu comme aide au sevrage à la cocaïne

Solène Pedron

20ème Forum des Jeunes Chercheurs

23-24 juin 2014, Besançon, France.

De plus, elle a obtenu une bourse de mobilité de l'Université de Franche-Comté pour se rendre à la Chicago Medical School, IL, USA du 6 octobre au 6 novembre 2014 (Pr Heinz Steiner) afin de poursuivre ses expériences sur la stimulation transcrânienne par courant continu dans un environnement international, stimulant et riche en terme de ressources. Elle a aussi effectué un stage d'un mois, financé par l'Institut de REcherche scientifique sur les Boissons (IREB), dans l'Unité INSERM ERI24 GRAP dirigée par le Professeur Mickaël Naassila (Groupe de Recherche sur l'Alcool et les Pharmacodépendances, mai 2014) afin de bénéficier de l'expertise de cette équipe dans le champ de la recherche sur les pharmacodépendances. Cette collaboration devrait déboucher prochainement sur une nouvelle publication en cours de rédaction.

Enfin, Solène a eu l'opportunité de présenter ses résultats dans divers congrès nationaux et internationaux tels que par exemple au Forum FENS (Federation of European Neuroscience Societies) à Milan (communication affichée, juillet 2014) et à la Cambridge & Luton International Conference on Mental Health à Cambridge, UK (Communication orale, septembre 2013). Les données que je vais présenter dans ce

chapitre sont les principaux résultats brillamment obtenus par Solène au cours de sa thèse.

F-2) Principaux résultats obtenus

a) Contexte général du projet

Notre travail s'inscrit dans un projet de plus grande ampleur portant sur l'efficacité de la tDCS (stimulation transcrânienne par courant continu) et de la rTMS (stimulations magnétiques transcrâniennes répétées) pour le traitement de la dépression d'une part et comme aide au sevrage chez des personnes dépendantes d'autre part (projet coordonné au niveau national par le Professeur Emmanuel Haffen). Une sous-partie de ce travail vise à mieux appréhender les mécanismes qui sous-tendent les effets bénéfiques de la tDCS sur la consommation de drogues (alcool, nicotine, cocaïne).

La tDCS est une technique de neurostimulation non invasive peu coûteuse et simple d'exécution consistant à faire passer un courant continu entre deux électrodes au travers de la boîte crânienne d'un sujet afin de moduler l'excitabilité des zones corticales traversées. Les zones se trouvant sous l'anode présentent une excitabilité augmentée (augmentation de l'apparition de PA spontanées pendant la stimulation) alors que les zones sous la cathode sont inhibées (diminution de l'apparition de PA spontanées) (Bindman *et al*, 1964; Nitsche and Paulus, 2000). Cette technique constitue de ce fait un outil de recherche fondamentale de choix de part ses capacités de neuromodulation.

Un nombre important d'études cliniques a révélé les bénéfices variés apportés par cette technique (Kuo *et al*, 2014). Par exemple, de manière non exhaustive, on peut citer : le soulagement des symptômes de la dépression (Brunoni *et al*, 2012; Valiengo *et al*, 2013), l'amélioration de la mémoire chez des personnes présentant ou non des déficits préalables (Bennabi *et al*, 2014; Filmer *et al*, 2014), l'augmentation des performances attentionnelles (Coffman *et al*, 2012; Sparing *et al*, 2009), le soulagement de la douleur (Mehta *et al*, 2015; O'Connell *et al*, 2014; Vaseghi *et al*, 2014), la modulation de l'excitabilité du cortex moteur (Nitsche *et al*,

2003; Nitsche and Paulus, 2001), la diminution des acouphènes (Langguth and De Ridder, 2013; Song *et al*, 2012), la réduction de l'envie de consommer des drogues chez des personnes dépendantes (den Uyl *et al*, 2015; Feil *et al*, 2010), le soulagement des addictions comportementales (Sauvaget *et al*, 2015), la diminution du craving pour la nourriture (Bravo *et al*, 2015) etc…).

Le choix du montage des électrodes est déterminé par les processus que l'on souhaite moduler. Concernant la dépression, la mémoire et l'addiction, les électrodes sont le plus souvent placées au niveau du cortex préfrontal dorsolatéral (de manière asymétrique ou bilatéralement).

Tableau 5 Principaux avantages et inconvénients de la tDCS.

Avantages	Inconvénients
- Non invasive	- Mécanisme d'action ?
- Peu onéreuse	- Effets à long terme ? Exemples: • Interactions avec d'autres traitements ? • Effets non contrôlés sur la cognition ?
- Utilisation simple	
- Portable	
- Effets indésirables mineurs	

De manière générale, l'usage de la tDCS présente de nombreux avantages, mais également quelques inconvénients synthétisés dans le Tableau 5. Les principaux désavantages de cette technique sont la méconnaissance de son mode d'action au niveau physiologique (Stagg *et al*, 2011) ainsi que le manque de recul quant à son utilisation répétée chez l'Homme. Par ailleurs, son possible impact délétère en cas d'utilisation dans des populations sensibles (ex : adolescents (Krishnan *et al*, 2015)) a trop peu été étudié ce qui impose la plus grande prudence (cf. Perspectives 2: tDCS lors de l'adolescence et trajectoire développementale du cortex).

Les résultats disponibles à ce jour indiquent néanmoins que la tDCS est bien tolérée. Elle induit moins d'effets secondaires que les traitements médicamenteux. Les effets secondaires mineurs reportés lors de la stimulation vont de très légères sensations de brûlure à des picotements et des démangeaisons (au niveau de

l'électrode) et se limitent à de faibles sensations de picotements et de démangeaisons après l'arrêt stimulation (Figure 29) (Kessler *et al*, 2012).

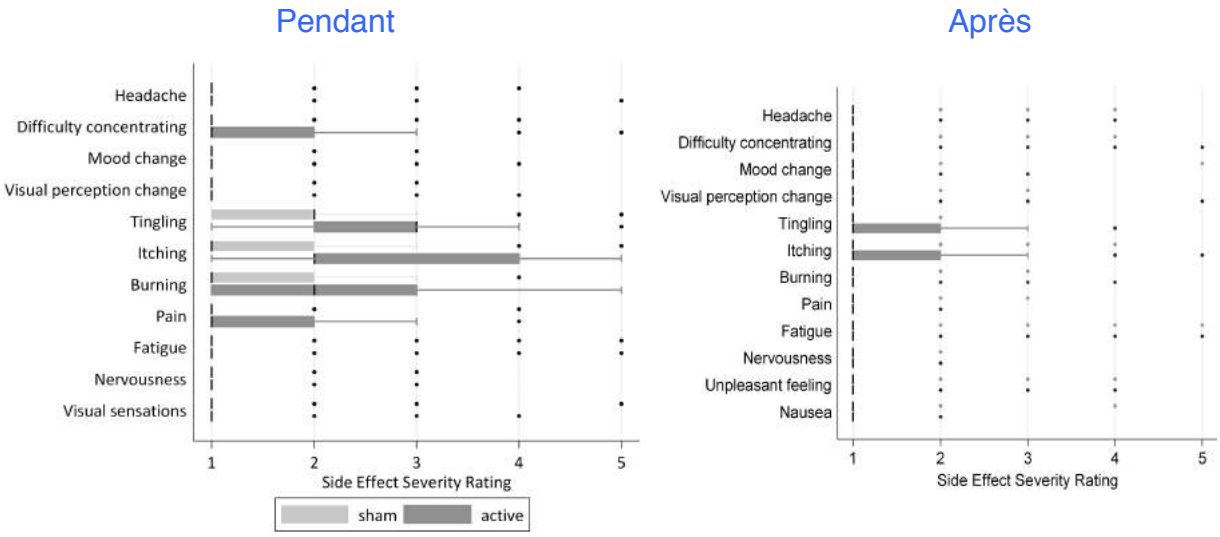


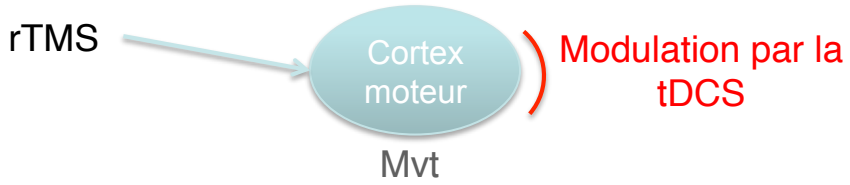
Figure 29 La tDCS est bien tolérée. Etude menée sur 137 sujets sains sur un total de 277 sessions. (Kessler *et al*, 2012). Sham : le courant n'est pas délivré ; Active : le courant est délivré.

b) Mécanismes d'action peu connus

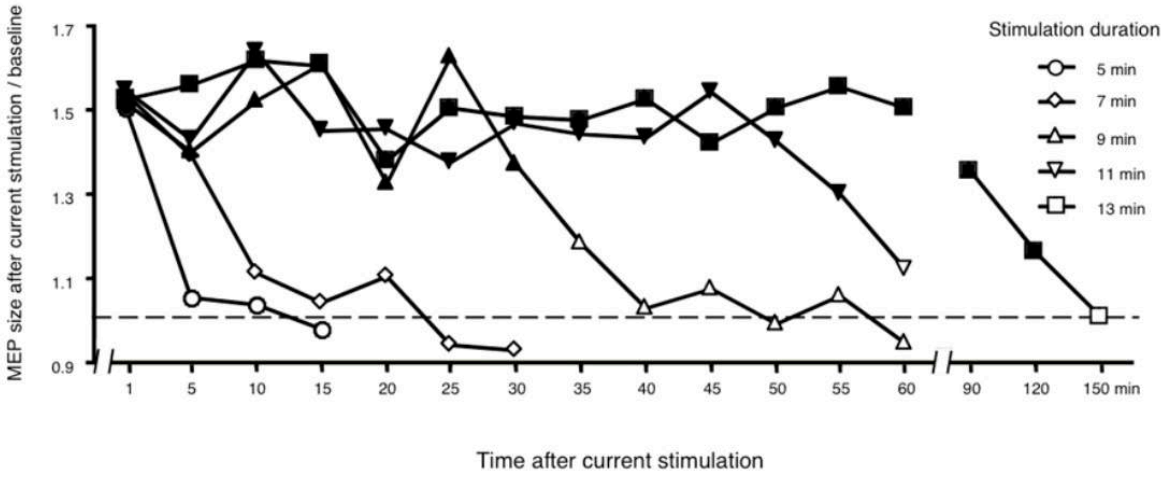
A partir d'expériences menées sur les potentiels évoqués moteurs (Figure 30A), Nitsche et collaborateur ont constaté que la tDCS facilite l'excitation du cortex moteur primaire (anode) ou l'inhibe (cathode) lors de la stimulation (appelé effet « on line »), et que ce phénomène perdure bien après l'arrêt de la stimulation (effet « off line », Figure 30B) (Nitsche *et al*, 2003; Nitsche *et al*, 2001). Ces effets on line et off line semblent recruter des mécanismes différents comme le suggère la Figure 30C.

Dans une série d'études pharmacologiques, l'équipe allemande a montré que le blocage des canaux Na^+ par la carbamazépine annule les effets on line d'une stimulation anodique (mais pas cathodique). D'autre part, les effets off line d'une stimulation (anodique et cathodique) sont supprimés par l'inhibition des récepteurs NMDA par le dextrométorphane suggérant une analogie entre ce mécanisme et celui de la « potentialisation à long terme » (Figure 30C). D'autres hypothèses ont également été énoncées comme par exemple la modification du débit sanguin par la tDCS dépendant de la polarité (Wachter *et al*, 2011) ou la modulation de l'expression d'un facteur neurotrophique : le Brain-Derived Neurotrophic Factor (BDNF) (Fritsch *et al*, 2010).

A



B



C

Blocage canaux	On line		Off line	
	anode	cathode	anode	cathode
Na^+ carbamazepine	X	-	X	-
Ca^{2+} Flumazenile	(X)	-	X	-
NMDA dextrometorphane	-	-	X	X

Figure 30 A) Principe du potentiel évoqué moteur : le cortex moteur primaire d'un sujet est stimulé en utilisant la rTMS. Un mouvement (Mvt) est donc produit (ex : muscle adducteur du petit doigt) et est enregistré par électromyographie (en mV). Il est alors possible de déterminer l'impact de la tDCS sur l'amplitude de ce phénomène et de réaliser des études pharmacologiques chez l'Homme. B) Maintien d'une facilitation du potentiel évoqué moteur (MEP) après la fin de la tDCS (courant anodique d'une durées de 5, 7, 9, 11 ou 13 minutes, 1 mA) : correspond à l'effet off line. Plus la stimulation est longue, plus l'effet off line perdure. C) Synthèses des résultats obtenus au moyen d'études pharmacologiques chez l'Homme en bénéficiant des données provenant des potentiels évoqués moteurs (d'après (Stagg and Nitsche, 2011))

Il est à noter que l'impact de la tDCS sur l'excitabilité corticale se révèle plus complexe qu'il n'y paraît au premier abord. Ainsi, on ne connaît pas réellement la trajectoire exacte empruntée par le courant entre les deux électrodes. Des études mettent également en évidence le fait qu'une stimulation n'induit pas forcément une réponse linéaire sur l'excitabilité corticale. De manière générale, plus l'intensité de stimulation est importante, plus le cortex sera activé (anode) ou inhibé (cathode) (Batsikadze *et al*, 2013). Cependant, une étude a révélé qu'une stimulation cathodique de 1 mA inhibe le cortex moteur, comme attendu, mais qu'une stimulation à 2 mA induit paradoxalement une facilitation de l'excitabilité de ce cortex (Batsikadze *et al*, 2013). D'autre part, les conséquences du courant électrique semblent dépendre de l'orientation des cellules par rapport au champ électrique (Kabakov *et al*, 2012) ce qui complique largement les choses lorsque l'on souhaite modéliser les effets de la tDCS. Enfin, le protocole de stimulation, notamment l'intervalle entre les stimulations lorsque celles-ci sont répétées a des répercussions sur les effets de la tDCS (Monte-Silva *et al*, 2013). Des études complémentaires seront donc nécessaires pour mieux comprendre/prédire les conséquences des stimulations sur la zone d'intérêt.

- Objectifs du projet

Notre objectif est de valider un modèle murin de tDCS et d'évaluer les mécanismes neurobiologiques sous-tendant ses effets bénéfiques sur la vulnérabilité aux drogues.

- Stratégie scientifique pour atteindre les objectifs

Nous avons développé en partenariat avec la société DIXI Medical (http://www.diximedical.net/, Jean-Pierre Darnis, Besançon) un modèle de tDCS chez la souris. Nous utilisons une électrode de stimulation d'une surface de contact de 3,5 mm² (Fabrication DIXI Medical) placée au niveau du cortex frontal gauche (centre de l'anode positionné 1 mm postérieurement et 1 mm vers la gauche par rapport au Bregma) et une contre-électrode en silicone d'une surface de contact de 10,5 cm² (Physiomed Elektro) placée au niveau du thorax (Liebetanz et al., 2009) (Figure 31). Une embase support de l'électrode est fixée sur la surface de l'os du crâne de

l'animal (ciment verre ionomère) au niveau du cortex frontal gauche une semaine avant le début des stimulations. L'électrode de stimulation (anode) est vissée à l'embase préalablement remplie de liquide physiologique lors des phases de stimulations. Les stimulations consistent en l'application d'un courant continu 2 x 20 min/jour pendant 5 jours (intensité = 0,2 mA) (Ferrucci *et al*, 2009; Rigonatti *et al*, 2008) en utilisant le même modèle de générateur et les mêmes paradigmes de stimulation qui sont utilisés en recherche clinique à Besançon et dans d'autres villes ou les études cliniques sont menées (NB : intensité /10 ; Eldith DC Stimulator®, The Magstim Company®, Whitland, UK). Les conditions expérimentales sont identiques pour le groupe témoin (implantation des embases, branchement des électrodes) mais la stimulation électrique n'est pas délivrée.

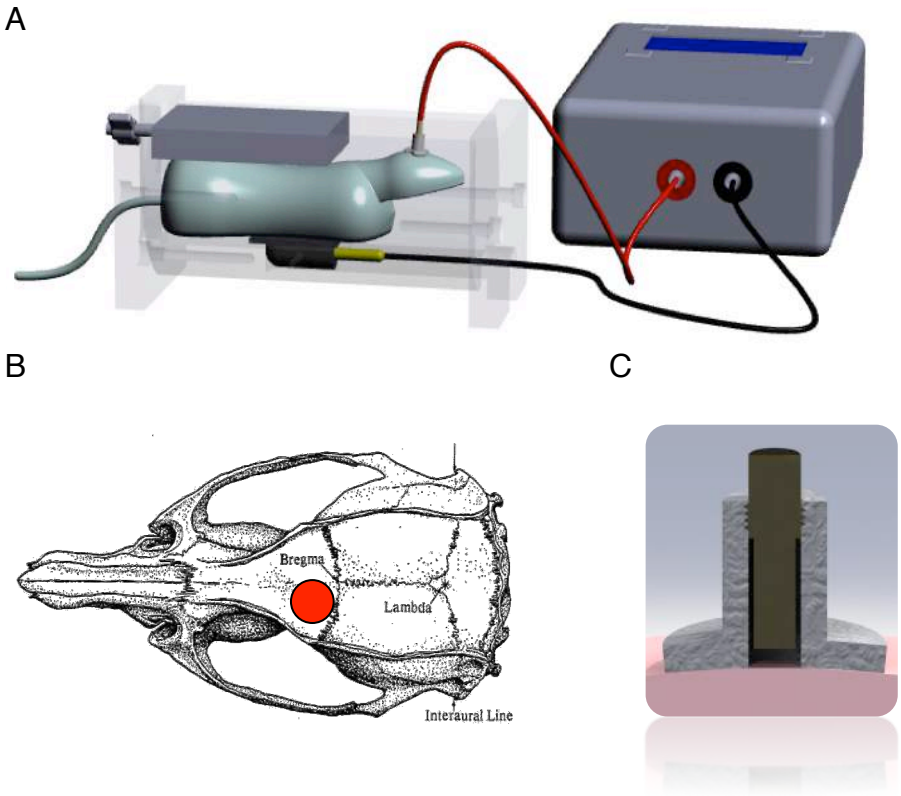


Figure 31 Modèle animal de tDCS développé à l'EA481 (Pedron *et al*, 2014). A) L'animal est placé dans une boîte de contention, l'anode est positionné au niveau du cortex frontal, la cathode plaquée contre le thorax. B) Le centre de l'anode est placée 1 mm postérieurement et 1 mm vers la gauche par rapport au Bregma. C) Coupe montrant l'électrode vissée dans l'embase (embase en gris, remplie de liquide physiologique avant la stimulation).

- Originalité des résultats attendus

Ce projet translationnel et novateur est à la jonction entre la recherche clinique et pré-clinique. Il permet l'intervention synergique de nombreuses équipes au niveau régional (clinique : CHU de Besançon (Emmanuel Haffen) et Centre d'investigation clinique et d'innovation technologique INSERM CIC-IT 808; électronique/mécanique : CNRS Institut FEMTO-ST; entreprise de biotechnologie : DIXI Medical; recherche fondamentale : EA481 (Dr Vincent Van Waes, Dr Yvan Peterschmitt) et EA3922 (Dr Pierre-Yves Risold) (SFR FED 4234). Il devrait permettre d'accélérer le développement d'une thérapeutique innovante comme aide au sevrage chez les personnes dépendantes et de mieux comprendre les mécanismes physiologiques à l'origine de ses effets.

c) Focus 1: Validation du modèle animal

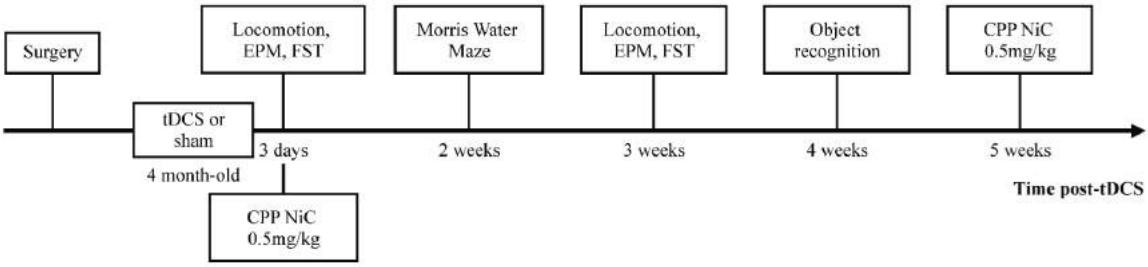
Dans un premier temps, nous avons caractérisé les comportements affectés par la tDCS à court (3 jours) et à long terme (3-5 semaines après la fin des stimulations) en utilisant une batterie de tests comportementaux. Le protocole expérimental et les principales données obtenues sont synthétisés dans la figure Figure 32.

De cette expérience, nous sommes parvenus à deux conclusions :

1) Notre modèle animal présente une bonne validité apparente. Les mêmes paramètres affectés chez l'Homme le sont également chez la souris. Les paramètres non impactés chez l'Homme ne le sont pas non plus chez l'animal.

2) Nous postulons (à partir de ces résultats et d'autres résultats non publiés et non présentés dans ce document) que les modifications comportementales n'apparaissent pas tout de suite à la fin des stimulations mais sont maximales après environ 3 semaines.

A



B

- **Locomotion (stress):** Videotracking
- « **Anxiété** » **:** Labyrinthe en croix surélevé
- « **Dépression** » **:** Test de la nage forcée
- **Mémoire spatiale:** Test de la piscine de Morris
- **Mémoire de travail:** Reconnaissance d'objet
- **Valeur hédonique:** Préférence de place conditionnée

C

Effet tDCS	Homme	Souris
Stress	-	-
Anxiété	-	-
Dépression	OUI	OUI
Mémoire spatiale	OUI	OUI
Mémoire de travail	OUI	OUI
« Craving » (nicotine)	OUI	OUI

D

Exemple mémoire de travail

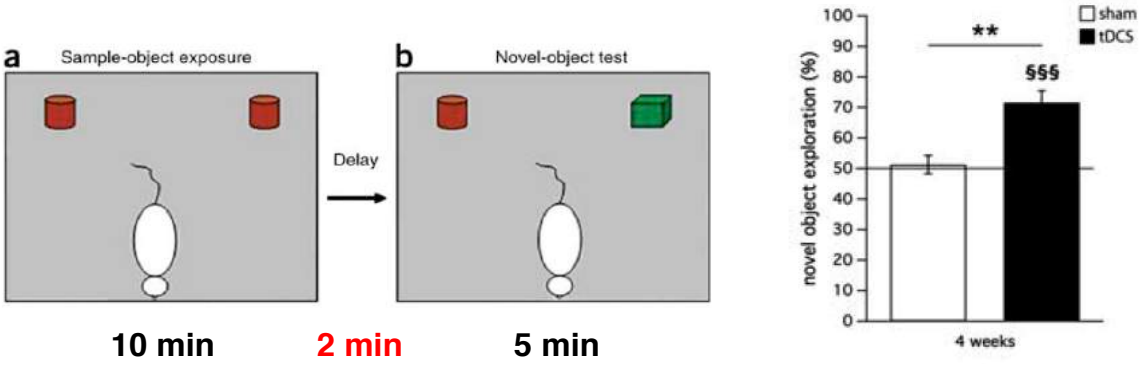


Figure 32 A) Protocole expérimental (caractérisation des comportements affectés par la tDCS). EPM : labyrinthe en croix surélevé, FST : test de la nage forcée, CPP : préférence de place conditionnée. B) Principaux tests comportementaux utilisés. C) Synthèse des résultats indiquant une bonne validité apparente de notre modèle. D) Exemple du test de reconnaissance de place effectué 4 semaines après la dernière stimulation pour évaluer la mémoire de travail. Les souris qui ont reçu la stimulation discriminent un nouvel objet remplaçant un objet exploré deux minutes auparavant (temps d'exploration du nouvel objet significativement supérieur à 50%, p<0.001). Ce n'est pas le cas des animaux témoins dans ces conditions expérimentales (Pedron *et al*, 2014).

d) Focus 2: Nicotine

Nous avons traité des souris pendant l'adolescence (adolescence chez la souris : ~J30-J60, (Spear, 2000)) en leur injectant de la nicotine (1 mg/kg) 2 fois par jour du jour post-natal 30 au jour post-natal 43. Cette exposition suivi d'un sevrage est une procédure induisant des conséquences délétères persistantes sur le comportement (augmentation des symptômes liés à la dépression à l'âge adulte, *i.e.* augmentation du comportement de résignation dans le test de la nage forcée) (Iniguez *et al*, 2009). Ces symptômes qui pourraient favoriser une rechute de la consommation de nicotine sont supprimés suite à l'administration d'antidépresseurs (typiques, ex : fluoxétine ; ou atypiques, ex : brupopion) ou à la suite d'une réexposition à la nicotine (Iniguez *et al*, 2009). Puisque la tDCS induit des effets antidépresseurs (cf. Focus 1), nous nous sommes demandés si les stimulations électriques pourraient également soulager ces symptômes. Nos résultats montrent qu'un traitement tDCS à l'âge adulte permet non seulement de supprimer les conséquences de l'exposition préalable à la nicotine sur les comportements associés à la dépression (Pedron *et al*, 2014), mais il abolit également l'augmentation drastique de la valeur hédonique de la nicotine (0,5 mg/kg) mesurée dans le test de préférence de place conditionnée (Figure 33) (Pedron *et al*, 2014).

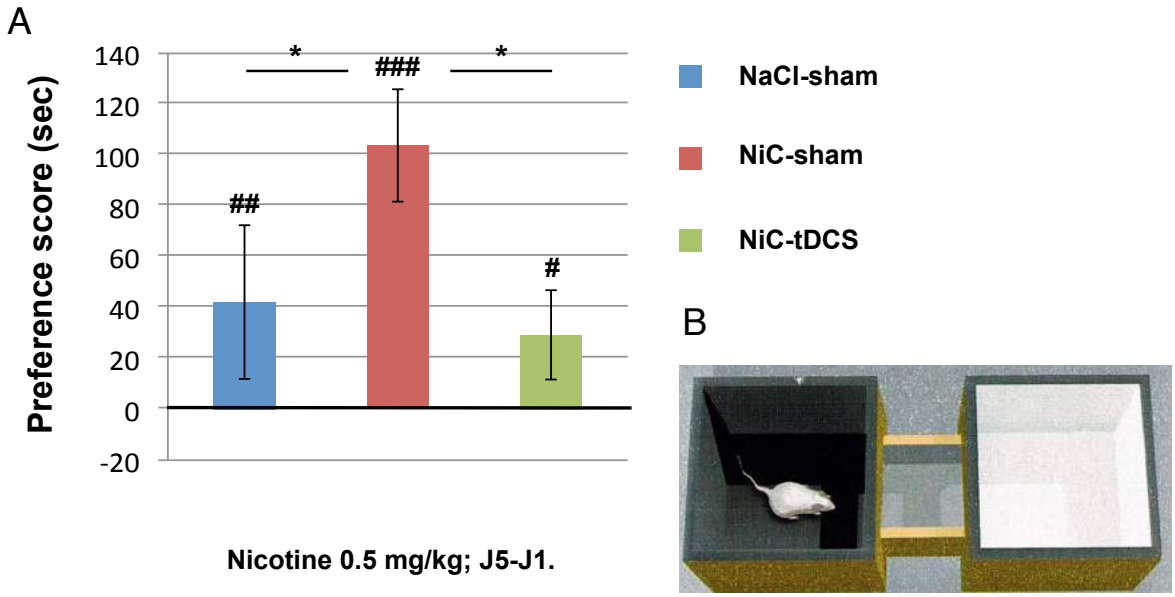


Figure 33 A) La nicotine (0,5 mg/kg) induit chez les animaux adultes préalablement exposés à la nicotine lors de l'adolescence une préférence de place très marquée (Nic-sham). Celle-ci revient à un niveau similaire à ceux d'animaux jamais été exposés à la drogue (NaCl-sham) 3 semaines après les stimulations électriques (Nic-tDCS). B) Test de préférence de place conditionnée, dans lequel l'animal associe son état interne lors de l'exposition à la substance à un environnement particulier.

La tDCS « gomme » donc les effets néfastes résultant d'un traitement chronique à la nicotine lors de l'adolescence (retour à des niveaux d'animaux jamais exposés à la nicotine). Elle pourrait de ce fait constituer un outil complémentaire dans l'arsenal thérapeutique disponible pour favoriser le sevrage tabagique.

e) Focus 3: Alcool

L'addiction à l'alcool est une pathologie chronique induisant une altération de circuits neuronaux, mais également des problèmes psychologiques et socio-environnementaux. La thérapeutique actuelle consiste en l'arrêt de consommation (sevrage), aidé ou non de traitements médicamenteux (*e.g.* naltrexone, acamprosate…) et/ou d'une psychothérapie. Cependant, outre de fréquents effets indésirables, aucun médicament ne permet d'éliminer définitivement les risques de rechutes.

Le but de cette étude était de déterminer si des stimulations anodiques répétées au niveau du cortex frontal permettent de diminuer l'auto-administration d'alcool de souris caractérisées comme «grandes buveuses». Elle a été réalisée au Laboratoire de Neurosciences Intégratives et Cliniques de Besançon et au sein du Groupe de Recherche sur l'Alcool et les Pharmacodépendances (GRAP INSERM ERI 24, Amiens) dirigé par le Pr. Michaël Naassila.

MATERIELS ET METHODES

Animaux

Nous avons utilisé des souris femelles de souche Swiss âgées de 12 semaines au début de l'auto-administration (adultes). Sur 54 souris au départ, nous avons sélectionné les 22 plus grandes consommatrices d'alcool («grandes buveuses») et les avons réparties en deux groupes expérimentaux :

- Groupe Sham (N=11) : chirurgie avec implantation de l'embase de l'électrode, pas de stimulation électrique.
- Groupe tDCS (N=11) : chirurgie avec implantation de l'embase de l'électrode, tDCS anodique au niveau du lobe frontal gauche.

Procédure d'auto-administration d'alcool

Ce test se déroule dans une cage de Skinner munie de 2 trous : un « actif » (un nombre fixé par l'expérimentateur d'explorations (= *nose-pokes*) de ce trou permet l'obtention de l'agent renforçateur, ici 20 μl d'alcool à 20%) et un « inactif » dont l'exploration ne provoque aucune conséquence. Une session d'auto-administration dure 1h et permet de comparer le nombre de visites des trous actif et inactif. Trois conditions sont étudiées : habituation, extinction et rechute (cf. Figure 34) :

A) Habituation: pendant plusieurs semaines, les souris sont habituées à fournir un travail selon un ratio fixe (RF1 à 3 ; de 1 à 3 *nose-pokes*) pour obtenir une dose d'alcool.

B) Extinction: dès la fin des stimulations, les souris sont replacées dans les cages d'auto-administration, mais quel que soit le travail fourni la dose d'alcool n'est pas délivrée. Cette phase est maintenue jusqu'à ce que les souris atteignent un niveau bas et stable de *nose-pokes* dans la fenêtre active.

C) Rechute: une dose « gratuite » d'alcool est offerte à la souris au début d'une session, puis le travail fourni pour obtenir de nouvelles doses d'alcool est mesuré (à raison de 2 *nose-pokes* pour obtenir une dose). Ce paramètre peut être déterminé sur une session ou sur plusieurs (pour les suivantes, il n'y a plus de « don » d'alcool préalable, mais l'alcool est disponible contre travail).

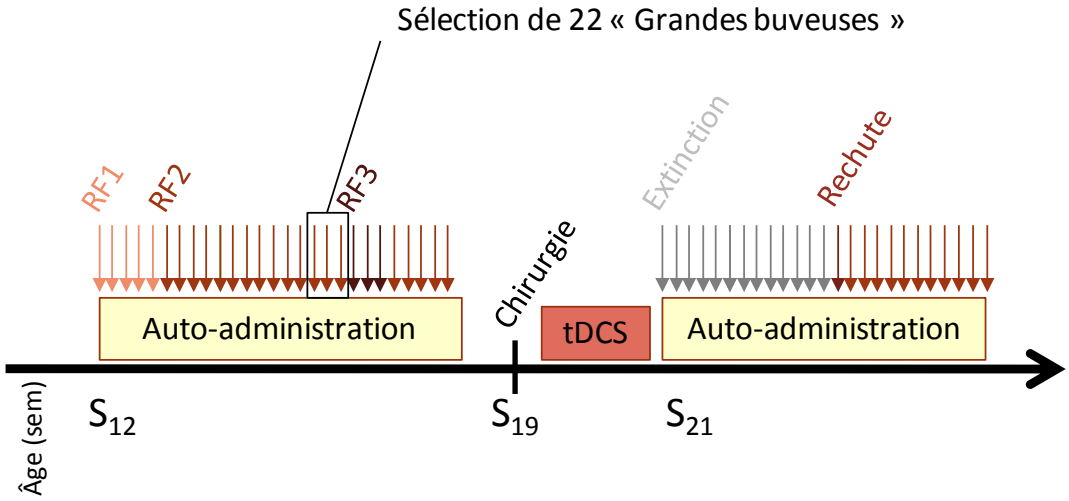


Figure 34 Protocole expérimental pour évaluer l'auto-administration d'alcool. Les souris ont été habituées 3 semaines à l'auto-administration, puis les 22 plus « grandes buveuses » d'alcool ont été sélectionnées et ont suivi le reste du protocole (stimulations anodiques ou sham, extinction puis évaluation de la rechute).

RESULTATS

La tDCS diminuait les comportements de rechute dans le test d'auto-administration. De manière générale, nos résultats montrent une diminution de la consommation d'alcool chez les souris ayant reçu la tDCS (ANOVA à mesures répétées : effet traitement $F_{(1,20)} = 5,43$; $p<0,05$). L'analyse *post-hoc* de Newman-Keuls montre une différence entre les deux groupes au moment de la première semaine de rechute (S25), les souris ayant reçu la stimulation active fournissant significativement moins d'efforts pour obtenir de l'alcool que les souris sham (NK, $p<0.05$; Figure 35).

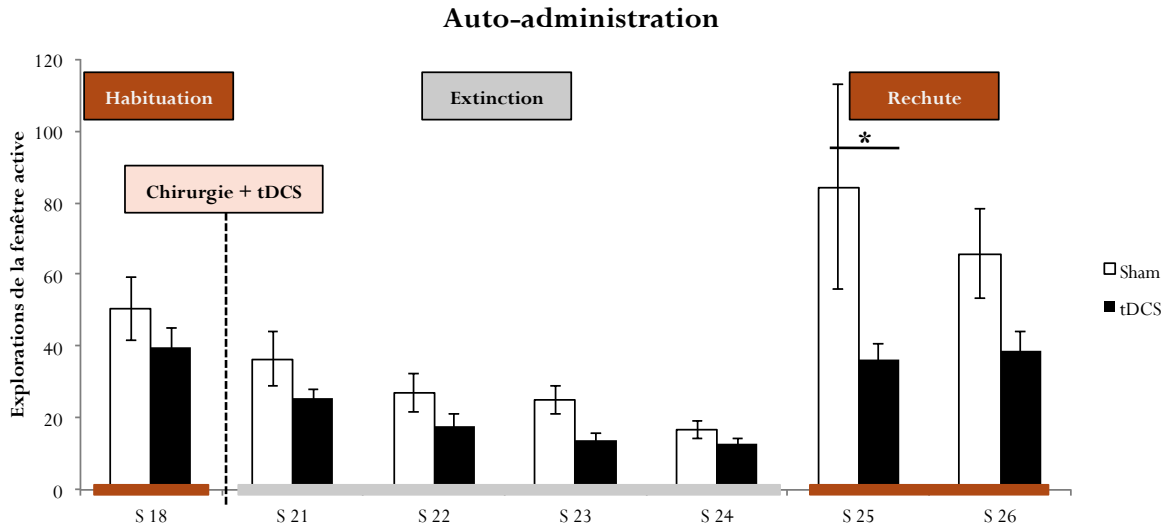


Figure 35 Nombre de visites du trou actif au cours des sessions d'auto-administration (sessions d'habituation, d'extinction puis de rechute) ; NK : * p<0.05 *vs.* Sham.

DISCUSSION

A la suite d'un sevrage forcé (extinction du comportement d'auto-administration), les animaux stimulés présentent une ré-acquisition (rechute) plus faible du comportement d'auto-administration d'alcool. La tDCS pourrait donc se révéler utile chez des patients alcoolo-dépendants sevrés à risque de rechute.

D'autres tests doivent être réalisés afin de confirmer les effets bénéfiques de la tDCS comme aide au sevrage. Les résultats présentés ici ont été obtenus sur une population de souris caractérisées comme « grandes buveuses ». Il serait désormais nécessaire d'examiner si la tDCS a un effet bénéfique sur une population d'animaux rendus dépendants par inhalation chronique de vapeurs d'alcool (procédé maitrisé

par le GRAP à Amiens, demande commune de financement pour ce projet à la Fondation pour la Recherche en Alcoologie, 2016, porteur du projet : VVW). Par ailleurs, les résultats obtenus dans notre étude concernent les 6 semaines suivant les stimulations. Il serait judicieux de vérifier sur quelle durée s'inscrivent ces effets, et si une ou plusieurs stimulations complémentaires permettrait de prolonger les conséquences bénéfiques de la tDCS.

f) Focus 4: Cocaïne

L'administration de cocaïne induit des modifications de l'expression génique dans les circuits cortico-striataux impliqués dans la compulsion. Nous avons évalué l'impact de la tDCS sur l'induction de Zif268 dans ces circuits (Zif268 : marqueur d'activité neuronale et facteur de transcription impliqué dans la plasticité synaptique).

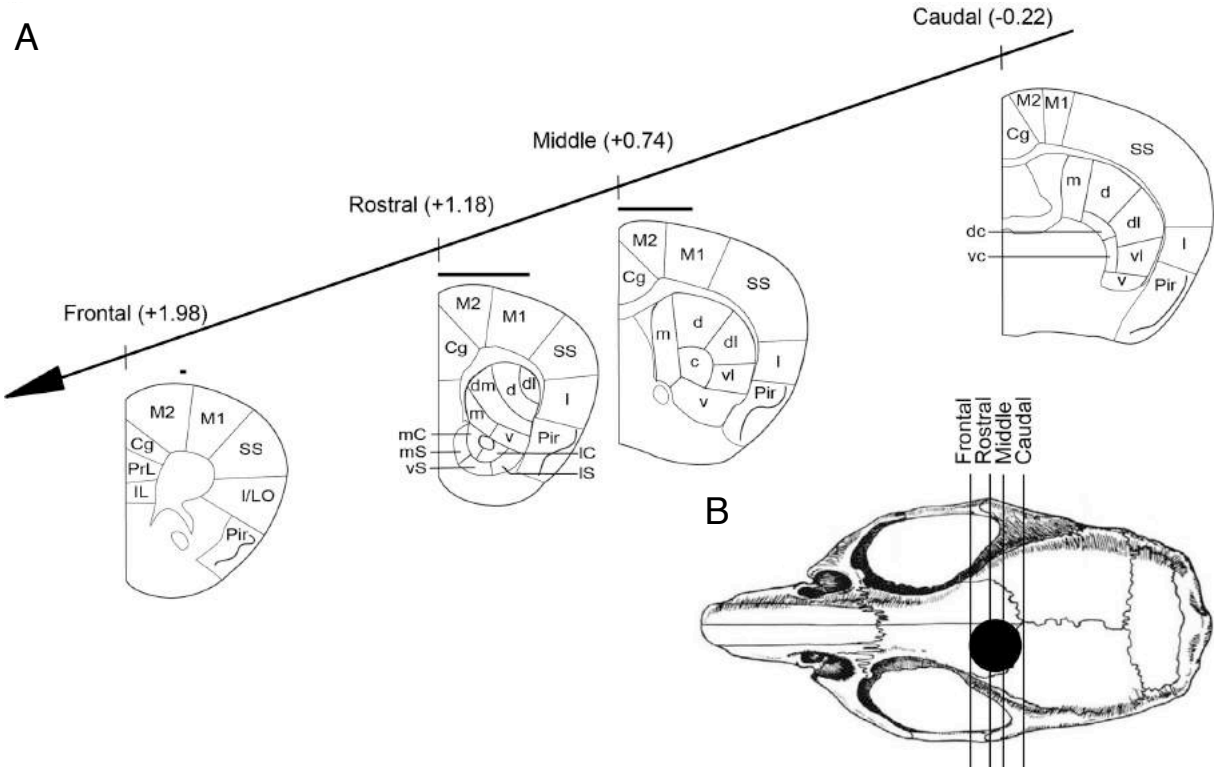


Figure 36 A) Régions du cortex et du striatum ou l'expression de Zif268 a été analysée (sur 4 niveaux : frontal, rostral, moyen et caudal). Trait noir : position de l'électrode. Voir Figure 22. B) Position de l'électrode (rond noir) par rapport aux différents niveaux de coupe.

L'induction de Zif268 par la cocaïne était évaluée par hybridation *in situ*. Vingt-deux régions corticales et 23 secteurs du striatum définis par leurs principales afférences corticales ont été analysés (répartis sur 4 niveaux de coupe, Figure 36).

Le protocole expérimental ainsi que les principaux résultats comportementaux sont synthétisés dans la figure Figure 37.

Un des résultats marquants que nous avons obtenu est que l'effet de la tDCS sur l'expression de Zif268 n'était pas latéralisé (*i.e.* pas de différence d'effet entre l'hémisphère droit et l'hémisphère gauche bien que l'anode soit placée de façon asymétrique 1 mm vers la gauche par rapport au bregma). Nous avons en conséquence moyenné les effets obtenus dans les hémisphères droit et gauche.

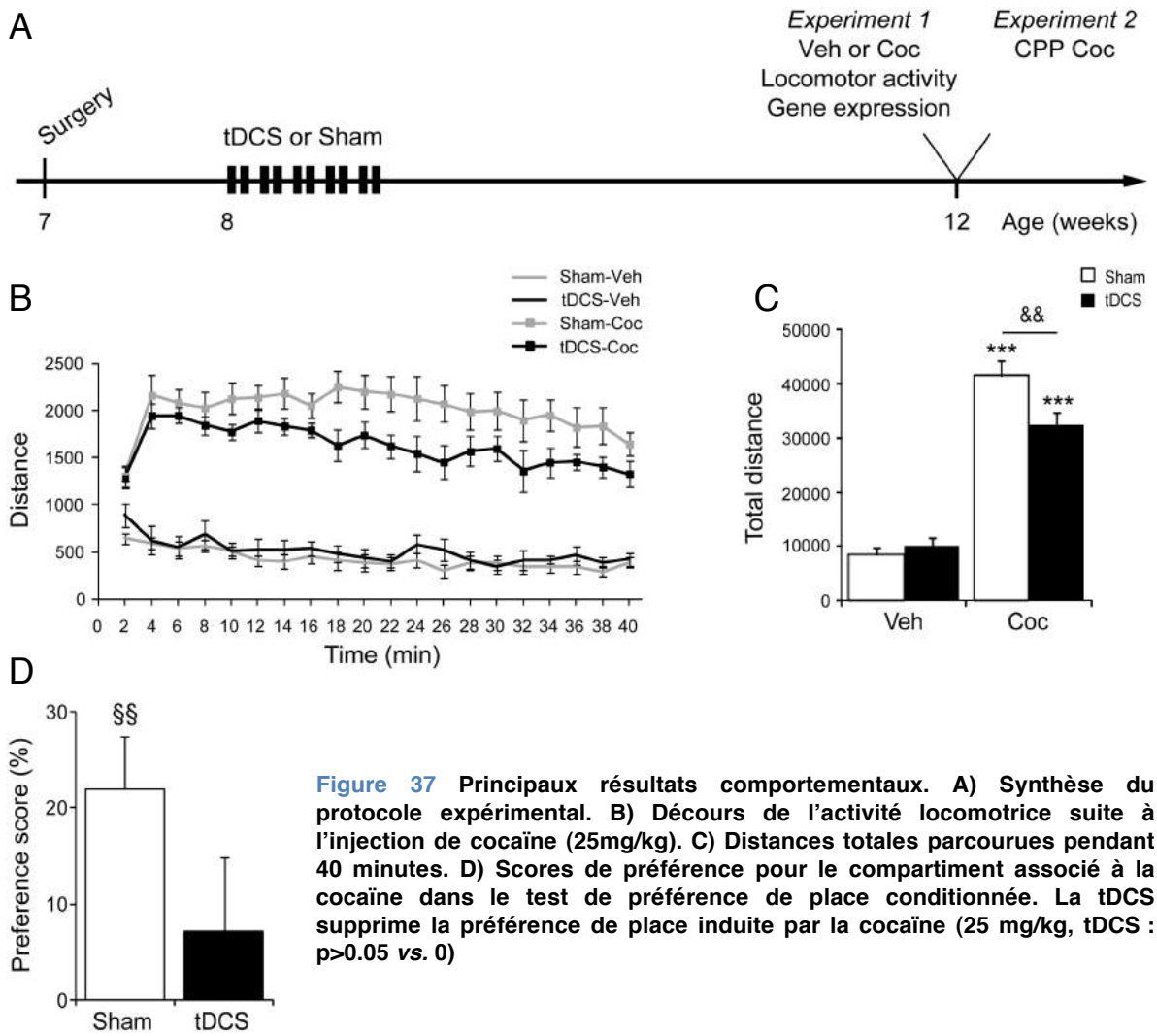


Figure 37 Principaux résultats comportementaux. A) Synthèse du protocole expérimental. B) Décours de l'activité locomotrice suite à l'injection de cocaïne (25mg/kg). C) Distances totales parcourues pendant 40 minutes. D) Scores de préférence pour le compartiment associé à la cocaïne dans le test de préférence de place conditionnée. La tDCS supprime la préférence de place induite par la cocaïne (25 mg/kg, tDCS : p>0.05 *vs.* 0)

La tDCS induisait une augmentation de l'expression basale de Zif268 (3 semaines après la dernière stimulation) principalement dans le striatum (sur les niveaux « rostral » et « moyen ») mais également dans le cortex (cortex piriforme et cortex cingulaire principalement) (résultats non présentés).

La tDCS réduisait aussi l'expression génique induite par la cocaïne (Figure 38).

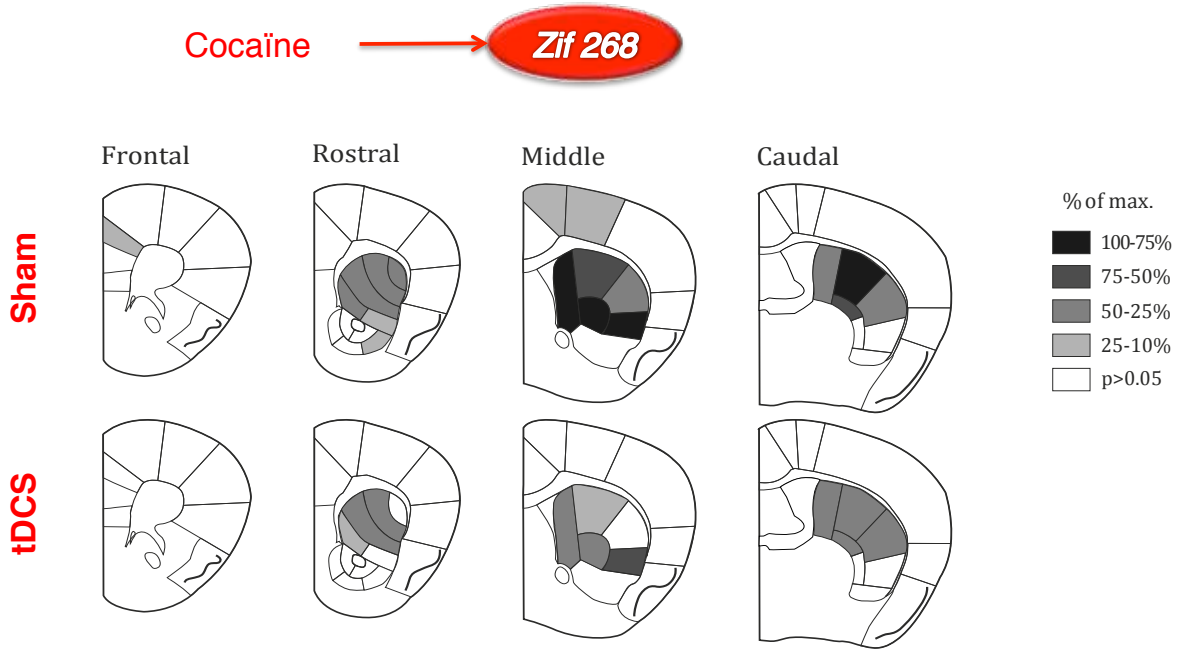


Figure 38 Cartographie de l'induction de Zif268 par la cocaïne. Les valeurs sont codées en utilisant une échelle de gris (augmentation maximale : 100%, noir). La tDCS diminue l'induction de Zif268 par la cocaïne dans le cortex et dans le striatum

On note que la tDCS affectait des circuits anatomiques spécifiques (en particulier les boucles sensorimotrices et associatives) plutôt que la zone située directement en regard de l'électrode.

En conclusion, nous avons montré pour la première fois que la tDCS réduit les effets moléculaires de la cocaïne dans les circuits cortico-striataux impliqués dans l'addiction (compulsion/habitudes, boucles sensorimotrices et associatives), les effets comportementaux de la cocaïne (activation locomotrice), ainsi que son effet hédonique (cf. article en annexe, en révision, Addiction Biology).

g) Perspective 1 : Neurogenèse hippocampique

La neurogenèse chez l'adulte serait impliquée dans les phénomènes d'apprentissage (Abrous and Wojtowicz, 2015; Deng *et al*, 2010; Drapeau *et al*, 2003; Dupret *et al*, 2008; Tronel *et al*, 2015) et dans la physiopathologie de la dépression (Miller and Hen, 2015; Sahay and Hen, 2007). La neurogenèse est donc un mécanisme candidat pouvant sous-tendre les effets pro-mnésiques et

antidépresseurs de la tDCS. Nous étudions actuellement l'impact de la tDCS sur la neurogenèse hippocampique chez l'adulte (en collaboration avec les Docteurs Yvan Peterschmitt et Pierre-Yves Risold). Nous avons dans un premier temps exploré la prolifération cellulaire (en utilisant comme technique l'immunohistochimie ; bromodeoxyuridine, BrdU ; Figure 39).

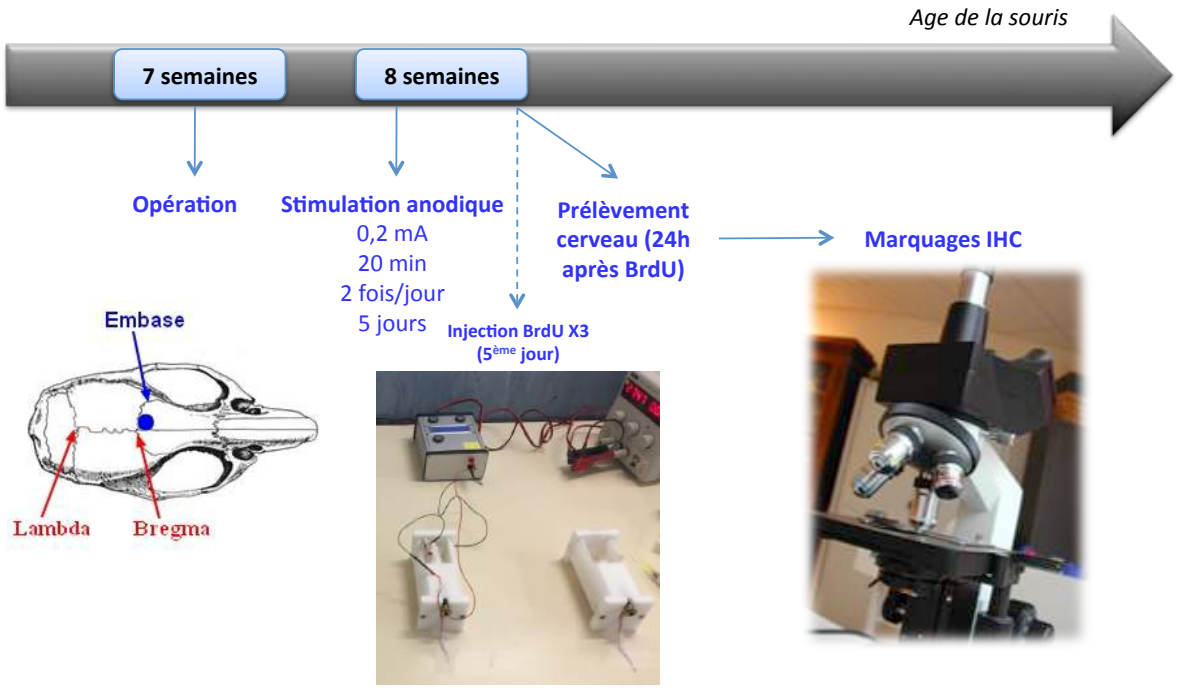


Figure 39 Protocole expérimental pour étudier l'effet de la tDCS sur la prolifération cellulaire dans le gyrus denté de l'hippocampe. La BrDU est injecté à trois reprises (6h d'intervalle entre les injections) lors du cinquième (et dernier) jour de stimulation électrique. Les cerveaux sont récupérés 24h plus tard (fixés) et le nombre de cellules BrdU-positives est compté (immunohistochimie).

Nos résultats préliminaires montrent que la prolifération cellulaire est augmentée par la tDCS aussi bien dans le gyrus denté droit que gauche (~47% d'augmentation en moyenne, Figure 40). Par ailleurs, cette augmentation est significative dans la partie ventrale (plutôt impliquée dans la régulation du stress et des émotions, ~51% d'augmentation) et dorsales (plutôt impliquée dans les fonctions cognitives, ~32% d'augmentation) (Fanselow and Dong, 2010; Kheirbek and Hen, 2011). Des études complémentaires portant sur la différenciation de ces nouvelles cellules et leur éventuelle intégration au sein de circuits préexistants sont en cours au laboratoire. Une étudiante que j'encadre en Master 2 « Physiologie, Neurosciences et Comportement » a été recrutée à plein temps pour l'année universitaire 2015-2016 afin de tenter de répondre à ces questions.

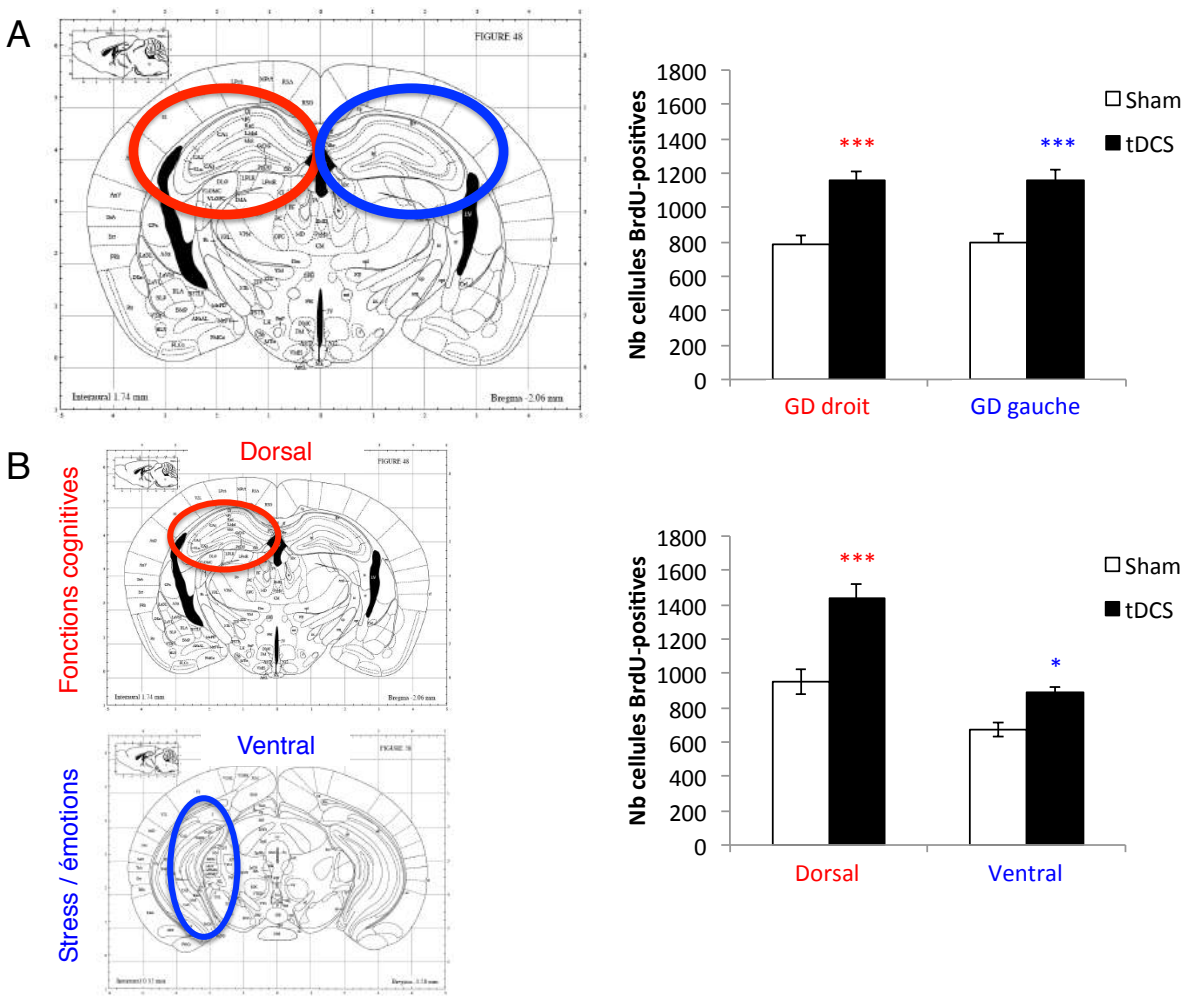


Figure 40 A) Nombre moyen de cellules BrdU-positives dans le gyrus denté droit et gauche en fonction du groupe expérimental. ***p<0,001 *versus* sham. B) Nombre moyen de cellules BrdU positives dans l'hippocampe dorsal et dans l'hippocampe ventral en fonction du groupe expérimental. ***p<0,001 et *p<0,05 *versus* sham.

h) Perspective 2 : tDCS lors de l'adolescence et trajectoire développementale du cortex

Pour finir, j'ai initié une collaboration avec le Professeur Kuei Tseng à la Chicago Medical School. La tDCS semble être bien tolérée chez l'adulte mais il existe peu d'études portant sur ses possibles effets à long terme chez l'adolescent (Krishnan *et al*, 2015). L'équipe du Professeur Tseng a démontré que les interneurones GABAergiques présents dans le cortex préfrontal évoluent de façon spécifique lors de l'adolescence. Les quantités d'interneurones GABAergiques à parvalbumine sont augmentées entre le jour postnatal 30 (début de l'adolescence) et le jour postnatal 70 (jeunes adultes) chez le rat. Au contraire, les taux

d'interneurones à calrétinine diminuent lors de la même période alors que les quantités d'interneurones à calbindine n'évoluent pas (Caballero *et al*, 2014). Cette trajectoire développementale particulière dans le cortex préfrontal est affectée si on expose le cerveau adolescent à des perturbations comme par exemple un traitement avec de la cocaïne. Ainsi, une exposition chronique à de la cocaïne lors de l'adolescence (1 injection/jour, 20 mg/kg i.p., pendant 5 jours, jours postnataux 35-40) supprime l'augmentation des interneurones à parvalbumine normalement observée pendant cette période (Cass *et al*, 2013).

Nous souhaiterions déterminer si l'exposition à la tDCS pendant l'adolescence affecte la trajectoire développementale de la maturation du cortex préfrontal (modification des quantités d'interneurones). Dans une première expérience, nous avons évalué si notre protocole de tDCS (20 minutes, 2 fois par jour, pendant 5 jours consécutifs, jours postnataux 35-39) module le nombre d'interneurones à parvalbumine et à calretinine dans le cortex préfrontal. Les cerveaux ont été récupérés au jour postnatal 42 et les quantités de neurones évaluées par immunohistochimie à fluorescence. Nos résultats, qui doivent être confirmés, semblent indiquer que la tDCS diminue les quantités d'interneurones à parvalbumine dans le cortex infralimbique. D'autres expériences complémentaires doivent être réalisées pour étoffer ces résultats préliminaires.

F-3) Publications résultant de ces travaux

Peer-reviewed publications:

1) Pedron S., Beverley J., Haffen E., Andrieu P., Steiner H, and **Van Waes V.** Transcranial direct current stimulation in mice produces long lasting attenuation of cocaine-induced gene regulation in striatum and cortex and reduces behavioral responses to cocaine.
En révision, Addiction Biology, 2016

2) Bennabi D.*, Pedron S.*, Haffen E., Monnin J., Peterschmitt Y., and **Van Waes V.**

Transcranial direct current stimulation for cognitive enhancement: from clinical research to animal models

Frontiers in Systems Neuroscience, Vol. 8, Art. 159, 2014

3) Pedron S., Monnin J., Haffen E., Sechter D., and **Van Waes V.**

Repeated transcranial direct current stimulation prevents abnormal behaviors associated with abstinence from chronic nicotine consumption

Neuropsychopharmacology Vol. 39 (4) p 981-988, 2014

Other publications:

1) Pedron S., Coune F., Haffen E., Andrieu P., Sechter D., Naassila M., Gonzalez-Marin M.C., and **Van Waes V.**

Effets de la stimulation transcrânienne par courant continu sur l'auto-administration d'alcool chez la souris.

Cahier de l'IREB n°22, in press, 2015.

2) Pedron S. and **Van Waes V.**

Effets de la stimulation transcrânienne par courant continu chez la souris: études comportementales

PUFC Presses Universitaires Franche-Comté, 2014

3) Pedron S., Monnin J., Andrieu P., Nicolier M., Millot J., Sechter D., Haffen E., and **Van Waes V.**

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PARTIE III : SELECTION DE PUBLICATIONS

Liste des publications

- **Publication #1**

Van Waes V., Enache M., Dutriez I., Lesage J., Morley-Fletcher S., Vinner E., Lhermitte M., Vieau D., Maccari S., and Darnaudéry M.

Hyporesponse of the hypothalamic-pituitary-adrenocortical axis after an ethanol challenge in prenatally stressed adolescent male rats

European Journal of Neuroscience Vol. 24(4), p1193-1200, 2006.

- **Publication #2**

Van Waes V., Enache M., Zuenna A.R., Mairesse J., Nicoletti F., Vinner E., Lhermitte M., Maccari S., and Darnaudéry M.

Ethanol attenuates spatial memory deficits and increases mGlu1a receptor expression in the hippocampus of rats exposed to prenatal stress

Alcoholism: Clinical and experimental research Vol. 33(8), p1346-1354, 2009.

- **Publication #3**

Van Waes V., Enache M., Berton O., Vinner E., Lhermitte M., Maccari S., and Darnaudéry M.

Effect of prenatal stress on alcohol preference and sensitivity to chronic alcohol exposure in male rats

Psychopharmacology Vol. 214(1), p197-208, 2011.

- **Publication #4**

Van Waes V., Beverley J., Marinelli M., and Steiner H.

SSRI antidepressants potentiate methylphenidate (Ritalin)-induced gene regulation in the adolescent striatum

European Journal of Neuroscience Vol. 32, p435-447, 2010.

- **Publication #5**

Steiner H. and **Van Waes V.**

Addiction-Related Gene Regulation: Risks of exposure to cognitive enhancers vs. other psychostimulants

Progress in Neurobiology Vol. 100, p60-80, 2013

- ## Publication #6

Van Waes V., Ehrlich S., Beverley J., and Steiner H.

Fluoxetine potentiation of methylphenidate-induced gene regulation in striatal output pathways: Potential role for 5HT1B receptor

Neuropharmacology, Vol. 89C, p77-86, 2015

- ## Publication #7

Van Waes V., Beverley J., Siman H., Tseng K.Y, and Steiner H.

CB1 cannabinoid receptor expression in the striatum: association with corticostriatal circuits and developmental regulation

Frontiers in Neuropharmacology Vol. 3, Art. 21, 2012

- ## Publication #8

Van Waes V., Tseng K.Y, and Steiner H.

GPR88: a putative signaling molecule predominantly expressed in the striatum; Cellular localization and developmental regulation

Basal Ganglia Vol. 1(2), p83-89, 2011

- ## Publication #9

Pedron S., Monnin J., Haffen E., Sechter D., and **Van Waes V.**

Repeated transcranial direct current stimulation prevents abnormal behaviors associated with abstinence from chronic nicotine consumption

Neuropsychopharmacology Vol. 39 (4), p981-988, 2014

- ## Publication #10

Pedron S., Beverley J., Haffen E., Andrieu P., Steiner H, and **Van Waes V.**

Transcranial direct current stimulation in mice produces long lasting attenuation of cocaine-induced gene regulation in striatum and cortex and reduces behavioral responses to cocaine

Addiction Biology, 2016, In Press

Publication 1

European Journal of Neuroscience, Vol. 24, pp. 1193–1200, 2006 doi:10.1111/j.1460-9568.2006.04973.x

Hypo-response of the hypothalamic-pituitary-adrenocortical axis after an ethanol challenge in prenatally stressed adolescent male rats

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Keywords: adrenocorticotropic hormone, alcohol, corticosterone, corticotropin-releasing hormone, maternal stress

Abstract

The period of adolescence and environmental factors, such as stress, are important in determining ethanol vulnerability in both humans and rats. Ethanol is a powerful activator of the hypothalamic-pituitary-adrenal (HPA) axis but attenuated responses of the HPA axis to ethanol have been described in populations with a high risk of ethanol abuse. In rats, prenatal stress leads to prolonged stress-induced corticosterone secretion and increases the vulnerability to drugs of abuse, such as amphetamine and nicotine in adulthood and 3,4-methylenedioxymethamphetamine in adolescent rats. The aim of the present study was to assess the impact of a prenatal stress on HPA axis responsiveness to a moderate dose of ethanol (1.5 g/kg i.p.) in adolescent male rats (28 days old). The parameters evaluated were plasma adrenocorticotropic hormone, plasma corticosterone and mRNA expression of HPA axis central markers (mineralocorticoid receptor, glucocorticoid receptor, corticotropin-releasing hormone and pro-opiomelanocortin). Contrary to prior expectations, our results demonstrate that prenatal stress blunts the HPA axis responsiveness to a moderate dose of ethanol in adolescent rats in spite of similar blood ethanol levels. These data suggest that prenatal stress may have the opposite effect on the response to stress depending on the attributes of the stressor stimulus. They thus raise questions about the possible impact of prenatal stress on the further development of ethanol vulnerability.

Introduction

The hypothalamo-pituitary-adrenocortical (HPA) axis, a major component of the stress response, modulates ethanol intake in both humans and animals (Phillips *et al.*, 1997; Brady & Sonne, 1999). Studies conducted in rodents suggest the existence of complex links between the HPA axis and ethanol vulnerability. Ethanol is a well-known powerful activator of the HPA axis (Rivier & Lee, 1996) and experimental manipulations of this axis modify spontaneous ethanol consumption. Indeed, the infusion of corticosterone in the ventral striatum facilitates ethanol intake (Fahlke & Hansen, 1999) whereas adrenalectomy reduces it (Lamblin & De Witte, 1996; Fahlke & Eriksson, 2000). In a similar way, ethanol intake is reduced in a dose-dependent manner by intraperitoneal injections of mifepristone, a glucocorticoid receptor (GR) antagonist (Koenig & Olive, 2004).

In rats, application of repeated restraint stress on pregnant dams induces a long-lasting alteration of the HPA axis in the offspring (for review see Maccari *et al.*, 2003). Prenatally stressed animals display reduced levels of both mineralocorticoid receptor (MR) and GR in the hippocampus (Henry *et al.*, 1994; Maccari *et al.*, 1995; Koehl *et al.*, 1999), as well as a prolonged stress-induced corticosterone secretion after restraint stress or novelty exposure (Maccari *et al.*, 1995; Morley-Fletcher *et al.*, 2003). Interestingly, previous works indicate that prenatal stress induces a greater vulnerability to several drugs of abuse. Thus, prenatally stressed rats exhibit a facilitation of amphetamine-induced sensitization (Henry *et al.*, 1995), an enhancement of amphetamine self-administration (Deminiere *et al.*, 1992), an increase in nicotine-induced locomotor activity (Koehl *et al.*, 2000) and an increase in motor alterations induced by 3,4-methylenedioxymethamphetamine ('ecstasy') (Morley-Fletcher *et al.*, 2004). Despite the fact that prenatal stress causes HPA axis disturbances and that this axis plays a significant role in ethanol intake, the impact of prenatal stress on the ethanol-induced HPA axis activation remains unclear (DeTurck & Pohorecky, 1987; Weinberg, 1987).

In humans, adolescence is a specific age known to be crucial for risk of ethanol abuse (Chung *et al.*, 2005). Similarly, a peculiar ontogenetic phase (28–60 days old) qualified as 'adolescence' is described in rats as a period of increased vulnerability to ethanol (Smith, 2003). During adolescence, rats display a relative insensitivity to many ethanol effects, including ethanol-induced corticosterone release (Silveri & Spear, 2004). This insensitivity could contribute to the increased ethanol intake reported in adolescent rats (Doremus *et al.*, 2005). Our study was designed to investigate whether prenatal stress can affect the neuroendocrine HPA axis response to a moderate dose of ethanol during the adolescence period in rats. For this purpose, we examined,

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in 28-day-old control and prenatally stressed rats, the impact of an ethanol challenge (1.5 g/kg) on the time course of plasma adrenocorticotropic hormone (ACTH) and corticosterone levels, as well as on the gene expression of several central markers of the HPA axis.

Materials and methods

Animals

Control and prenatally stressed male rats (28 days old) obtained from litters bred in our laboratory (Villeneuve d'Ascq, France) were used. Rats were individually housed in a temperature- (22 ± 2 °C) and humidity- (60%) controlled animal room on a 12-h light/dark cycle (light on at 07:00 h) with *ad libitum* access to food and water. Manipulation of the animals was conducted in accordance with the principles of laboratory animal care published by the French Ethical Committee and the rules of the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Prenatal stress procedure

Thirty nulliparous female Sprague Dawley rats (Harlan, France), weighing 200–225 g, were group-housed for at least 10 days before mating. Subsequently, females were individually housed overnight with a sexually experienced male rat (400 g) and vaginal smears were examined on the following morning. The day on which the smear was sperm positive was determined as embryonic day 0. Each pregnant female was then single-housed and randomly assigned to control or stress groups. Control dams ($n = 15$) were left undisturbed whereas stressed dams ($n = 15$) were subjected to a repeated restraint stress procedure as previously described (Maccari *et al.*, 1995; Morley-Fletcher *et al.*, 2004). The stress procedure consisted of restraining the pregnant dam in a transparent cylinder (7.5 cm diameter, 19 cm long) under a bright light (650 lux) for 45 min three times daily from day 11 of pregnancy until delivery. Stress sessions were conducted during the light phase but the schedule of sessions was not fixed in order to reduce a possible habituation to repeated restraint stress. After weaning (postnatal day 21), male offspring from litters with similar sex ratios were housed individually. A maximum of two males per litter were used for each treatment to avoid any litter effect.

Ethanol treatment

Adolescent prenatally stressed and control male rats (28 days old) received an i.p. injection of ethanol (1.5 g/kg, 20% v/v diluted in NaCl 0.9%; ethanol from Flourent-Brabant, France) or an equivalent volume of physiological saline and were killed by decapitation 30 min ($n = 42$), 60 min ($n = 38$) or 240 min ($n = 40$) later. The dose of 1.5 g/kg was chosen as a moderate dose to challenge the HPA axis based on previous works (DeTurck & Pohorecky, 1987; Ryabinin *et al.*, 1995). Animals removed from their home cage and immediately decapitated ($n = 19$) were used for basal conditions. Experiments were performed between 08:00 and 12:00 h to avoid circadian variations of the plasma corticosterone and ACTH concentrations (Koehl *et al.*, 1999). To minimize the stress, the injection conditions had been simulated repeatedly during the week preceding the experiment by daily handling of the animals and pressure exerted on their belly with a pointed metal object. A preliminary study was performed to control for non-specific corticosterone increases after the ethanol administration. Rats were injected i.p. with 3 g/kg ethanol (20% v/v) diluted in a saline solution with or without a local anaesthetic agent (10% lidocaine, Sigma-Aldrich, France). No difference in corticosterone levels was observed 30 min after the injection between animals injected with or without the addition of lidocaine (NaCl, 9.1 μg/dL; NaCl + lidocaine, 8.0 μg/dL; ethanol, 73.6 μg/dL; ethanol + lidocaine, 73.1 μg/dL; sham injection, 2.4 μg/dL). In consequence, local anaesthesia was not performed to avoid skews which could be caused by a possible interaction between stress and lidocaine metabolism (Saranteas *et al.*, 2002).

Plasma and tissue collections

Trunk blood samples (approximately 4 mL) were collected in chilled tubes containing 40 μL of 5% EDTA (Sigma Aldrich) and centrifuged at 2000 ***g*** for 15 min at 4 °C. Aliquots of plasma were stored at −20 °C until the assays. For *in situ* hybridization, the brain and pituitary gland were removed in the animals decapitated 60 or 240 min after the injection and then immediately frozen on dry ice. Brains and pituitary glands were stored at −80 °C until sectioning.

Hormone assays

Plasma corticosterone levels were determined with a radioimmunoassay kit (Kit ImmunChem™ Corticosterone ^{125}I RIA, ICN Biomedicals, France) using a highly specific corticosterone antiserum. The minimum level of detection was 0.1 μg/dL and the intra- and inter-assay coefficients of variation were 6.6 and 11.4%, respectively.

Plasma ACTH levels were determined with a radioimmunoassay kit (RSL ^{125}I hACTH, Biomedicals, France). The ACTH antibody cross-reacts 100% with $ACTH_{1\ 39}$ and $ACTH_{1\ 24}$ but < 1% with other pro-opiomelanocortin (POMC) derivatives. The detection threshold was 5 pg/mL and the intra- and inter-assay coefficients of variation were 7.3 and 10.6%, respectively.

In situ *hybridization*

Coronal sections (12 μm thick) of the brain through the hypothalamic paraventricular nucleus (PVN) (ranging from −1.3 to −2.12 mm posterior to bregma, according to the atlas of Paxinos & Watson, 1998) and the hippocampus (ranging from −2.12 to −3.80 posterior to bregma) as well as sections from the pituitary gland were made at −20 °C with a cryostat (CM3050 S, Leica, France). The sections were mounted onto gelatin-coated slides, dried on a slide warmer and kept at −80 °C. *In situ* hybridization was performed as previously described (Lesage *et al.*, 2001).

The corticotropin-releasing hormone (CRH) probe was a 770-bp fragment of the rat CRH gene subcloned into pGEM4 (supplied by Dr K.E. Mayo, North-Western University, USA) and linearized with *Hind*III (antisense probe). The POMC probe was a 397-bp fragment of the rat POMC gene subcloned into pSP65 (supplied by Dr M. Grino, INSERM UMR 626, Marseille, France) and linearized with *Bam*HI (antisense probe). The MR and GR probes were 513- and 674-bp fragments of rat complementary DNA clones encoding the 3′ regions of MR and GR messenger RNA, subcloned into pGEM4 and pGEM3, respectively (supplied by Dr J. Seckl, University of Edinburgh, Edinburgh, UK) and linearized with *Hind*III and *Ava*I, respectively (antisense probes). Riboprobes were labelled using [^{35}S]-dUTP (1300 Ci/mmol; Amersham Biosciences, Germany) with the Sp6/T7 Transcription Kit (Roche Diagnostics, Germany). Controls included hybridization with sense probes and no specific hybridization signals were observed under these conditions. For each probe, all of the slides were exposed together on one X-ray film (Biomax-MR,

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Kodak, France). Autoradiograms were digitized during the same session.

Quantification of the hybridization signal

The impact of ethanol on the HPA axis was evaluated on the MR and GR mRNA expression in the hippocampus (CA1, CA2, CA3 and dentate gyrus), on the CRH mRNA expression in the PVN and on the POMC mRNA expression in the adenopituitary. POMC mRNA expression was also assessed in the intermediate lobe of the pituitary as a control area not directly involved in HPA axis function. Four sections per brain area and six sections of the pituitary gland from each animal were analysed. Hybridization signals were quantified on the autoradiogram films as previously described (Lesage *et al.*, 2001). The optical density of the hybridized signal was measured using a GS-700 densitometer coupled with computer-assisted image analysis using MULTI-ANALYST software (Biorad Laboratories, France). Optical densities for the probe signal and for the background of tissue, expressed as optical density/mm^2, were measured on the same section. Data were then expressed as percentages of control values.

Blood ethanol levels

Blood ethanol levels were measured using a gas chromatograph coupled with a flame ionization detector (5890 series II, Hewlett Packard, France). Acetonitrile was used as the internal standard. To 100 μL plasma were added 100 μL NaOH (0.5 M), 100 μL zinc sulphate (30%) and 50 μL acetonitrile (pure). The whole was centrifuged at 1000 ***g*** for 10 min at 4 °C, the supernatant was collected and 3 μL of the latter was injected into the gas chromatograph. The limit of quantification of the method was 0.01 g/L.

Statistics

All data are presented as means ± SEM. Hormonal measures and hybridization were analysed using three-way ANOVA with group (control, prenatal stress), treatment (NaCl, ethanol) and time postinjection (hormones, 30, 60 and 240 min; mRNA, 60 and 240 min) as between-subject variables. For the hybridization study, control and prenatally stressed animals were first compared in NaCl-treated animals, using data expressed as percentages of the control group. The ethanol effect was then assessed on data expressed as a percentage of the respective NaCl groups (control NaCl or prenatal stress NaCl). Blood ethanol levels were compared by two-way ANOVA with group (control, prenatal stress) and time postinjection (30, 60 and 240 min) as between-subject variables. For each ANOVA, single or multiple R^2 was reported to indicate the percentage of variance explained by the model. These analyses were followed by posthoc analyses with Newman-Keuls tests for specific comparisons. Student's *t*-tests were employed to compare the percentages of mRNA with 100% (i.e. no change). An independent Student's *t*-test was also used to examine initial body weight differences between control and prenatal stress groups. Correlations were calculated using Pearson's test. Significance was set at $P < 0.05$.

Results

Body weight

Rats prenatally exposed to stress showed a significant reduction of their body weight at 28 days compared with control rats (control, 71.29 ± 1.25 g; prenatal stress, 67.64 ± 1.21 g; Student's *t*-test, $t = 2.33$, d.f. = 115, $P < 0.05$).

Plasma adrenocorticotropic hormone and corticosterone levels after ethanol injection

Plasma ACTH (Fig. 1A) and corticosterone (Fig. 1B) levels in the animals not injected (i.e. basal) were similar in prenatally stressed and control rats (see left panels in Fig. 1). As shown in the right panels in Fig. 1, ethanol injection significantly increased both plasma ACTH (ANOVA, $F_{1,98} = 40.78$, $P < 0.001$; $R^2 = 0.14$) and corticosterone ($F_{1,102} = 61.77$, $P < 0.001$; $R^2 = 0.17$) levels. However, the effect of ethanol differed between prenatally stressed and control rats (ACTH 60 min post injection, group × treatment effect, $F_{1,30} = 10.06$, $P < 0.01$, $R^2 = 0.57$; corticosterone, group × treatment × time effect, $F_{2,102} = 3.01$, $P = 0.054$, $R^2 = 0.71$). At 60 min after the ethanol injection, ACTH (Newman-Keuls, $P < 0.001$) and corticosterone (Newman-Keuls, $P < 0.001$) levels were lower in prenatally stressed rats compared with control rats. Plasma ACTH and plasma corticosterone were positively correlated in the control ($r = 0.86$, $P < 0.001$) and prenatal stress ($r = 0.78$, $P < 0.001$) groups.

mRNA levels of central hypothalamic-pituitary-adrenal axis markers in NaCl-treated animals

Prenatal stress decreased MR ($F_{1,27} = 3.95$, $P = 0.06$; $R^2 = 0.10$) and GR ($F_{1,27} = 4.74$, $P < 0.05$; $R^2 = 0.11$) mRNA levels (expressed as percentages of control group) in the hippocampus (Table 1). Subfield analysis indicated that the expression of MR

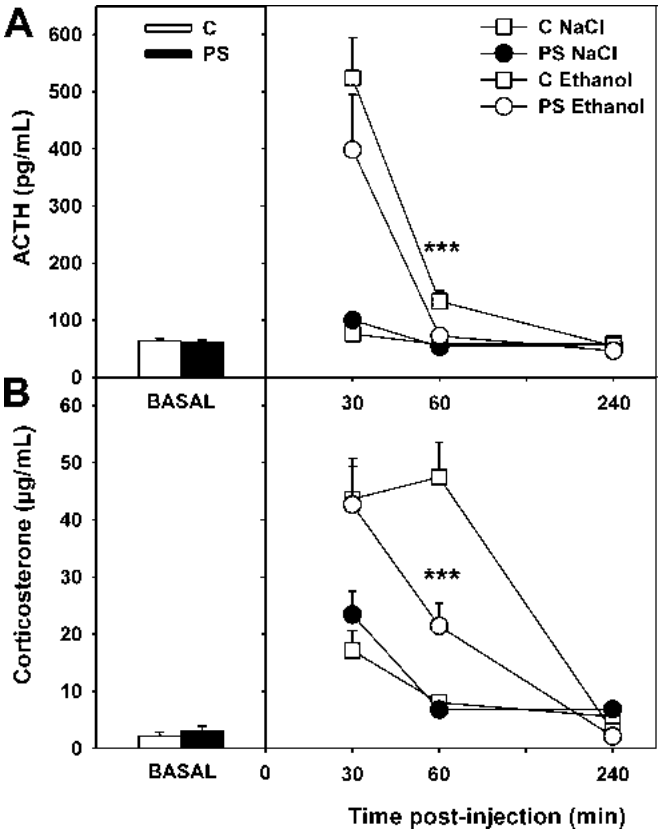

FIG. 1. (A) Plasma adrenocorticotropic hormone (ACTH) levels (pg/mL) and (B) plasma corticosterone levels (μg/dL) in control (C) and prenatal stress (PS) groups, in basal condition (left panel, $n = 19$) or 30 min ($n = 42$), 60 min ($n = 38$) and 240 min ($n = 40$) after ethanol (1.5 g/kg) or NaCl (0.9%) intraperitoneal injection (right panel). ***$P < 0.001$ control ethanol vs. prenatal stress ethanol.

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TABLE 1. Semiquantitative analysis of MR and GR mRNA levels in control and prenatal stress groups treated with NaCl in the whole hippocampus and in its different subfields

	MR mRNA levels (percentage of control group)		GR mRNA levels (percentage of control group)	
	Control	Prenatal stress	Control	Prenatal stress
Whole hippocampus (HPC)	100 ± 2.7	92 ± 3.02 ($P = 0.06$)	100 ± 4.4	88 ± 3.6*
CA1	100 ± 3.2	92 ± 5.51	100 ± 3.8	93 ± 3.3
CA2	100 ± 2.7	93 ± 3.47	100 ± 5.7	84 ± 5.2*
CA3	100 ± 3.8	89 ± 5.47*	100 ± 5.3	84 ± 4.4*
Dentate gyrus	100 ± 2.1	92 ± 4.31 ($P = 0.06$)	100 ± 4.1	92 ± 3.0

GR, glucocorticoid receptor; MR, mineralocorticoid receptor. *$P < 0.05$ control ($n = 16$) vs. prenatal stress ($n = 15$) groups.

mRNA in prenatally stressed rats was significantly reduced in the CA3 ($F_{1,27} = 4.55$, $P < 0.05$; $R^2 = 0.11$) and tended to be decreased in the dentate gyrus ($F_{1,27} = 3.87$, $P = 0.06$; $R^2 = 0.08$). The expression of GR mRNA was significantly lower in prenatally stressed animals in the CA2 ($F_{1,27} = 4.68$, $P < 0.05$; $R^2 = 0.09$) and CA3 ($F_{1,27} = 5.61$, $P < 0.05$; $R^2 = 0.13$). The PVN CRH mRNA levels were similar between control and prenatally stressed animals (control NaCl, 100 ± 3.1%; prenatal stress NaCl, 103 ± 4.5%; $F_{1,27} = 1.02$, $P = 0.65$). Control NaCl and prenatal stress NaCl groups exhibited similar POMC mRNA levels in the anterior pituitary (control NaCl, 100 ± 4.0%; prenatal stress NaCl, 108 ± 4.1%, $F_{1,28} = 2.27$, $P = 0.14$) and intermediate lobe (control NaCl, 100 ± 2.8%; prenatal stress NaCl, 107 ± 3.5%, $F_{1,25} = 2.29$, $P = 0.14$).

Effect of ethanol administration on hippocampal mineralocorticoid receptor and glucocorticoid receptor mRNA levels

Whatever the group or time, when considering the whole hippocampus, ethanol challenge had no effect on GR ($F_{1,56} = 0.43$, $P = 0.51$) and MR ($F_{1,56} = 1.50$, $P = 0.22$) mRNAs (data not shown). In contrast, ethanol modified MR mRNA in the dentate gyrus of the hippocampus ($F_{1,56} = 5.04$; $P < 0.05$, $R^2 = 0.07$, Fig. 2A and B). This effect mainly reflected a significant decrease in dentate gyrus MR mRNA after ethanol in controls (control group, $F_{1,28} = 6.49$, $P < 0.05$, $R^2 = 0.16$; prenatal stress group, $F_{1,28} = 0.57$, $P = 0.45$, Fig. 2A). The effect of ethanol on mRNA levels was similar at 60 and 240 min after the injection ($F_{1,28} = 0.04$, $P = 0.84$; control ethanol vs. 100%, 60 min, $P < 0.05$, 240 min, $P = 0.057$).

Effect of ethanol administration on paraventricular nucleus corticotropin-releasing hormone mRNA levels

As shown in Fig. 3A, the expression of CRH mRNA in the PVN was differently affected by ethanol according to the group and time (group × treatment × time effect, $F_{1,53} = 3.85$, $P = 0.05$; $R^2 = 0.28$). Posthoc analysis showed that ethanol administration elicited a transient increase in CRH mRNA levels 60 min post-treatment in the control group (Newman-Keuls, control ethanol 60 min postinjection vs. all other groups, $P < 0.01$; control ethanol 60 min vs. 100%, $P < 0.001$, Fig. 3A and B), whereas CRH mRNA levels remained unchanged in the prenatal stress group.

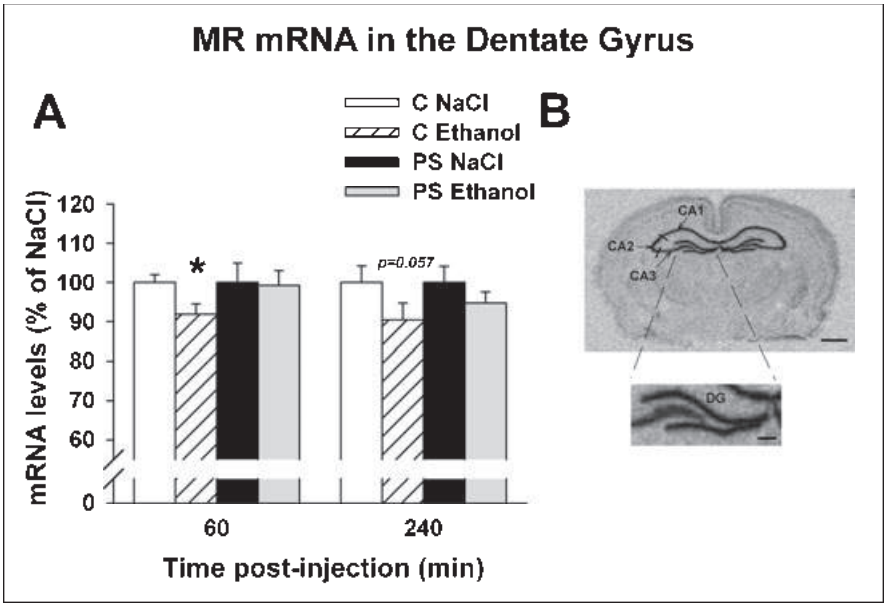

FIG. 2. (A) Semiquantitative analysis of dentate gyrus (DG) mineralocorticoid receptor (MR) mRNA levels (expressed as percentages of respective NaCl groups) in control (C) and prenatal stress (PS) groups, 60 and 240 min after the ethanol (1.5 g/kg) or the NaCl (0.9%) intraperitoneal injection ($n = 7$–9 per group). *$P < 0.05$ control ethanol vs. 100%. (B) Photomicrographs of brain coronal sections (bregma AP −3.6 mm) showing the *in situ* hybridization signal for MR mRNA in the different hippocampus (HPC) subfields (top, whole HPC, scale bar, 1.5 mm; bottom, dentate gyrus, scale bar, 0.25 mm).

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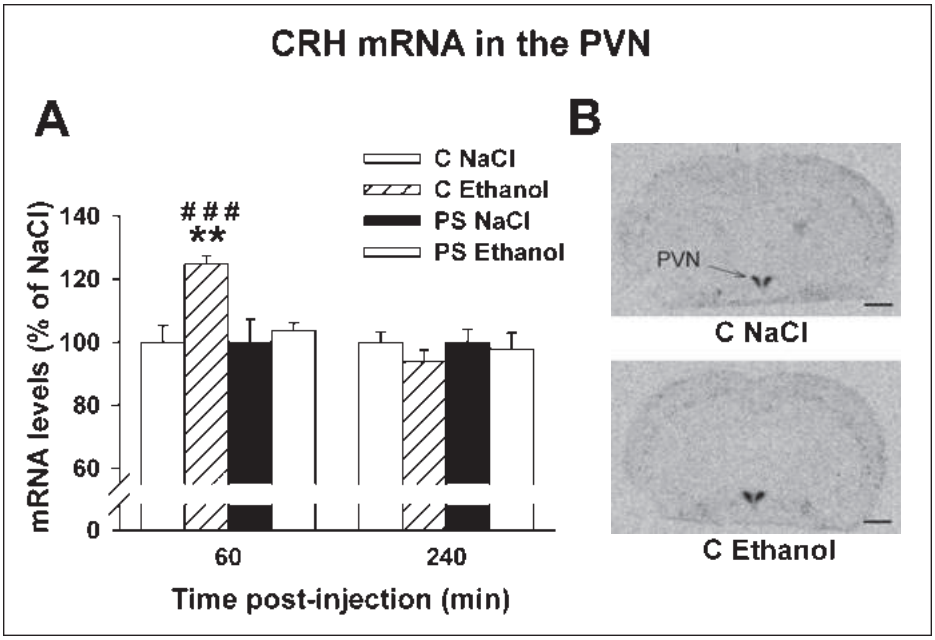

FIG. 3. (A) Semiquantitative analysis of paraventricular nucleus (PVN) corticotropin-releasing hormone (CRH) mRNA levels (expressed as percentages of respective NaCl groups) in control (C) and prenatal stress (PS) groups, 60 and 240 min after the ethanol (1.5 g/kg) or the NaCl (0.9%) intraperitoneal injection ($n = 7$–9 per group). $^{**}P < 0.01$ control NaCl vs. control ethanol; $^{###}P < 0.001$ control ethanol vs. 100%. (B) Photomicrographs of brain frontal sections (bregma AP −1.8 mm) showing the *in situ* hybridization signal for CRH mRNA in control NaCl (top) and control ethanol (bottom) groups 60 min postinjection. Scale bar, 1.5 mm.

Effect of ethanol administration on pituitary pro-opiomelanocortin mRNA levels

Anterior pituitary POMC mRNA levels were differently affected by the ethanol administration in control and prenatal stress groups (group × treatment effect, $F_{1,55} = 7.47$, $P < 0.01$; $R^2 = 0.20$, Fig. 4A). Ethanol administration induced a long-lasting increase in the expression of POMC mRNA in the anterior pituitary in control animals (control ethanol vs. all other groups, $P < 0.05$; control ethanol vs. 100%, 60 min, $P < 0.05$, 240 min, $P < 0.05$), whereas prenatally stressed rats were not affected (Fig. 4A and C). In the intermediate lobe, POMC mRNA levels were not altered by the ethanol challenge ($F_{1,46} = 0.84$, $P = 0.36$, Fig. 4B).

Blood ethanol levels after acute alcohol administration

As shown in Fig. 5, the blood ethanol levels decreased with time ($F_{2,51} = 99.75$, $P < 0.001$, $R^2 = 0.78$) and were undetectable 240 min after the injection. Exposure to a prenatal stress did not influence the pharmacokinetic of blood ethanol elimination ($F_{1,28} = 0.33$, $P = 0.57$). Subsequent analysis indicated that blood ethanol levels (30 and 60 min postinjection) were positively correlated to plasma ACTH values in prenatally stressed animals (30 min, $r = 0.81$, $P < 0.01$; 60 min, $r = 0.68$, $P < 0.05$). No significant correlations were reported in the control group.

Discussion

The present study was performed to investigate the HPA axis response of prenatally stressed adolescent rats to a single moderate dose of ethanol. Our results indicate that ethanol administration caused a rapid activation of the HPA axis in adolescent rats. Interestingly, this activation was attenuated in prenatally stressed rats.

We showed that ethanol injection produced an increase in ACTH and corticosterone plasma concentrations in adolescent rats. These results are in accordance with previous works performed in developing and mature animals (Rivier & Lee, 1996; Silveri & Spear, 2004). After ethanol administration, the gene expression of key markers of the HPA axis was modified in the control group whereas the prenatal stress group was unaffected. Thus, in control animals, ethanol challenge enhanced CRH and POMC mRNA levels, indicating that ethanol stimulates the expression of these two genes. Rivier and co-workers have previously reported an increase in CRH and POMC heteronuclear RNA levels in the PVN and anterior pituitary, respectively, after an acute ethanol administration but this was not clearly accompanied by enhanced mRNA levels in adult animals (Rivier & Lee, 1996; Ogilvie *et al.*, 1998; Lee *et al.*, 2004). Our study is the first to show that a rapid (60 min) increase in CRH and POMC mRNA level takes place after an acute administration of a moderate dose of ethanol in adolescent rats. Furthermore, we reported that the change of the POMC mRNA levels was restricted to the adenopituitary, which releases the ACTH, suggesting a specific effect of ethanol on HPA axis activation in this area. The study of corticosteroid receptor gene expressions after ethanol challenge revealed that MR mRNA levels were slightly but significantly reduced in the dentate gyrus of the control group. A recent report demonstrated that an acute dose of ethanol decreases the cell proliferation in the dentate gyrus of adolescent animals (Crews *et al.*, 2006). These results could reflect the high sensitivity of this brain area to the toxic effect of ethanol, even after an acute administration.

Previous works in the literature have demonstrated that prenatal stress induces alterations of the HPA activity in response to stressful stimuli in infant (Henry *et al.*, 1994), adolescent (Morley-Fletcher *et al.*, 2003), adult (Maccari *et al.*, 1995) and ageing (Vallee *et al.*, 1999) animals. Indeed, prenatally stressed rats exhibit a long-lasting increase in corticosterone levels after novelty (Henry *et al.*, 1994;

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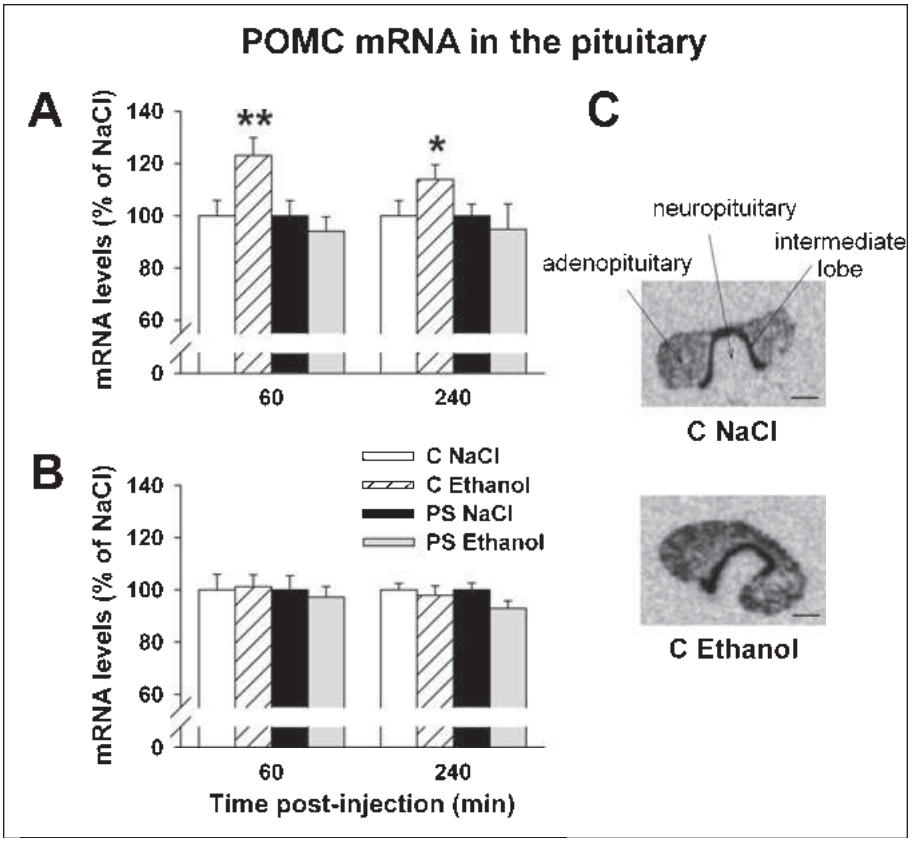

FIG. 4. Semiquantitative analysis of pro-opiomelanocortin (POMC) mRNA levels (expressed as percentages of respective NaCl groups) (A) in the anterior pituitary and (B) in the intermediate lobe of the pituitary in control (C) and prenatal stress (PS) groups, 60 and 240 min after the ethanol (1.5 g/kg) or the NaCl (0.9%) intraperitoneal injection ($n = 6$–9 per group). $^{**}P < 0.01$ and $^{*}P < 0.05$ control ethanol vs. 100%. (C) Photomicrographs of pituitary gland sections showing the *in situ* hybridization signal for POMC mRNA in control NaCl (top) and control ethanol (bottom) groups 60 min postinjection. Scale bar, 0.5 mm.

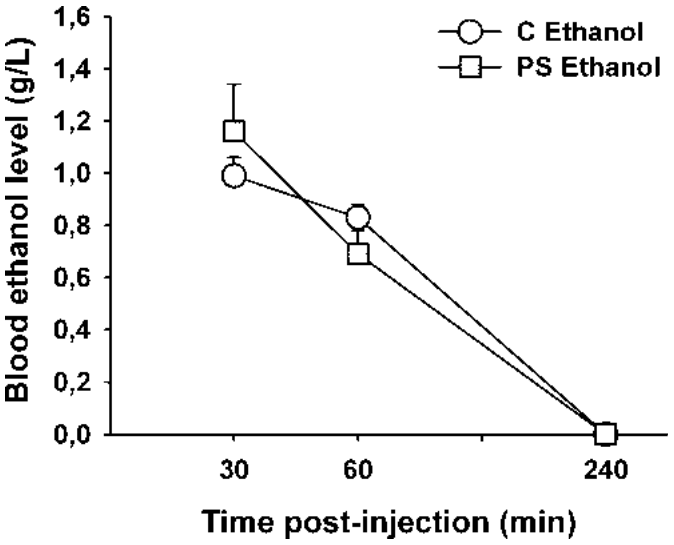

FIG. 5. Blood ethanol level (g/L) 30, 60 and 240 min after the intraperitoneal injection of ethanol (1.5 g/kg) in control (C) and prenatally stressed (PS) rats ($n = 9$–10 per group).

Maccari *et al.*, 1995) or restraint stress exposure (Morley-Fletcher *et al.*, 2003), suggesting an impairment of the negative feedback processes. This hypothesis is confirmed by binding analysis showing a decrease in the MR and GR density in the hippocampus of prenatally stressed rats (Maccari *et al.*, 1995; Barbazanges *et al.*, 1996; Koehl *et al.*, 1999). According to these data our *in situ* hybridization study reveals, for the first time, a global reduction of the relative quantities of mRNA coding MR and GR in the hippocampus of adolescent rats exposed to stress *in utero*. Furthermore, we suggest a possible dissociation between the hippocampal subfields in the alteration of MR and GR observed after prenatal stress.

Despite the reduced corticosteroid receptor mRNA levels, we did not observe an alteration of the negative HPA axis feedback processes after the ethanol injection in the prenatal stress group. In contrast, we report a lower increase in plasma ACTH and corticosterone levels following an ethanol injection in animals exposed to stress during the prenatal period. Furthermore, mRNA levels of central components of the HPA axis were not affected by ethanol administration in stressed animals. Blood ethanol levels were positively correlated to plasma ACTH values in prenatally stressed but not in control animals. This result could reflect the high interindividual variability observed in blood ethanol levels in the prenatal stress group after the administration of 1.5 g/kg ethanol. However, the assessment of the kinetic of blood ethanol levels indicates that the metabolic rate of ethanol was not affected by prenatal stress, suggesting that the blunted response in prenatally stressed rats is related to differences in their HPA axis and/or central nervous system response to ethanol rather than simply to varying levels of circulating ethanol. Considering previous studies on the impact of prenatal stress on HPA axis response to stress, the blunted HPA axis response to ethanol was unexpected. It could be hypothesized that prenatal stress induces an opposite effect on ACTH

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and corticosterone release according to the type of stimulation considered. Although an indirect action of ethanol cannot be excluded, the PVN has been proposed as being the primary site of ethanol action on the HPA axis (Redei *et al.*, 1988; Lee *et al.*, 2004). Therefore, differences in central mechanisms of stress integration, which are dependent on the stressor attributes, could explain our results. Indeed, 'processive' stressors (brain-generated), like restraint stress, require interpretation by higher brain structures than the HPA axis whereas 'systemic' stressors, like ethanol, involve an immediate physiological threat relayed directly to the PVN (Emmert & Herman, 1999; Herman *et al.*, 2003).

Our data obtained in prenatally stressed rats confirm and extend previous results obtained in adult animals by DeTurck & Pohorecky (1987) with another maternal stress model consisting of repeated handling of the pregnant dams during the last week of pregnancy. Moreover, as another study has shown that maternal restraint stress during the last 3 days of pregnancy does not affect the HPA response to ethanol in the offspring (Weinberg, 1987), our work underlines the importance of the temporal window during which the stress takes place in the prenatal stress-induced long-term effect on HPA axis reactivity to ethanol. The impact of the prenatal stress on the HPA axis response to an ethanol challenge could result from foetal programming of the HPA axis function by glucocorticoids (Barbazanges *et al.*, 1996). However, an indirect postnatal effect of prenatal stress exposure, via altered maternal care, cannot be excluded. Indeed, a recent report from Smith *et al.* (2004) shows that gestational stress (chronic restraint stress) impairs maternal care in rats. Furthermore, postnatal manipulations, such as handling or maternal separation, modulate the effect of prenatal ethanol exposure on the HPA axis (Ogilvie & Rivier, 1997) and maternal separation increases the ethanol preference in the offspring (Huot *et al.*, 2001).

Increased HPA reactivity to stress has been associated with a higher propensity to drug self-administration in animals (Piazza *et al.*, 1991). In contrast, adolescent animals which exhibit a dampened HPA response to several drugs of abuse, such as amphetamine, cocaine, morphine or ethanol (Bailey & Kitchen, 1987; Laviola *et al.*, 1995, 2002; Silveri & Spear, 2004), have been described as more sensitive to some of the rewarding effects of drugs (Smith, 2003; Doremus *et al.*, 2005). We describe here in prenatally stressed rats a hypo-response of the HPA axis to a moderate dose of ethanol (1.5 g/kg). Interestingly, clinical studies have reported that attenuated cortisol and ACTH responses to ethanol are associated with an increased risk for the development of alcoholism (Schuckit *et al.*, 1987, 1988). A recent report demonstrated that heavy social drinkers have a blunted cortisol response to a dose of 0.8 g/kg of alcohol (King *et al.*, 2006). Corticosterone presents reinforcing properties in rats (Deroche *et al.*, 1993; Piazza *et al.*, 1993) and its release after ethanol consumption may contribute to the rewarding effect of ethanol. In this context, it could be hypothesized that animals stressed during the prenatal period will need higher amounts of ethanol to obtain an equivalent ethanol appetitive effect. Prenatal stress in rats is associated with an increase in psychostimulant self-administration (Deminiere *et al.*, 1992) and change in dopaminergic function (Henry *et al.*, 1995). However, the processes underlying ethanol self-administration differed from psychostimulants. For example, contrary to cocaine self-administration, ethanol intake is unaffected by mesolimbic dopamine depletion (Roberts & Koob, 1982; Rassnick *et al.*, 1993). Therefore, it could be relevant to assess ethanol self-administration and the HPA response to psychostimulants in the model of adolescent rats exposed to prenatal stress.

In conclusion, this study shows that prenatal stress blunts the HPA response to an ethanol challenge in adolescent animals. These data suggest that prenatal stress may affect the response to stress in opposite ways depending on the attributes of the stressor stimulus. These findings indicate that prenatal stress could be a useful animal model for investigating the role of HPA function on alcohol vulnerability during adolescence.

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Abbreviations

ACTH, adrenocorticotropic hormone; CRH, corticotropin-releasing hormone; GR, glucocorticoid receptor; HPA, hypothalamo-pituitary-adrenocortical; MR, mineralocorticoid receptor; POMC, pro-opiomelanocortin; PVN, paraventricular nucleus.

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Publication 2

Ethanol Attenuates Spatial Memory Deficits and Increases mGlu1a Receptor Expression in the Hippocampus of Rats Exposed to Prenatal Stress

Vincent Van Waes, Mihaela Enache, Annarita Zuena, Jérôme Mairesse, Ferdinando Nicoletti, Elisabeth Vinner, Michel Lhermitte, Stefania Maccari, and Muriel Darnaudéry

Background: Although it is generally believed that chronic ethanol consumption impairs learning and memory, results obtained in experimental animals are not univocal, and there are conditions in which ethanol paradoxically improves cognitive functions. In the present work, we investigated the effects of prenatal stress and of chronic ethanol exposure during adulthood on spatial memory in rats.

Methods: Rats were subjected to a prenatal stress delivered as 3 daily 45-minute sections of restraint stress to the mothers during the last 10 days of pregnancy (PRS rats). After 7 months of ethanol exposure (ethanol 10%, oral intake), memory performances were evaluated in a spatial discrimination test in control and PRS male rats. Then, the oxidative damages and the expression of metabotropic glutamate (mGlu) receptors were assessed in their hippocampus.

Results: Chronic ethanol exposure resulted in a reduced performance in a spatial recognition task in control animals. Unexpectedly, however, the same treatment attenuated spatial memory deficits in rats that had been subjected to prenatal stress. This paradigm of ethanol administration did not produce detectable signs of oxidative damage in the hippocampus in either unstressed or PRS rats. Interestingly, ethanol intake resulted in differential effects in the expression of mGlu receptor subtypes implicated in mechanisms of learning and memory. In control rats, ethanol intake reduced mGlu2/3 and mGlu5 receptor levels in the hippocampus; in PRS rats, which exhibited a constitutive reduction in the levels of these mGlu receptor subtypes, ethanol increased the expression of mGlu1a receptors but did not change the expression of mGlu2/3 or mGlu5 receptors.

Conclusion: Our findings support the idea that stress-related events occurring before birth have long-lasting effects on brain function and behavior, and suggest that the impact of ethanol on cognition is not only dose- and duration-dependent, but also critically influenced by early life experiences.

Key Words: Maternal Restraint Stress, Spatial Recognition, Alcohol, Lipid Peroxidation, Glutamate.

CHRONIC ETHANOL CONSUMPTION and abuse is a growing health problem around the world (Cargiulo, 2007) and a major cause of mortality in the socioeconomically developed world. Moreover, diseases linked to chronic ethanol abuse, such as certain cancers, diabetes, psychosocial conflict and cognitive disorders, have an enormous social and economic cost (Harwood et al., 1998; Reynaud et al., 2001). Prolonged ethanol consumption is associated with cognitive disorders, especially related to memory, in humans (Fama et al., 2004; Oscar-Berman et al., 2004). However, studies of the cognitive outcome associated with moderate chronic ethanol consumption have yielded complex and heterogeneous results in humans as well as in rodents, leading to a consideration of vulnerable phenotypes. Indeed, memory performance has variously been reported to be impaired (Farr et al., 2005; Matthews and Morrow, 2000), unaffected (Fadda et al., 1999; Gal and Bardos, 1994; Homewood et al., 1997), or even improved (Krazem et al., 2003a,b; Robles and Sabria, 2008; Steigerwald and Miller, 1997) in experimental animals following chronic ethanol exposure. Several factors such as the dose, the duration of ethanol exposure, or the mode of treatment (withdrawal episodes) determine the impact of ethanol on memory. However, little is known about the repercussions of factors linked to the history of an individual on the effects of alcohol in the long term.

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In the present work, we tested the hypothesis that early life events influence the long-term effects of ethanol on memory.

In rats, the administration of repeated restraint stress to pregnant dams is a well-documented model known to induce long-lasting physiological and behavioral alterations in the offspring (Darnaudery and Maccari, 2008; Maccari and Morley-Fletcher, 2007). Rats from mothers stressed during gestation (prenatal restraint stress or PRS rats) have decreased hippocampal neuroplasticity (Lemaire et al., 2000) associated with decreased levels of hippocampal mGlu2/3 and mGlu5 receptors (Zuena et al., 2008). They also display exacerbated age-related learning and memory impairments (Darnaudery et al., 2006; Vallee et al., 1999). Finally, PRS rats exhibit greater vulnerability to several drugs of abuse, including ethanol (Darnaudery et al., 2007; Deminiere et al., 1992; Kippin et al., 2007; Koehl et al., 2000; Morley-Fletcher et al., 2004; Yang et al., 2006b). Recently, we have demonstrated that adolescent PRS rats present a blunted activation of both the hippocampal antioxidant defense system (Enache et al., 2007) and of the hypothalamic-pituitary-adrenal axis (Van Waes et al., 2006) in response to an acute ethanol challenge. Nevertheless, the effects of chronic ethanol exposure in PRS rats remain to be determined.

The aim of the present study was to determine the impact of chronic ethanol treatment on spatial recognition performance in middle-aged (8- to 9-month old) PRS rats. In parallel, in order to study the neurobiological mechanisms involved in this behavioral response, we quantified oxidative stress as well as group I (mGlu1a and mGlu5) and II (mGlu2/3) mGlu receptor levels in the hippocampus of rats following chronic ethanol exposure.

MATERIALS AND METHODS

Animals

Twenty-seven male control and PRS rats obtained from litters bred in our animal facility were used (Villeneuve d'Ascq, France). Rats were individually housed in a temperature (22 ± 2°C) and humidity (60%) controlled animal room on a 12 hour light/dark cycle (lights on at 7 AM), with *ad libitum* access to food throughout the experiment, and free access to water except during the forced chronic ethanol treatment. Animal manipulations were conducted in accordance with the rules of European Communities Council Directive of 1986 (86/609/EEC) and following the Institute for Laboratory Animal Research "Guide for the Care and Use of Laboratory Animals."

PRS Procedure

Fourteen nulliparous female Sprague-Dawley rats weighing 200 to 225 g (Harlan, Gannat, France) were group-housed (5 females per cage) in the animal facility for at least 10 days before mating. Subsequently, females were individually housed overnight with a sexually experienced male rat (400 g) and vaginal smears were examined on the following morning. The day on which the smear was sperm positive was determined to be embryonic day 0. Each pregnant female was then single-housed and randomly assigned to control or stress groups. Control dams ($n = 7$) were left undisturbed throughout gestation, whereas stressed dams ($n = 7$) were subjected to a repeated restraint stress procedure as previously described (Maccari et al., 1995; Van Waes et al., 2006). Briefly, the stress procedure consisted of restraining the pregnant dam in a transparent cylinder (7.5 cm diameter, 19 cm long) under a bright light (6500 lux) for 45 minutes 3 times daily from day 11 of pregnancy until delivery. Stress sessions were conducted during the light phase but at differing periods of the day (9:00 AM, 1:00 and 5:00 PM ± 2 hours; separated by a 2- to 4-hour interval between sessions) in order to reduce possible habituation to repeated restraint stress. After weaning (postnatal day 21), male offspring from litters with similar numbers of males and females were selected for the study and housed individually. One male per litter was used in each experimental group to avoid any litter effect.

Chronic Oral Ethanol Administration

From postnatal day 28 to 40, animals were exposed to an oral free-choice paradigm (water vs. ethanol 10% v/v, ethanol from Flourent-Brabant, Tressin, France) for progressive habituation to ethanol. They were then subjected to forced consumption for the following 9 months, during which the only available drink was 10% ethanol solution (Fig. 1). Water groups had free access to tap water throughout the experiment.

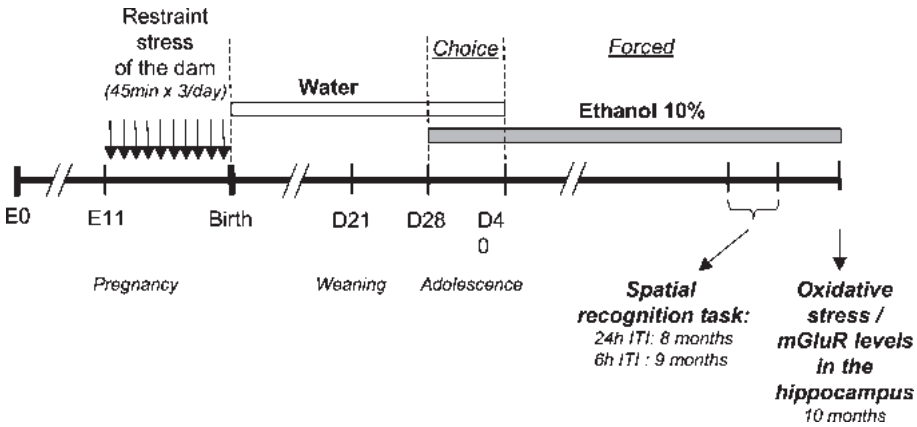

Fig. 1. Diagram of the experimental design. The PRS procedure consisted of restraining the pregnant dam (Sprague-Dawley rats) in a transparent cylinder under a bright light for 45 minutes thrice daily from day 11 of pregnancy until delivery. Male offspring were exposed from postnatal day 28 to 40 to an oral free choice paradigm (water vs. 10% ethanol) for progressive habituation to ethanol. They were subsequently exposed to an oral forced-consumption period during which the only drink available was a 10% ethanol solution (no withdrawal period). At 8 and 9 months of age, animals were submitted to a spatial recognition test in a Y-maze. At 10 months, rats were killed and oxidative stress and mGlu receptor levels were assessed in the hippocampus.

Spatial Recognition Memory

Rats (8 to 9 months old) were tested using a 2-trial memory task between 9 AM and 3 PM, in a Y-maze as previously described (Dellu et al., 1992). The Y-maze consisted of 3 identical arms illuminated by a dim light (45 lux) and enclosed by 36 cm high side walls. Each arm was equipped with infrared beams and the Y-maze was linked to a computer. Numerous visual cues were placed on the wall of the testing room and were kept constant throughout behavioral testing. The floor of the maze was covered with dirty sawdust from the home cages of several animals, and was mixed between sessions in order to eliminate olfactory cues. The task consisted of 2 trials separated by a variable time interval. During the first trial (acquisition phase), 1 arm of the Y-maze was closed and animals were placed in the center and allowed to explore the two other arms for 10 minutes. During the inter-trial interval (ITI, 6 or 24 hours), rats were housed in their home cages in a room different from the test room. During the second trial (test phase), the animals had free access to all 3 arms. The parameter evaluated was the time spent in the novel arm (the one closed during the first trial) during the first 3 minutes of the test phase. This parameter was expressed in percentages and was compared with the percentage of random/chance exploration of the 3 arms (i.e., 33% for each arm). The animals were determined to have discriminated between the novel arm and the 2 familiar arms if the percentage of time spent in this arm was significantly superior to 33%. Memory performance was tested using 2 ITI (6 and 24 hours) separated by an interval of 1 month.

Plasma and Tissue Collection

Animals were killed by decapitation between 9 AM and 1 PM, 1 month after the end of the behavioral assessment, in order to avoid transient changes in mGlu receptors levels in the hippocampus induced by the behavioral task itself (Riedel et al., 2000).

Trunk blood samples (approximately 4 ml) were collected in chilled tubes containing 40 μl of 5% EDTA and centrifuged at 2000 *g* for 15 minutes at 4°C. Aliquots of plasma were stored at −80°C until the assay of blood ethanol levels. Brains were removed from the skull and quickly chilled with cold NaCl (0.9%). Right and left hippocampi were dissected on ice, frozen immediately in liquid nitrogen and stored at −80°C until mGlu receptor and MDA assays were performed. Right and left hippocampi were randomly assigned to the mGlu receptor assay or the MDA assay to avoid laterality biases.

Oxidative Stress in the Hippocampus

The levels of hippocampal malondialdehyde (MDA), a metabolite of lipid peroxidation (Esterbauer et al., 1991) and thus a marker of oxidative stress in the hippocampus, were determined after chronic ethanol treatment. Hippocampi were homogenized on ice in 50 mM phosphate-buffered saline (pH 7.0) containing 0.1 mM EDTA to yield a 5% homogenate (w/v). Homogenates were then centrifuged at 6000 *g* for 10 minutes at 4°C to remove nuclei and debris. The supernatants were separated and used for the MDA assay. Total protein concentrations were determined using a BCA assay kit (Pierce, Brebières, France). MDA levels were estimated using the NWK-MDA01 assay kit (Northwest Life Science Specialties, Vancouver, Canada). The assay was based on the reaction of MDA with thiobarbituric acid (TBA) to form an MDA-TBA2 adduct that absorbs strongly at 532 nm. Homogenates (0.250 ml) were mixed with butylated hydroxytoluene (0.250 ml), phosphoric acid (0.250 ml), and TBA (0.250 ml) and incubated at 60°C for 60 minutes. After centrifugation (1000 *g* for 3 minutes), the MDA concentration of samples was determined with a spectrophotometer by simple absorption at 532 nm. Using tetramethoxypropane, a standard curve (0, 1, 2, 3, 4 μM) was prepared and the values of the homogenates were determined from this curve. Assays were carried out in duplicate and the results were expressed as MDA equivalents (μmol/g protein). The lower limit of detection by this method was 0.1 μmol of MDA.

Blood Ethanol Levels

Blood ethanol levels were measured using a gas chromatograph coupled with a flame ionization detector (Hewlett Packard 5890 series II GC, HP Les Ulis, ZI Courtaboeuf, France). Acetonitrile was used as an internal standard. To 100 μl plasma were added 100 μl NaOH (0.5 m), 100 μl zinc sulfate (30%), and 50 μl acetonitrile (pure). The whole was centrifuged at 1000 *g* for 10 minutes at 4°C, the supernatant was collected and 3 μl of the latter was injected into the gas chromatograph. The lower limit of detection of the method was 0.01 g/l.

Western Blot Analysis of Hippocampal mGlu Receptor Levels

Hippocampi were homogenized on ice in 500 μl of HEPES (5 mM)/sucrose (320 mM) buffer pH 7.4, containing 1% SDS, 50 mM NaF, 5 μl of phosphatase inhibitor cocktail I, 5 μl of phosphatase inhibitor cocktail II, and 5 μl of protease inhibitor cocktail I (Sigma-Aldrich, Lyon, France). Protein concentrations were determined using a BCA assay kit (Pierce). Samples were then mixed with Laemmli buffer and adjusted to a final protein concentration of 3 mg/ml. Equal amounts of protein (30 μg) were separated by SDS-PAGE (8% polyacrylamide) and electro-transferred to nitrocellulose membranes overnight (40 V). Ponceau red was used to check the efficacy of the transfer. Membranes were blocked for 60 minutes in T-TBS [0.1 M Tris–HCL, 0.5 M NaCl, 0.05% (v/v) TWEEN-20] containing 1% (w/v) nonfat milk and 1% (w/v) bovine serum albumin. Subsequently, blots were incubated overnight with anti-mGlu1a (1:500), anti-mGlu5 (1:1000), or anti-mGlu2/3 (1:250) receptor IgG (Upstate Biotechnology, Guyancourt, France) in blocking solution at 4°C. Antibody binding was revealed by incubation with a 1:10,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG (Amersham Biosciences, Buckinghamshire, UK) and an enhanced chemiluminescence system (Amersham Biosciences). To ensure that the amount of protein loaded in each lane was equivalent, the blots were also probed with anti-actin IgG (1:1000; Sigma-Aldrich) and incubated with 1:5,000 horseradish peroxidase-conjugated goat anti-mouse IgG (Amersham Biosciences, Buckinghamshire, UK). Chemiluminescence was detected by exposure of a Kodak autoradiography film. Assays were carried out in duplicate. The results obtained were expressed as a ratio of optical densities (target/actin).

Statistics

Statistical analyses were conducted using 2-way ANOVA, with group (Control vs. PRS) and treatment (Water vs. 10% Ethanol) as between-subject variables. Planned comparisons were used for post-hoc analysis. Student's *t*-tests were used to compare groups 2 by 2 or to compare the means of each group with a standard value (i.e., 33% for the Y maze). Relations between memory performance and mGlu levels in the hippocampus were evaluated using Pearson's correlation test. All data are presented as mean values ± SEM. Significance was set at $p < 0.05$.

RESULTS

Chronic Ethanol Treatment

Chronic ethanol treatment significantly diminished food intake [treatment effect, $F(1,23) = 8.8$, $p < 0.01$; Water:

26.4 ± 0.8; Ethanol: 21.3 ± 0.8 g of food/d] and 10 month-old alcoholized animals weighed significantly less than water rats [treatment effect, $F(1,23) = 6.57$, $p < 0.05$; Water: 580 ± 14 g; Ethanol: 540 ± 10 g]. However, no differences were detected between Control and PRS rats for these parameters [group × treatment effects, food intake, $F(1,23) = 0.12$, $p = 0.72$; body weight, $F(1,23) = 1.03$, $p = 0.33$, data not shown]. During the free choice period (habituation), Control and PRS animals consumed the same quantity of ethanol (Control: 2.46 ± 0.48; PRS: 2.43 ± 0.39. Mean values here and below are expressed in g of ethanol/kg body weight/d). Then, the mean ethanol intake was stable throughout the forced treatment and similar between Control and PRS rats (Control: 4.96 ± 0.34; PRS: 5.03 ± 0.18). Despite a significant ethanol consumption (around 5 g/kg/d), blood ethanol levels, assessed at the end of the treatment during the light phase, were below detection limits, confirming that animals drink in bouts rather than continuously, and most intake occurs during the circadian dark when they eat (Dole et al., 1985). In a separate set of chronically alcoholized rats (10% for 2 months), blood ethanol levels were determined 2 hours after the beginning of the dark phase. To control the fluid consumption, ethanol bottle was removed (for 2 hours) at the beginning of the dark period and was then presented to the animals for 15 minutes before the first blood sampling. The mean ethanol intake was 2.85 ± 0.17 g/kg. The time course of blood ethanol levels was: 0.77 ± 0.14 g/l (0 minutes postingestion), 1.04 ± 0.27 g/l (15 minutes postingestion), 1.09 ± 0.19 g/l (45 minutes postingestion), 1.08 ± 0.17 g/l (75 minutes postingestion), and 0.64 ± 0.17 g/l (105 minutes postingestion). 240 minutes after the ethanol presentation, blood ethanol levels were undetectable. Since all the experiments were carried during the light period (more than 2 hours after the end of the dark period), it could be assumed that rats have not been tested in an intoxicated state; therefore the results obtained reflected more the effect of chronic alcoholization rather than the consequences of an acute alcoholization.

Chronic Ethanol Consumption Alters Spatial Recognition in Control Rats, but Attenuates Memory Impairment in PRS Rats

During the acquisition phase, the total amount of time spent in each arm was similar across experimental groups, showing that all animals exhibited an equivalent exploration of the 2 arms [treatment effect, $F(1,23) = 0.33$, $p = 0.57$; group effect, $F(1,23) = 0.44$, $p = 0.51$ for an ITI of 6 hours, and treatment effect, $F(1,23) = 0.34$, $p = 0.56$; group effect, $F(1,23) = 2.14$, $p = 0.16$ for an ITI of 24 hours respectively, data not shown].

After 6 hours of retention (Fig. 2), the Control Water group significantly distinguished between the novel arm and the other arms ($p < 0.05$ vs. 33%), unlike the PRS Water group, which exhibited random exploration of the 3 arms ($p = 0.76$ vs. 33%). The time spent in the novel arm was differentially affected by ethanol, depending on the group [group × treatment effect, $F(1,23) = 11.80$, $p < 0.01$]. Planned comparison analysis revealed that Control Water group performance was better than the performance of both the Control Ethanol and the PRS Water groups (Control Water vs. Control Ethanol, $p < 0.01$; Control Water vs. PRS Water, $p < 0.01$), but not different from the performance of the PRS group exposed to ethanol. This suggests that chronic ethanol exposure had opposing impacts on spatial recognition performance in Control and PRS animals. Indeed, after chronic alcohol exposure, spatial recognition was impaired in Control rats ($p = 0.67$ vs. 33%), whereas PRS animals showed a significant preference for the novel arm in comparison with chance exploration of the 3 arms ($p < 0.05$ vs. 33%). After a 24-hour delay, all groups exhibited random exploration of the 3 arms of the Y-maze [treatment effect,

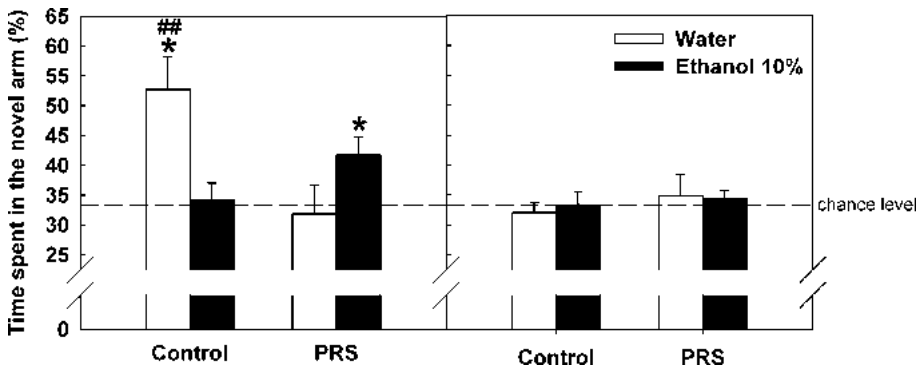

Fig. 2. Time spent in the novel arm of the Y-maze (expressed in percentages) after 6 and 24 hours inter-trial intervals (ITI). Groups were thought to recognize the arm as novel if the percentage of time spent in the novel arm was significantly above random/chance, i.e., 33% (* $p < 0.05$ vs. 33%). After 6 hours of retention, the time spent in the novel arm was differentially affected by ethanol according to the group (group x treatment effect, $F(1,23) = 11.80$, $p < 0.01$). Ethanol induced memory deficits in control animals ($p = 0.67$ vs. 33%). PRS rats presented constitutive spatial memory impairment ($p = 0.76$ vs. 33%), but this deleterious effect was attenuated by ethanol treatment ($p < 0.05$ vs. 33%). After the 24 hours delay, all groups exhibited random exploration of the 3 arms of the Y-maze. Dotted line: chance level. $n = 6$ to 7 per group. ## $p < 0.01$, Control Water vs. Control Ethanol and PRS Water.

$F(1,23) = 0.61$, $p = 0.44$; group effect, $F(1,23) = 0.03$, $p = 0.86$; group × treatment effect, $F(1,23) = 0.11$, $p = 0.75$].

Chronic Ethanol Consumption Differentially Affects the Expression of Hippocampal mGlu Receptor Subtypes in Control and PRS Rats

Searching for potential molecular/cellular correlates for the behavioral data obtained above, we first considered whether chronic ethanol consumption differentially damaged the hippocampus of control and PRS rats. Assessment of lipid peroxidation by measurements of MDA levels did not show any effect of chronic ethanol either in control or PRS rats. MDA levels were also identical between control and PRS rats treated with water (Control Water: 1.26 ± 0.31 μmol/g; Control Ethanol: 1.57 ± 0.30 μmol/g; PRS Water: 1.33 ± 0.17 μmol/g; PRS Ethanol: 1.44 ± 0.23 μmol/g). We then examined the expression of group I (mGlu1a and mGlu5) and group II (mGlu2 and mGlu3) mGlu receptors, which have been implicated in the regulation of synaptic plasticity (Balschun et al., 2006; Lu et al., 1997; Riedel et al., 1996; Steckler et al., 2005).

As shown in Table 1, PRS by itself significantly decreased mGlu1a ($t = 2.31$, df = 11, $p < 0.05$), mGlu5 ($t = 2.99$, df = 11, $p < 0.05$), and mGlu2/3 ($t = 2.18$, df = 11, $p < 0.05$) receptor protein levels in the hippocampus of 10-month-old rats. Ethanol treatment differentially affected hippocampal levels of mGlu1a receptors in the 2 groups of animals [Fig. 3*A*, group × treatment effect, $F(1,23) = 4.17$, $p = 0.05$]. Ethanol treatment increased mGlu1a receptor levels in PRS animals (PRS Water vs. PRS Ethanol, $p < 0.05$), but did not change receptor levels in control rats ($p = 0.54$). Both PRS (Fig. 3*B*, group effect, $F(1,23) = 15.92$, $p < 0.001$) and ethanol [treatment effect, $F(1,23) = 8.19$, $p < 0.01$] decreased mGlu5 receptors levels in the hippocampus. However, the effect of ethanol was observed in control rats ($p < 0.01$), but not in PRS rats ($p = 0.28$). Ethanol also reduced hippocampal mGlu2/3 receptor levels [Fig. 3*C*, treatment effect, $F(1,23) = 4.34$, $p < 0.05$], and, again, this effect was seen exclusively in control rats (Control: $p < 0.05$, PRS: $p = 0.72$). We noted a trend for a positive correlation between the spatial memory performance in the Y-maze and the mGlu1a receptor levels in the hippocampus of PRS animals ($r = 0.52$, $p = 0.058$). No other significant correlations were found between mGlu receptor subtypes and spatial recognition performance.

Table 1. Impact of the PRS Procedure on Hippocampal mGlu Receptor Levels in the Hippocampus (expressed in percentage of control animals)

	mGlu Receptor levels in the hippocampus (% of control group)	
	Control water	PRS water
mGlu1a	100 ± 3.17	90.70 ± 2.56*
mGlu5	100 ± 5.14	77.58 ± 5.36*
mGlu2/3	100 ± 3.57	88.78 ± 3.65*

A significant decrease in mGlu1a, mGlu5, and mGlu2/3 receptor levels was observed in rats exposed to PRS (Control $n = 6$, PRS $n = 7$; Student's *t*-test: $^*p < 0.05$ Control vs. PRS).

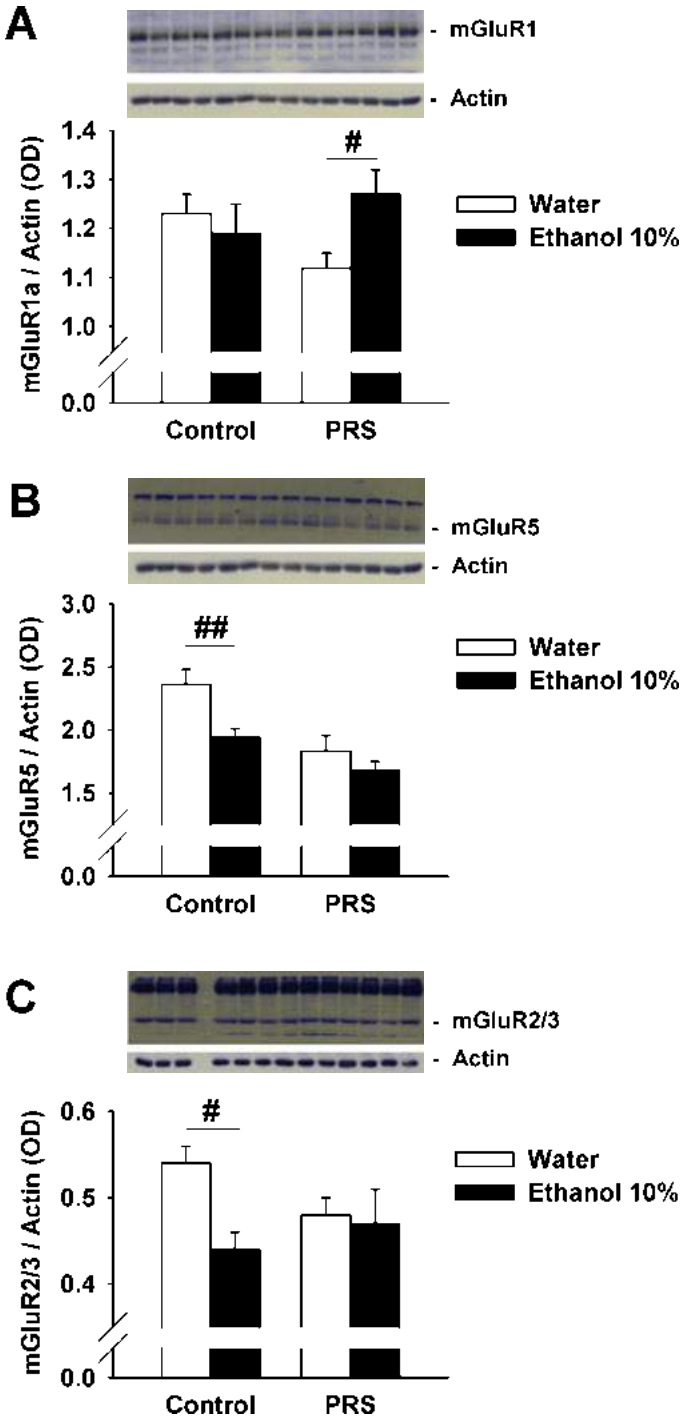

Fig. 3. Impact of chronic ethanol exposure on mGlu1a (**A**), mGlu5 (**B**), and mGlu2/3 (**C**) receptor levels in the hippocampus of control and PRS rats (expressed in optical density, OD). Hippocampal mGlu5 and mGlu2/3 receptors were significantly decreased by ethanol in control rats while mGlu1a receptor levels were increased by ethanol in PRS rats. A scan of 1 film used to quantify mGlu receptor levels is shown for each receptor ($n = 6$ to 7 per group, planned comparisons: #$p < 0.05$, ##$p < 0.01$).

DISCUSSION

The present study shows that chronic ethanol consumption induces memory impairment in control animals, whereas it attenuates the memory deficit observed in PRS rats. This is not associated with detectable levels of oxidative stress in the hippocampus, either in control or in PRS rats. However, the expression of mGlu receptor subtypes involved in memory processes is differentially affected, with ethanol treatment decreasing mGlu5 and mGlu2/3 receptor levels in control rats and increasing mGlu1a receptor levels in PRS rats. Finally, since the rats were not under the acute effect of ethanol when they were tested for memory or when we assessed mGlu receptor expression (blood ethanol levels were undetectable during the light phase), we can assume that the reported changes mainly reflect the impact of chronic ethanol exposure.

It is generally observed that chronic ethanol consumption results into cognitive impairment in humans. However, data obtained from experimental animals are not unequivocal. Some studies report deleterious effects of chronic ethanol administration (Beracochea et al., 1992; Fadda and Rossetti, 1998; Farr et al., 2005; Matthews and Morrow, 2000), whereas others fail to reveal any memory deficit (Fadda et al., 1999; Gal and Bardos, 1994; Homewood et al., 1997; Krazem et al., 2003a; Robles and Sabria, 2008). Several variables, including the presence and extent of a withdrawal period, can strongly influence the effect of ethanol consumption on cognitive function. For example, Lukoyanov and colleagues (1999) have shown that rats chronically exposed to ethanol for 13 months develop cognitive impairment only after 6 weeks of withdrawal. Despite an absence of withdrawal period in our study, we found a significant impairment of spatial memory in the alcoholized control rats. Unexpectedly, however, a similar chronic exposure to ethanol in PRS rats attenuated their spatial memory deficits. In a previous experiment, we have shown that the kinetic of blood ethanol levels after ethanol administration (1.5 g/kg, i.p.) was not affected by PRS, and thus it can be assumed that the opposite behavioral changes observed here do not reflect varying levels of circulating ethanol (Van Waes et al., 2006). Chronic ethanol exposure produced a marked decrease in the food intake and bodyweight. However, these alterations are probably not involved in the ethanol effect on memory because alcoholization decreased food intake and bodyweight in both control and PRS rats. Furthermore, a recent work demonstrates that the deleterious impact of chronic ethanol consumption on learning and memory in rodent is not related to changes in caloric intake (Farr et al., 2005).

Studies in nonhuman primates and rodents have reported alterations in the structure and function of the hippocampus as a consequence of prenatal stress (Coe et al., 2003; Son et al., 2006). Several works have shown learning and/or memory impairment in offspring of dams stressed during pregnancy (Kapoor et al., 2009; Meunier et al., 2004; Mueller and Bale, 2007; Szuran et al., 2000; Wu et al., 2007; Yaka et al., 2007; Yang et al., 2006a; Zuena et al., 2008). However, the timing of the prenatal stress (early vs. mid- or late-gestation; e.g., Mueller and Bale, 2007), the type and/or the severity of the prenatal stress and the sex of the offspring (e.g., Zuena et al., 2008), and the age of the offspring at time of memory assessment (juvenile, adult or aged) appear to be critical. In rats, for example, an intense maternal stress (10 unpredictable, 1 second, 0.8 mA foot shocks per day during gestational days 13 to 19) impairs spatial learning and memory in the Morris water maze in the offspring (Yang et al., 2006a); in contrast, a mild prenatal stress (induced by a mild restraint in a small cage for 30 minutes daily from gestation day 15 to 17) enhances active avoidance and radial maze learning performance (Fujioka et al., 2001). Furthermore, maternal exposure to a chronic restraint stress during the late gestation (3 times daily for 45 minutes) has generally few effects on performance in young male adults (Li et al., 2008; Vallee et al., 1999; Zuena et al., 2008), but exacerbates the memory disorders observed during aging (Darnaudery et al., 2006; Vallee et al., 1999). Accordingly, we observed a decrease in spatial memory performance in 9-month-old male PRS rats. As opposed to control animals, PRS rats did not differentiate between the novel arm and the two other arms after an ITI of 6 hours. The processes involved in the effects of PRS on cognition are still unknown. From a behavioral point of view, variations in maternal care during the postnatal period could be involved in the detrimental effects of PRS (Maccari et al., 1995). Indeed, some studies suggest that PRS also affects maternal behavior (Champagne and Meaney, 2006; Smith et al., 2004) and poor maternal care is associated with learning/memory deficits in adult offspring (Liu et al., 2000). At a cellular level, PRS could impair spatial memory via a number of mechanisms, including a reduction in neurogenesis in the dentate gyrus (Lemaire et al., 2000), and the abnormal activation of the protein kinase C (PKC) pathway, as reflected by a reduction in the expression of PKCβ1 levels in the hippocampus (Wu et al., 2007). Interestingly, the impaired memory performance we have found in PRS rats was associated with the reduced expression of the 2 mGlu receptor subtypes (mGlu1a and mGlu5) coupled with polyphosphoinositide (PI) hydrolysis (Pin and Duvoisin, 1995), a transduction pathway that leads to the activation of PKC (Kikkawa et al., 1987). The mGlu1 and mGlu5 receptors are involved in the induction of activity-dependent forms of synaptic plasticity (e.g., long-term potentiation and long-term depression), as well as in the retention of new information in hippocampus-dependent learning paradigms (Balschun et al., 2006; Lu et al., 1997; Riedel et al., 1996; Steckler et al., 2005). The expression of mGlu2/3 receptors was also reduced in the hippocampus of PRS rats. These 2 receptor subtypes (here detected using a common antibody) are negatively coupled to adenylyl cyclase (Pin and Duvoisin, 1995), but their activation amplifies the PI response mediated by mGlu1/5 receptors through a cross-talk mechanism likely mediated by the $\beta\gamma$ subunits of the Gi protein (Genazzani et al., 1993; Schoepp et al., 1999). Previous studies suggest that learning can affect mGlu receptor expression. Riedel and colleagues (2000) have shown that mGlu5

receptors are strongly increased in the CA1 and dentate gyrus of the hippocampus 10 days after a learning task. Thus, we cannot exclude that the higher levels of mGlu5 receptors in control rats reflect a long-lasting response to their better performance in the spatial memory test, although there was a 1-month interval between the execution of the test and measurements of mGlu5 receptor expression. Present results confirm that expression of mGlu5 and mGlu2/3 receptors is reduced in the hippocampus of PRS rats (Zuena et al., 2008) and extend the reduction to mGlu1a receptors, at least to this group of aged PRS rats.

Surprisingly, the memory deficit observed in PRS rats was attenuated by chronic ethanol intake. These data are in line with data obtained in mice by Krazem and colleagues, who used a paradigm of ethanol treatment similar to that used by us (12% ethanol as the sole source of fluid during 5 months, with no withdrawal). This particular treatment reverses the memory deficits associated with aging (Krazem et al., 2003a) and attenuates the age-related decline of hippocampal neurogranin (Krazem et al., 2003b), a PKC substrate playing a central role in the regulation of hippocampal synaptic plasticity and spatial learning (Pak et al., 2000). Interestingly, Krazem and colleagues have found a "bidirectional effect" of chronic ethanol exposure on spatial memory, depending on the age (Krazem et al., 2003a). They showed that ethanol impairs performance (relational memory) in 7- to 8-month-old mice (adult animals), but attenuates memory deficits (relational memory and long term retention) in 21- to 23-month-old mice (aged animals). Similarly to our findings, the restoration of memory following ethanol exposure was not complete. Furthermore, cognitive tasks in Krazem's study (place discrimination task in a 8-arm radial maze) and our study (spontaneous recognition Y maze task) require the integrity of the hippocampus (Conrad et al., 1996; Etchamendy et al., 2003). Taken together, these data suggest that chronic exposure to ethanol might be detrimental in subjects with "normal" cognitive function, but become beneficial if memory is impaired, as occurs in aged or PRS animals. A recent study demonstrates that the positive impact of low-dose ethanol consumption on memory may be mediated by NMDA receptors in the hippocampus (Kalev-Zylinska and During, 2007). We show here that chronic ethanol consumption also affects the expression of specific mGlu receptor subtypes in the hippocampus. In control rats, chronic ethanol exposure lowered the expression of mGlu2/3 and mGlu5 receptors. These data are in agreement with the reduction of mGlu5 receptor mRNA levels found in the hippocampus of rats following a 2-month daily intake of 5% ethanol (Simonyi et al., 2004). It is also noteworthy that a prenatal ethanol exposure leads, in the adult life, to a long-lasting memory impairment (Savage et al., 2002) and a reduced expression of mGlu5 receptors in the hippocampal dentate gyrus (Galindo et al., 2004). Remarkably, ethanol treatment in PRS rats increased mGlu1a receptor levels, but failed to reduce mGlu5 and mGlu2/3 receptor levels in the hippocampus, as it did in control rats. In the PRS group, the spatial performance tended to positively correlate with the hippocampal mGlu1a receptor levels. It will be interesting to examine whether (1) knock-down of mGlu1a receptors in the hippocampus prevents the improving effect of ethanol on memory in PRS rats; and (2) overexpression of mGlu1a receptors occludes the effect of ethanol on spatial recognition. Finally, the roles of NMDA and mGlu receptors in the beneficial effects of ethanol on cognition may be convergent because the NR2 subunit of NMDA receptors is physically and functionally linked to mGlu1 or mGlu5 receptors through a chain of anchoring proteins that include PSD95, Shank, and the long isoforms of Homer (Tu et al., 1999).

In conclusion, the same chronic ethanol treatment produced differential effects in control and PRS animals both on memory in the Y maze and expression of mGlu receptors in the hippocampus. Although the underlying mechanisms are unknown, our data suggest that chronic alcohol consumption might have dual effects on memory and highlight the importance of early life experiences in the impact of ethanol on some cognitive functions.

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ORIGINAL INVESTIGATION

Effect of prenatal stress on alcohol preference and sensitivity to chronic alcohol exposure in male rats

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Abstract
Rationale In rats, prenatal restraint stress (PRS) induces persistent behavioral and neurobiological alterations leading to a greater consumption of psychostimulants during adulthood. However, little is known about alcohol vulnerability in this animal model.
Objectives We examined in adolescent and adult male Sprague Dawley rats the long-lasting impact of PRS exposure on alcohol consumption.
Methods PRS rats were subjected to a prenatal stress (three daily 45-min sessions of restraint stress to the mothers during the last 10 days of pregnancy). Alcohol preference was assessed in a two-bottle choice paradigm (alcohol 2.5%, 5%, or 10% versus water), in both naïve adolescent rats and adult rats previously exposed to a chronic alcohol treatment. Behavioral indices associated with incentive motivation for alcohol were investigated. Finally, plasma levels of transaminases (marker of hepatic damages) and *ΔFosB* levels in the nucleus accumbens (a potential molecular switch for addiction) were evaluated following the chronic alcohol exposure.
Results Alcohol preference was not affected by PRS. Contrary to our expectations, stressed and unstressed rats did not display signs of compulsive alcohol consumption. The consequences of the alcohol exposure on locomotor reactivity and on transaminase levels were more prominent in PRS group. Similarly, PRS potentiated alcohol-induced *ΔFosB* levels in the nucleus accumbens.
Conclusion Our data suggest that negative events occurring in utero do not modulate alcohol preference in male rats but potentiate chronic alcohol-induced molecular neuroadaptation in the brain reward circuitry. Further studies are needed to determine whether the exacerbated *ΔFosB* upregulation in PRS rats could be extended to other reinforcing stimuli.

Keywords Maternal restraint stress · Ethanol · Locomotor reactivity to novelty · Gene regulation · AST/ALT · Corticosterone

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Introduction

Alcoholism is a complex multifactorial disease governed by both genetic and environmental factors (Kaufman et al. 2007). To develop the most effective treatment approaches against this disease, accurate knowledge and understanding of risk factors involved in alcohol use disorders are essential. Alcohol abuse is highly comorbid with stress-related psychopathologies such as depression, anxiety, or post-traumatic stress disorder (Breese et al. 2005; Driessen et al. 2001; Jacobsen et al. 2001; Spanagel et al. 1995). Stress exposure during adulthood stimulates alcohol consumption, both in human and laboratory animals (Brady and Sonne 1999; Breese et al. 2005; Siegmund et al. 2005; Vengeliene et al. 2003). Although these effects appear transient and reversible when exposure occurs in the adult, they can be long lasting when stress is experienced during critical developmental windows. For instance, in rodents, early maternal separation affects alcohol intake in operant self-administration and free choice procedures in adolescents and during adulthood (Cruz et al. 2008; Huot et al. 2001; Lancaster 1998; Ploj et al. 2003). However, these effects are dependent on the protocol used for maternal separation (Jaworski et al. 2005). To date, it is unknown whether the prenatal period constitutes an equally sensitive window (Darnaudery et al. 2007; DeTurck and Pohorecky 1987; Weinberg 1987).

Repeated restraint stress of the pregnant dams is a well-documented model of prenatal stress in rats (prenatal restraint stress (PRS)). Rats born from dams stressed during the last 10 days of gestation (PRS rats) display a decreased ability to cope with stress (Darnaudery and Maccari 2008; Maccari and Morley-Fletcher 2007), an exacerbated anxiety-like behavior (Vallee et al. 1997), a hyper-response to an intense footshock stress (Louvart et al. 2005), and depressive-like disturbances (Morley-Fletcher et al. 2003). At a neurobiological level, PRS rats show several alterations of neurotransmitter systems involved in the alcohol response, such as glutamatergic (Barros et al. 2004; Berger et al. 2002; Van Waes et al. 2009; Zuena et al. 2008), GABAergic (Barros et al. 2006), and dopaminergic (Adrover et al. 2007; Barros et al. 2004; Berger et al. 2002; Henry et al. 1995) dysfunctions. At a hormonal level, adolescent PRS male rats exhibit a reduced hypothalamic-pituitary-adrenocortical axis response to an acute alcohol administration (Van Waes et al. 2006), a phenomenon previously described in human populations with a high risk of alcohol abuse (King et al. 2006; Schuckit et al. 1987, 1988), as well as in alcohol-dependent rats (Richardson et al. 2008). Together, these data raise questions about the incentive motivation for alcohol in rats submitted to PRS, especially since these animals also show a greater propensity to consume other drugs of abuse, including amphetamine (Deminiere et al. 1992) and cocaine (Kippin et al. 2007; Thomas et al. 2009).

In this study, we first determined the impact of PRS on alcohol consumption in male rats by assessing the spontaneous alcohol preference during adolescence, a specific period of high vulnerability to alcohol (Garcia-Burgos et al. 2009; Maldonado et al. 2008; Spear 2000). Then, we examined whether PRS may lead to compulsive alcohol consumption after several months of oral alcohol intake. In parallel, we evaluated in stressed and unstressed rats the consequences of the long-term exposure to alcohol on several parameters associated with drug intake. We focused on the locomotor response to novelty and anxiety-like behavior since these parameters are positively correlated with alcohol self-administration (Nadal et al. 2002; Spanagel et al. 1995). We assessed the plasma aspartate aminotransferase (AST)/alanine aminotransferase (ALT) ratio, a metabolic marker of abusive alcohol consumption associated with liver damages (Conigrave et al. 2003). Finally, we evaluated in the nucleus accumbens the levels of the transcription factor *ΔFosB*, a potential sustained molecular switch for addiction (Nestler et al. 2001). Because *ΔFosB* is also modulated by stress (Marttila et al. 2007; Perrotti et al. 2004), we additionally checked plasma corticosterone levels during the alcohol exposure in the two groups of rats.

Materials and methods

Animals

Ninety-eight control and PRS male Sprague Dawley rats, obtained from litters bred in our animal facility, were used (Villeneuve d'Ascq, France). After weaning (postnatal day (PND) 21), rats were individually housed in a temperature (22±2°C)- and humidity (60%)-controlled animal room on a 12-h light/dark cycle (lights on at 7:00 a.m.), with ad libitum access to food throughout the experiment and free access to water, except during the forced chronic alcohol treatment. Animal manipulations were conducted in accordance with the rules of European Communities Council Directive of 1986 (86/609/EEC) and following the Institute for Laboratory Animal Research "Guide for the Care and Use of Laboratory Animals." All procedures used during this study were reviewed and approved by a local ethical committee (Université Nord de France). Significant efforts were also made to minimize the total number of animals used while maintaining statistically valid group numbers.

Prenatal stress procedure

Fifteen nulliparous female Sprague Dawley rats weighing 200–225 g (Harlan, Gannat, France) were group-housed (five females per cage) in the animal facility for at least 10 days before mating. Subsequently, females were

individually housed overnight with a sexually experienced male rat (400 g), and vaginal smears were examined on the following morning. The day on which the smear was sperm positive was determined to be embryonic day 0. Each pregnant female was then single-housed and randomly assigned to control or stress groups. Control dams (N=7) were left undisturbed throughout gestation, whereas stressed dams (N=8) were subjected to a repeated restraint stress procedure as previously described (Maccari et al. 1995; Van Waes et al. 2006). Briefly, the stress procedure consisted of restraining the pregnant dam in a transparent cylinder (7.5 cm diameter, 19 cm long) under a bright light (6,500 lx) for 45 min three times daily from day 11 of pregnancy until delivery. Stress sessions were conducted during the light phase but at differing periods of the day in order to reduce possible habituation to repeated restraint stress (9:00 a.m., 1:00 p.m., and 5:00 p.m.±2 h; separated by a 2–4-h interval between sessions). After weaning (PND 21), male offspring from litters with similar numbers of males and females were selected for the study and singly housed throughout the experiment. They were assigned to one of the eight experimental groups: control rats treated with alcohol 2.5% (N=14), 5% (N=13), 10% (N=13), or water (N=10) and PRS rats treated with alcohol 2.5% (N=13), 5% (N=13), 10% (N=12), or water (N=10). A maximum of two males per litter were used in each experimental group to avoid any litter effect.

Alcohol exposure

The experimental design is summarized in Fig. 1. The preference for the different alcohol concentrations versus water was evaluated in a two-bottle choice paradigm during adolescence (PND 28–38) in naive rats and during adulthood (PND 148–158) after a chronic forced alcohol treatment (PND 38–148). During these periods, each rat had the choice between tap water and alcohol (2.5%, 5%, or 10% *v*/*v* alcohol solutions, alcohol from Flourent-Brabant, Tressin, France). From day 38 to day 148 and from day 158 to 10 months old, animals were submitted to a forced alcohol treatment during which the only drink available was alcohol (at the same concentration than during the two-bottle choice paradigm). Water groups had free access to tap water throughout the experiment.

Alcohol intake and alcohol preference

Mean alcohol intake was expressed as the total amount of alcohol consumed per day related to body weight (g/kg b.w./day). The alcohol preference was calculated every 2 days in the two-bottle choice paradigm by weighting the bottles. The bottle position (right or left) was changed after each measure to avoid position bias. Alcohol preference was expressed as a percentage of alcohol intake relative to total fluid intake and was calculated according to the formula: (alcohol intake (milliliters)/total fluid drinking (milliliters))×100.

To evaluate whether PRS rats presented compulsive alcohol consumption following the chronic alcohol treatment, we focused on three parameters previously identified as behavioral indices of dependence (DSM-IV-TR, American Psychiatric Association 2000). First, we determined whether animals increased their total fluid intake (to maintain an equal total amount of alcohol ingested in g/kg b.w./day) upon a twofold reduction in the concentration of the available solution. The mean alcohol intake was measured

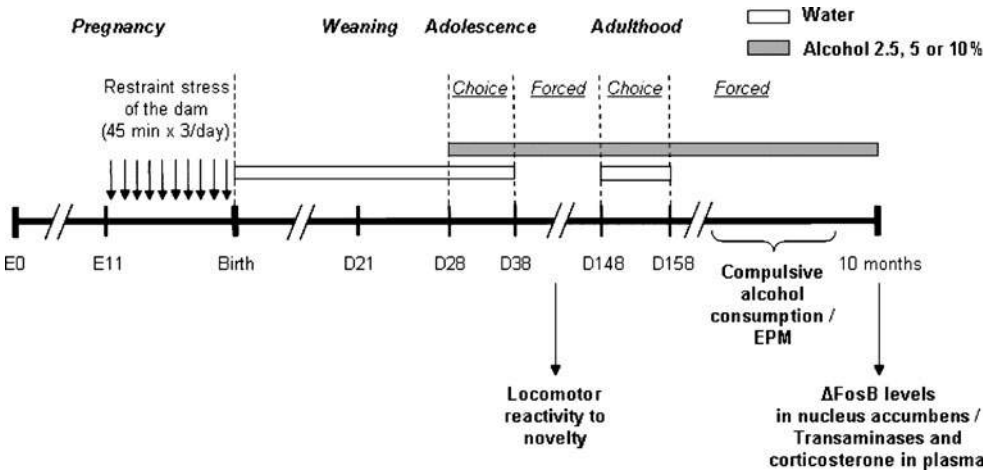

Fig. 1 Experimental design. Prenatal restraint stress and control animals were submitted to a chronic alcohol treatment beginning postnatal day 28. During choice periods, animals could choose to drink from a bottle of tap water or a bottle of alcohol (2.5%, 5%, or 10%). During the forced treatment periods, the only fluid available was alcohol (2.5%, 5%, or 10%). During the forced alcohol exposure, locomotor reactivity to novelty, compulsive alcohol consumption, and anxiety-like behavior in an elevated plus maze (EPM) were assessed. After 9 months of alcohol consumption, we determined corticosterone and transaminase levels in the plasma and the *ΔFosB* levels in the nucleus accumbens

under this condition (PND 202–203) and compared to the mean alcohol intake under basal condition (PND 197–201). Second, we studied the alcohol deprivation effect, which is defined as a temporary rise in the voluntary intake of alcohol over baseline drinking conditions when alcohol is reinstated after a period of deprivation and is hypothesized to be an animal model for alcohol craving (Heyser et al. 1997; Mormede et al. 2004; Rodd-Henricks et al. 2000; Wolffgramm and Heyne 1995). To monitor this effect, we compared PRS and control rats for their alcohol intake after a 24-h alcohol withdrawal period (PND 160). Finally, we hypothesized that in comparison with naive animals, dependent animals would show a relative neglect in response to the presentation of an alternative reward. To test this hypothesis, we used a concurrent reward schedule where the rat had the choice to consume alcohol or another powerful natural reward, i.e., sucrose. Alcohol preference was then evaluated in stressed rats when alcohol was in competition with a 1% sucrose solution (PND 278–279).

Locomotor reactivity to novelty and anxiety-related behavior

Locomotor reactivity to novelty (PND 110) was assessed during 30 min in a novel environment consisting in a transparent Plexiglas cage (18×30×18 cm high). Activity was automatically monitored by photocell beams and recorded via a computer system (Imétronic, Pessac, France). The test was performed between 9:00 a.m. and 1:00 p.m.

Anxiety-related behavior was assessed in an elevated plus maze (PND 193). The apparatus was 60 cm above the floor; it consisted of four arms radiating outward from a central square (15×15 cm). Two were open (50×15 cm), and two were closed with sidewalls (40 cm high). Each rat was placed on the central platform facing an open arm and allowed to freely explore the maze for 5 min. Behavior was recorded by video, and the percentage of time spent in each arm was scored. The test was performed between 9:00 a.m. and 1:00 p.m.

Plasma and tissue collections

During the forced alcohol treatment (PND 139), blood samples (1 ml) were collected from tail nicks (between 9:00 a.m. and 1:00 p.m.). Blood was collected in chilled tubes containing 10 µl of 5% EDTA and centrifuged at 2,000 g for 15 min at 4°C. Aliquots of plasma were stored at −80°C until the determination of the plasma alcohol concentrations.

At 10 months of age, animals were killed by decapitation (between 9:00 a.m. and 1:00 p.m.). Trunk blood samples (approximately 4 ml) were collected in chilled tubes containing 40 µl of 5% EDTA and centrifuged at 2,000 g for 15 min at 4°C. Aliquots of plasma were stored at −80°C until the assays of corticosterone levels and ratio of AST/ALT activities. Adrenal glands were dissected and weighted. Brains were removed from the skull and quickly chilled with cold NaCl (0.9%). Nuclei accumbens (1.8 mm punches, ranging from 2.7 to 0.7 mm anterior to bregma (Paxinos and Watson 1998)) were collected from 2-mm-thick coronal brain sections (matrix: Bioseb, Vitrolles, France), frozen immediately on dry ice and store at −80°C until they were assessed for *ΔFosB* levels. For each animal, the two nuclei accumbens were pooled.

Biological assessment in plasma

Blood alcohol levels (BAL) were measured using a gas chromatograph coupled with a flame ionization detector (Hewlett Packard 5890 series II GC). Acetonitrile was used as an internal standard. One hundred microliters NaOH (0.5 M), 100 µl zinc sulfate (30%), and 50 µl acetonitrile (pure) were added to 100 µl of plasma and centrifuged at 1,000 g for 10 min at 4°C. Three microliters of the resulting supernatant was injected into the gas chromatograph. The lower limit of detection of the method was 0.01 g/l.

Plasma corticosterone levels were determined using a radioimmunoassay kit (Kit ImmunChem™ Corticosterone ^{125}I RIA, ICN Biomedicals, Orsay, France) including a highly specific corticosterone antiserum. The lower limit of detection was 0.1 µg/dl. The average intra-assay coefficient of variation was 8.8%.

AST and ALT activities (Conigrave et al. 2003) were assayed in 150 µl of plasma using a fully automated enzyme spectrophotometric system and commercial kinetic UV tests (AST OSR6109 and ALT OSR6107, respectively, Olympus, Rungis, France). Ratios of AST/ALT activities were calculated and then expressed as a percentage of respective water groups (Control water or PRS water).

FosB immunoblotting

Western blotting was performed as described previously (Chen et al. 1997; Werme et al. 2002). Nuclei accumbens were homogenized on ice in 500 µl of HEPES (5 mM)/sucrose (320 mM) buffer pH7.4, containing 1% sodium dodecyl sulfate (SDS), 50 mM NaF, 5 µl of phosphatase inhibitor cocktail I, 5 µl of phosphatase inhibitor cocktail II, and 5 µl of protease inhibitor cocktail I (Sigma Aldrich, Lyon, France). Protein concentrations were determined using a bicinchoninic acid assay kit (Pierce, Brebières, France). Samples were then mixed with Laemmli and adjusted to a final protein concentration of 3 mg/ml. Equal amounts of boiled protein samples (30 µg) were separated by SDS-polyacrylamide gel electrophoresis (10% poly-

acrylamide gel) and electro-transferred to polyvinylidene fluoride membrane (Hybond-P, Amersham Bioscience, Orsay, France) overnight (40 V). The blots were blocked in TBS-Tween buffer (100 mM Tris, 0.9% NaCl, and 0.1% Tween 20) containing 5% of non-fat dry milk for 1 h at room temperature. All the antibodies dilutions were prepared in TBS-Tween buffer containing 2% of non-fat dry milk. The blots were incubated overnight at 4°C in a 1:200 dilution of a rabbit polyclonal antiserum raised against a middle region of Δ*FosB* (amino acids 75–150, SC7203, SantaCruz Biotechnology, Le Perray en Yvelines, France) and were washed three times for 50 min each in TBS-Tween. Blots were then incubated for 1 h at room temperature in a 1:5,000 dilution of donkey anti-rabbit antibody conjugated to horseradish peroxidase (NA934V, Amersham Bioscience, Orsay, France). The blots were washed three times for 50 min each with TBS-Tween and one time in TBS alone during 5 min, and then developed with the enhanced chemiluminescence system (Amersham Bioscience, Orsay, France). To ensure that each line was loaded with an equivalent amount of protein, the blots were probed again, according to the same protocol, with anti-β-actin serum (1:10,000; mouse IgG2a isotype, Sigma Aldrich, Lyon, France) overnight at 4°C and rabbit anti-mouse antibody conjugated to horseradish peroxidase (1:5,000; nif825, Amersham Bioscience, Orsay, France). The film autoradiograms (Kodak, Sigma Aldrich, Lyon, France) were captured using a light table (Northern Light, Imaging Research, St Catharines, Ontario, Canada) and a Sony XC-77 CCD camera. Levels of *FosB* (50 kD band), *ΔFosB* (~35–37 kD band), and β-actin were quantified by densitometry (TotalLab TL 120, Nonlinear Dynamics, Durham, NC, USA). *FosB*/β-actin and *ΔFosB*/β-actin ratios were calculated and then expressed as a percentage of respective water groups (control water or PRS water). To specifically identify Δ*FosB* band, immunoblots from brain samples were compared to protein extract from rat adrenal pheochromocytoma (PC12) cells (Clontech, Mountain View, CA, USA) transduced with a Δ*FosB*-expressing viral vector (Berton et al. 2007).

Statistics

All data were presented as means±SEM. Alcohol preference, alcohol intake, body weight, adrenal weight as well as alcohol, corticosterone, and transaminase levels in plasma were analyzed using two-way analysis of variance (ANOVA) with group (control, PRS) and concentrations (2.5, 5, 10%) as between-subjects factors. The alcohol dependence was assessed with three-way ANOVA with group (control, PRS) and concentrations (2.5%, 5%, and 10%) as between-subjects factors and (1) ethanol intake in basal situation and after the reduction of the concentration, (2) ethanol intake in basal situation and after 24 h of deprivation, and (3) ethanol preference in competition with water and in competition with sucrose as within-subjects factors. Locomotor activity was analyzed using three-way ANOVA with group (control, PRS) and concentrations (2.5%, 5%, and 10%) as between-subjects factors, and time (5, 10, 15, 20, 25, and 30 min) as within-subjects factors. *FosB and ΔFosB* levels in the nucleus accumbens were evaluated using analysis of covariance (ANCOVA; to explore a potential dose–response effect) with group (nominal variable; control, PRS) and concentrations (numeric variable; 2.5%, 5%, and 10%) as between-subjects factors, followed by linear regressions. Newman–Keuls test and planned comparisons were used as post hoc analysis. Student's *t* tests were used to compare initial body weight, initial sucrose preference, basal *ΔFosB* levels, and basal AST/ALT ratio between PRS and control groups. Significance was set at $p<0.05$.

Results

Prenatal stress affected neither the spontaneous alcohol preference nor the motivation for alcohol after a chronic alcohol exposure

The mean body weight of rats was similar between PRS and control groups prior to exposure to alcohol (PND 28 in grams, 70.9±1.4 and 71.0±1.4, respectively) and when rats were tested for alcohol preference during adulthood (PND 148, Table 1). The 10% alcohol treatment tended to decrease the bodyweight in both PRS and control groups (PND 148, concentration effect: $F(3,89)=2.75$, $p<0.05$; 0% versus 10%: $p=0.054$, no interaction). The total fluid intake was similar between PRS and control rats, both during adolescence and adulthood. PRS procedure had no effect on alcohol preference (Fig. 2a) or on alcohol intake (Fig. 2b) at any of the concentration tested. To examine the development of alcohol dependence after chronic exposure to alcohol, we used three experimental conditions: (1) abrupt reduction of the alcohol concentration, (2) alcohol withdrawal for 24 h, and (3) competition between alcohol and an alternative reward (sucrose 1%). As shown in Fig. 3, none of these three experimental conditions led to a significant group effect. When alcohol concentrations were diminished by 50% during the chronic forced alcohol treatment (Fig. 3a), rats did not raise their fluid intake sufficiently to maintain the previous levels of alcohol ingestion. Indeed, the reduction of the alcohol amount led to a drop of the alcohol intake in g/kg b.w./day (alcohol reduction effect, $F(2,57)=254.66$, $p<0.001$; concentration effect, $F(2,57)=183.84$, $p<0.01$; alcohol reduction×concentration effect, $F(2,57)=6.81$, $p<0.01$). The alcohol

Table 1 Mean bodyweight (postnatal day 148), ethanol intake, blood alcohol levels (BAL), time spent in the open arm of the elevated plus maze, plasma corticosterone levels, and adrenal weight assessed during the forced ethanol treatment in control and prenatal restraint stress (PRS) rats

	Control				PRS				ANOVA
	0%	2.5%	5%	10%	0%	2.5%	5%	10%	
Body weight (g)	447±10	441±9	435±9	412±10	440±15	451±10	432±9	429±7	Concentration effect p=0.054
Ethanol intake (g/kg b.w./day)	0±0	1.7±0.1	3.3±0.2	4.7±0.2	0±0	1.6±0.1	2.8±0.2	5.0±0.2	Concentration effect p<0.001
BAL (g/l)	0±0	0.02±0.02	0.11±0.08	0.31±0.27	0±0	0.22±0.22	0.42±0.32	0.89±0.88	NS
Time spent in the open arms (%)	14±3	18±4	18±5	11±3	9±2	13±4	15±4	9±1	Group effect p=0.09
Corticosterone levels (μg/dl)	1.8±0.4	1.3±0.2	1.6±0.4	1.4±0.2	1.3±0.1	1.3±0.4	0.8±0.1	1.9±0.6	NS
Adrenal weights (R+L, mg/kg)	92±10	99±5	101±6	104±7	96±7	94±3	99±4	93±4	NS

Except a tendency to reduce the amount of time spent in the elevated plus maze, PRS procedure had no impact on the different parameters studied

reduction×concentration interaction was explained by a weaker decrease in alcohol intake in the 10% group compared to the two others (mean percentage of decrease: 52%, 51%, and 26% for the 2.5%, 5%, and 10% groups, respectively). Nevertheless, the reduction of alcohol ingestion was highly significant in every group (N.K.: alcohol reduction; 2.5%, p<0.001; 5%, p<0.001; and 10%, p<0.001). As shown in Fig. 3b, 24 h of alcohol deprivation differently affected the alcohol intake depending on the concentration (deprivation×concentration effect: $F(2,63)$=5.70, p<0.01). Alcohol intake was higher after the deprivation for the 5% and 10% alcohol solution and remained stable for the 2.5% alcohol solution (N.K.: 2.5%, p=0.37; 5%, p<0.01; and 10%, p<0.01). As shown in Fig. 3c, alcohol preference was abolished when the 2.5% and 5% alcohol concentrations were in competition with a 1% sucrose solution (sucrose×concentration effect: $F(2,68)$=34.48, p<0.001; 2.5%, p<0.001; and 5%, p<0.001). The preference for the 10% alcohol concentration in competition with water or sucrose 1% remained low. A

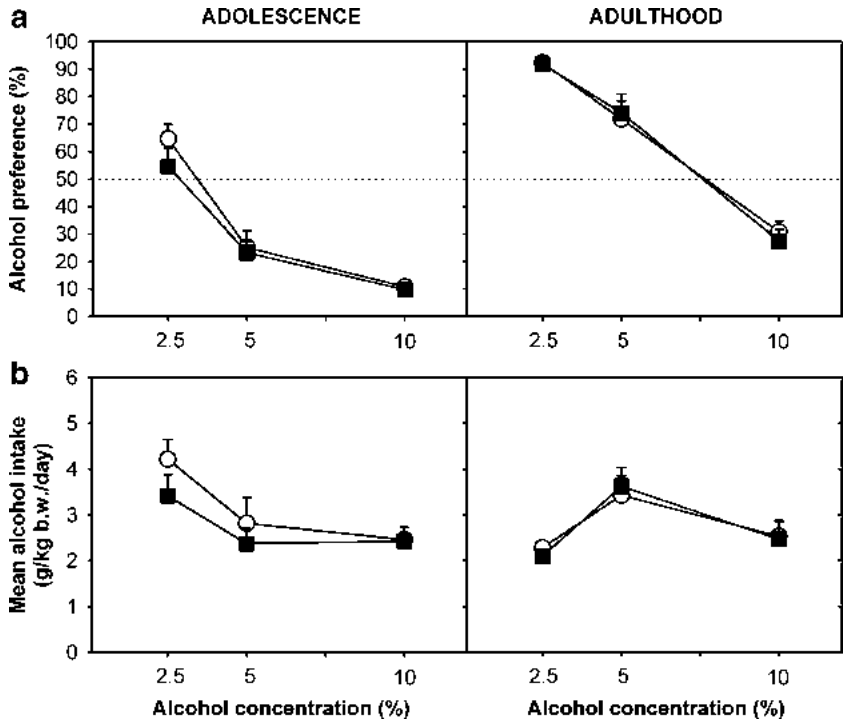

Fig. 2 Spontaneous alcohol preference in naïve adolescent (*left panels*) and in adult rats previously submitted to a forced alcohol exposure (*right panel*). **a** Alcohol preference (percent) and (**b**) mean alcohol intake (grams/kilogram of body weight per day) for different alcohol concentrations (2.5%, 5%, or 10%) in control (*circles*) and prenatal restraint stress (*squares*) rats. *Dotted lines* show no preference for alcohol over water. N=10–14 per group

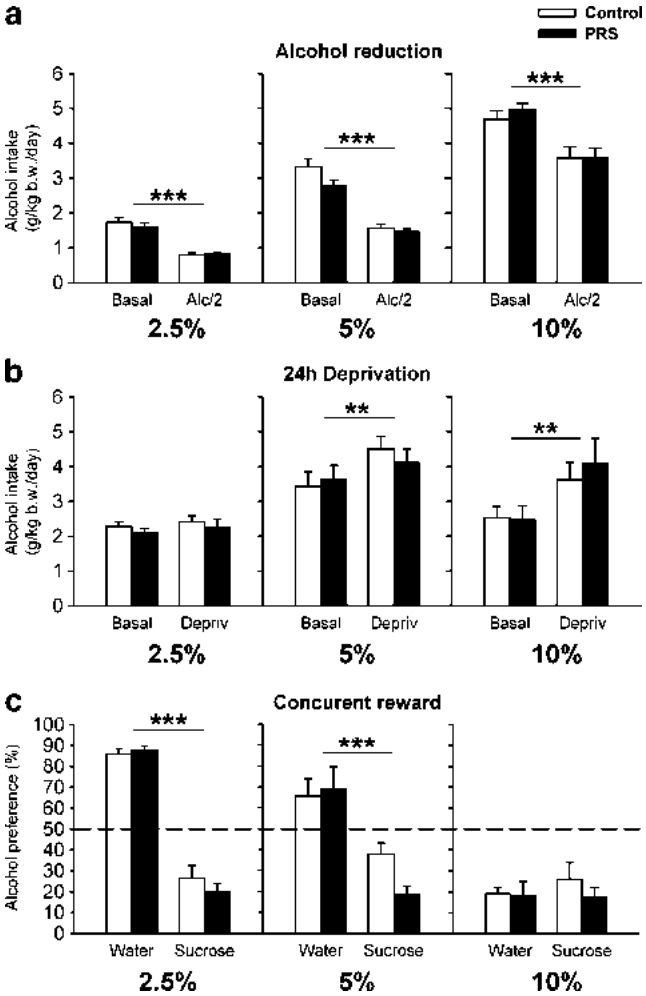

Fig. 3 Study of compulsive alcohol consumption following the chronic alcohol exposure in adult control (*open squares*) and prenatal restraint stress (*closed squares*) rats. **a** Mean alcohol intake related to body weight measured during the forced alcohol treatment under basal condition, or after a reduction per two of the alcohol concentration (Alc/2). ***$p<0.001$ different from basal intake. **b** Mean alcohol intake related to body weight measured in a two-bottles choice paradigm (free choice) under basal condition or after 24 h alcohol deprivation (*Depriv*). **$p<0.01$ different from basal intake. **c** Alcohol preference (percent) when alcohol was in competition with water or with sucrose. ***$p<0.001$ different from water. $N=10–14$ per group

complementary experiment was carried out with a different set of alcohol-naïve rats to establish that the preference for sucrose 1% versus water was similar in control and PRS rats (preference for sucrose 1% versus water, control rats, 78%±7; PRS rats, 87%±3).

Chronic alcohol intake (2.5% and 5%) suppresses hyper-reactivity to novelty associated with prenatal stress

As shown in Fig. 4, reactivity to novelty was differently affected by the treatment in PRS and control animals (group×concentration effect, $F(3,90)=3.37$, $p<0.05$). PRS

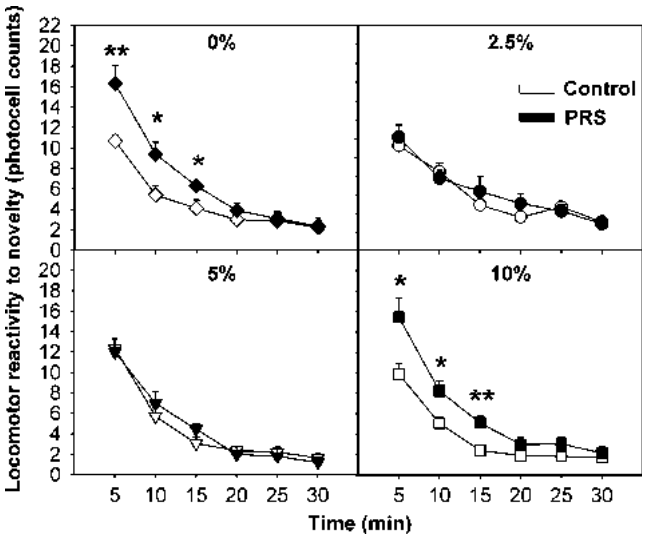

Fig. 4 Locomotor reactivity to novelty (photocell counts) in control (*open squares*) and prenatal restraint stress (PRS) (*closed squares*) rats during the forced treatment (alcohol 2.5%, 5%, 10%, or water). *$p<0.05$ and **$p<0.01$ control versus PRS. $N=10–15$ per group

rats exposed to water or to alcohol 10% displayed an elevated locomotor reactivity to novelty when compared to their respective control groups (group×time effect, water, $F(5,90)=3.26$, $p<0.01$; alcohol 10%, $F(5,115)=3.45$, $p<0.01$). Planned comparison revealed that this effect was significant during the first 15 min of the test (water, 5 min, $p<0.01$; 10 min, $p<0.05$; 15 min, $p<0.05$; and alcohol 10%, 5 min, $p<0.05$; 10 min, $p<0.05$; 15 min, $p<0.01$). No difference was reported between PRS and control rats treated with alcohol 2.5% and 5%.

Chronic alcohol consumption increases ΔFosB levels in the nucleus accumbens of rats exposed to stress in utero but has no effect on other parameters associated with stress or anxiety

Throughout the forced treatment, the mean alcohol intake increased with the alcohol concentration (concentration effect: $F(3,74)=330.62$, $p<0.001$, 10%>5%>2.5%, Table 1) but remained similar between control and PRS rats. PRS and alcohol treatment had no effect on the expression of full-length *FosB* protein (50 kD band) in the nucleus accumbens (mean optical density in water-exposed groups, control rats, 0.69±0.09; PRS rats, 0.72±0.11; and Fig. 5a). Similarly, *ΔFosB* (35–37 kD band) was unaffected by PRS in alcohol-naive animals (mean optical density in water groups, control rats, 1.00±0.23; PRS rats, 1.06±0.15). On the other hand, *ΔFosB* levels, expressed as a percentage of respective water groups, were differently affected by alcohol in PRS and control rats (Fig. 5b, ANCOVA, group effect, $F(1,58)=4.44$, $p<0.05$). In PRS rats, *ΔFosB* levels were enhanced in a dose-dependent manner by the chronic alcohol exposure

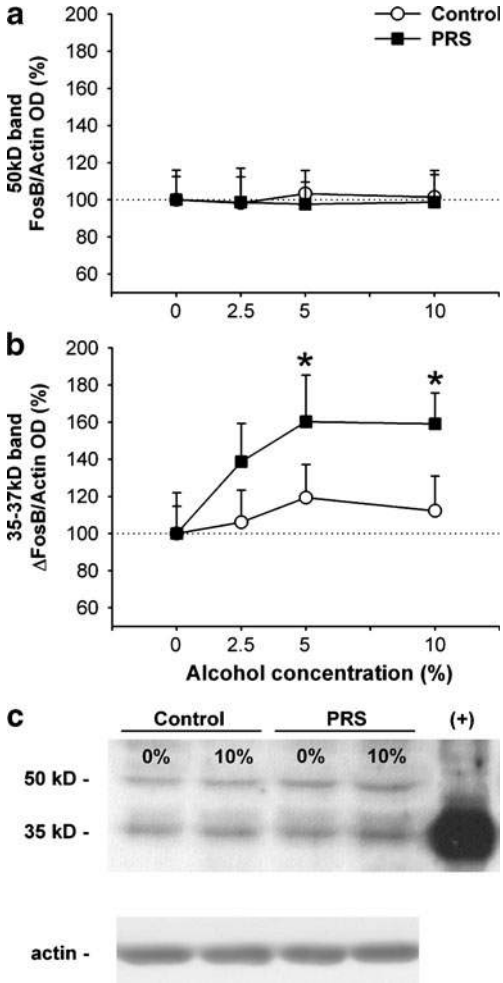

Fig. 5 **a** *FosB* (50 kD band) and (**b**) *ΔFosB* (35–37 kD band) immunoreactivity in the nucleus accumbens (*FosB/β-Actin* and *ΔFosB/β-Actin* optical density (OD)) expressed as percentage of respective water group in control (*circles*) and prenatal restraint stress (PRS) (*squares*) rats after 9 months of water exposure or 2.5%, 5%, or 10% alcohol exposure. N=6–9 per group. *p<0.05 versus PRS water. **c** Representative immunoblots of *FosB* immunoreactivity in the nucleus accumbens of PRS and control rats after 9 months of water exposure (control 0% and PRS 0%) or 9 months of 10% alcohol exposure (control 10% and PRS 10%). As a positive control (*plus sign*), we used a cell extract from PC12 cells transduced with a viral vector expressing *ΔFosB*

(linear regression, p<0.05). In contrast, alcohol had no effect on *ΔFosB* levels in control animals (linear regression, p= 0.62). Planned comparison revealed that the 5% and the 10% alcohol treatments significantly augmented *ΔFosB* levels in the nucleus accumbens of PRS rats (PRS water versus PRS 5%, p<0.05; PRS water versus PRS 10%, p<0.05).

As shown in Table 1, BAL assessed during the diurnal period was low and often below detection limit. BAL, basal plasma corticosterone levels (assessed during the first part of the light cycle) and adrenal weight were not affected by either the alcohol treatment or by the prenatal stress procedure. The percentage of time spent in the open arms of the elevated plus maze tended to be decreased in PRS animals (group effect, $F(1,88)=2.96$, p=0.09). Locomotor reactivity to novelty was similar between water and alcohol 10% treated rats, and between alcohol 2.5% and 5% treated rats (see Fig. 4). To determine if this dissociation could reflect differences in anxiety-like behavior in these animals, we compared the time spent in the open arms in animals exposed to alcohol 2.5% and 5% with those of rats exposed to water and alcohol 10%. Planned comparison revealed that animals exposed to the lowest alcohol concentrations were less anxious than the others animals (time in the open arms, alcohol 2.5%+alcohol 5% versus water+alcohol 10%, p<0.05).

Chronic alcohol consumption increases the AST/ALT ratio in rats exposed to prenatal stress

Prenatal stress significantly decreased the AST/ALT ratio in alcohol-naïve animals (control rats, 2.30±0.21; PRS rats, 1.77±0.13; Student's t test: t=2.12, df=17, p<0.05). In addition, the AST/ALT ratio, expressed as a percentage of respective water groups, was differently affected by alcohol in PRS and control animals (Fig. 6, group effect: $F(1,77)$= 15.32, p<0.001). Planned comparisons indicate that alcohol raised AST/ALT ratios in PRS rats at all tested doses (2.5%, p<0.05; 5%, p<0.01; and 10%, p<0.05), whereas it had no significant effect in control rats.

Discussion

The goal of our study was to investigate the effect of prenatal stress on alcohol vulnerability in adolescent and adult male rats. Our results reveal that alcohol preference was not affected by prenatal stress. In contrast, PRS group

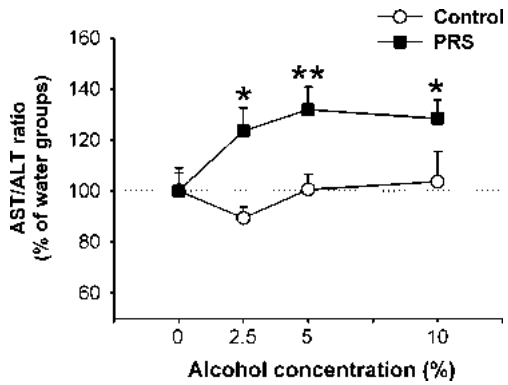

Fig. 6 Aspartate aminotransferase (AST)/alanine aminotransferase (ALT) ratio in plasma, expressed as percentage of respective water group, in control (*circles*) and prenatal restraint stress (*squares*) rats after 9 months of water exposure or 2.5%, 5%, or 10% alcohol exposure. N=9–13 per group. *p<0.05 and **p<0.01 alcohol versus water

did exhibit a higher sensitivity to the effects induced by the chronic alcohol exposure.

We confirm in the present study that prenatal stress exacerbates the locomotor reactivity to novelty in adult animals (Deminiere et al. 1992; Louvart et al. 2005). This behavior is associated with a higher sensitivity to the reinforcing effects of psychostimulants (Deminiere et al. 1989; Piazza et al. 1989) and correlates with oral alcohol self-administration in an operant paradigm (Nadal et al. 2002). However, we failed to show any impact of prenatal stress on alcohol preference, in agreement with data obtained in a previous work (Weinberg 1987). Furthermore, despite chronic exposure to alcohol (9 months at the end of the experiment), animals did not exhibit signs of dependence. We base this conclusion on three lines of evidence. First, we did not observe an inelastic drinking behavior, i.e., a compulsive behavior that is not under control (Spanagel and Holter 1999). Indeed, rats did not magnify their fluid intake to maintain a constant amount of alcohol ingestion upon reduction in the concentration presented. Second, we found that the temporary suppression of access to alcohol had little effect on subsequent intake, indicating an absence of craving. Finally, we showed that rats displayed a marked preference for a 1% sucrose solution when presented side by side with alcohol. Resistance to concurrent choice of preferred flavors, such as sucrose (Terenina-Rigaldie et al. 2004), has been typically used in the field as an index of alcohol motivation. This phenomenon extends to other highly addictive drugs of abuse (Lenoir et al. 2007). Although the reduction in sucrose preference is also considered a marker for anhedonia in animal model of depression (Bessa et al. 2009), we did not observe any decrease in the sucrose intake between PRS and control naïve animals. Altogether, our data illustrate the difficulty in inducing a compulsive drinking behavior using oral alcohol administration in rats, despite relative long-lasting treatments. This limitation is also reported in other studies, even when repeated deprivation phases are introduced or if rats are genetically selected for high voluntary consumption of alcohol (Mormede et al. 2004). The dissociation between the high alcohol intake and the lack of dependence signs reported in our study confirms that drug intake does not necessarily reflect the motivation for the drug (Zernig et al. 2007). To date, the inhalation of alcohol vapors (Aufrere et al. 1997; Le Bourhis and Aufrere 1983; Rogers et al. 1979) or liquid diets (Lieber and De Carli 1973) appear to be the most powerful procedures to induce a robust dependence in rats. In both of these models, the experimenters directly control animals' BAL, a paradigm that is very useful to study the consequences of dependence, but is of limited value when the objective is to determine the factors responsible for the vulnerability to addiction (Ahmed 2005).

Prenatal stress did not affect spontaneous alcohol consumption, but it modulated the behavioral and pharmacological effects of alcohol. Exacerbated locomotor reactivity to novelty induced by PRS was abolished by the 2.5% and 5% alcohol treatments, but not by the 10% alcohol concentration. This differential effect could reflect an anxiolytic effect of alcohol at the lowest concentrations, at which alcohol is appealing. This slight anxiolytic effect appears to be sufficient to normalize the exacerbated locomotor activity of PRS rats, but is insufficient to modulate locomotor activity in control rats. BAL assessed during the light period of the cycle was very low across all concentrations, indicating that the animals were not intoxicated during behavioral tasks. This suggests that the change in the response to novelty in PRS rats likely reflects a long-lasting effect of chronic alcohol intake rather than the acute level of intoxication during the test.

Chronic alcohol consumption produces brain region-selective changes in expression of inducible transcription factors such as *c-Fos*, *FosB*, and *Zif 268* (Bachtell et al. 1999; Vilpoux et al. 2009). *ΔFosB* is a truncated transcript of the *FosB* gene (Nestler et al. 2001). Induction of *ΔFosB* protein in the striatum is weak during initial drug exposures. However, it gradually accumulates upon repeated drug administration, in part because of its unique protein stability. This pattern of induction has been reported for several drugs of abuse, but limited data is available regarding the effect of alcohol (McClung et al. 2004; Vilpoux et al. 2009). Recently, Perrotti and collaborators (using immunohistochemistry) have shown that chronic, but not acute, administration of alcohol (7% alcohol liquid diet for 17 days) induces an increase of *ΔFosB* levels in the brain (Perrotti et al. 2008). This augmentation was detected in various areas, but the most dramatic increases were reported in the dorsal striatum, as well as in the core of the nucleus accumbens. In our study, *ΔFosB* induction by alcohol in the nucleus accumbens of control animals did not reach significance. The method used for *ΔFosB* detection (western blot versus immunochemistry) but also the mode and the duration of alcohol exposure could explain the lack of significant effect of alcohol in our study. Furthermore, in contrast to Perrotti's data (8–18 g/kg/day alcohol intake, BAL up to 2 g/l; Perrotti et al. 2008), we did not report significant rise of BAL during light period after the exposure to alcohol (max alcohol intake, 5 g/kg/day; max BAL, 1.4 g/l), confirming that animals drink during the dark period of the cycle and in bouts rather than continuously (Dole et al. 1985).

Interestingly, however, our protocol of alcohol exposure produced a dose-dependent elevation of *ΔFosB* in the nucleus accumbens of PRS rats, whereas prenatal stress exposure per se did not regulate the expression of this protein. *ΔFosB* can be induced in the nucleus accumbens

by several form of chronic stress (Perrotti et al. 2004). Alcohol preferring rats appeared more vulnerable to stress, since repeated saline injections induced a significant greater *FosB*/*ΔFosB* immunoreactivity in these animals in comparison to non-preferring rats (Marttila et al. 2007). PRS animals are more vulnerable to stress (Darnaudery and Maccari 2008; Maccari and Morley-Fletcher 2007), and it could be hypothesized that forced alcohol administration constitutes a chronic stressful situation. However, diurnal basal corticosterone and adrenal gland weight were similar in control and PRS rats at the end of the chronic alcohol exposure, and alcohol did not differentially regulate anxiety between groups. These findings suggest that the elevation of *ΔFosB* levels observed in PRS rats is related to the alcohol treatment itself. Further studies are needed to determine whether this result is specific to alcohol or whether it could be generalized to others reinforcing stimuli such as psychostimulants or even palatable food. To our knowledge, the present study is the first to demonstrate that early environmental manipulations can interact with the molecular effect of alcohol later in life. It is interesting because other data also indicate that environmental enrichment during early stages of life is able to change the consequences of repeated cocaine administration on striatal *ΔFosB* levels. Indeed, *ΔFosB* levels were upregulated by cocaine in mice reared in enriched environment but downregulated after the same treatment in the ones reared in a standard environment (Solinas et al. 2009).

Does the increase in *ΔFosB* levels in the nucleus accumbens could produce a greater vulnerability to ethanol consumption? Transgenic mice that overexpress *ΔFosB* display augmented locomotor responses to cocaine and sensitivity to the rewarding effects of cocaine and morphine in place conditioning test (Kelz et al. 1999; Zachariou et al. 2006). Moreover, mice expressing *ΔFosB* self-administer more cocaine in a progressive ratio procedure, suggesting that *ΔFosB* may sensitize animals to the incentive motivational properties of this drug (Colby et al. 2003). The link between *ΔFosB* levels in the nucleus accumbens and the rewarding effect of alcohol seems more complex. Indeed, dissociation between alcohol preference and saccharin preference has been reported in *FosB* knockout mice. The permanent elimination of *FosB* gene products does not alter alcohol intake but enhances the preference for sweet solution in mice (Korkosz et al. 2004). Several experimental procedures, such as a lesion of the subthalamic nucleus, have been shown to increase motivation for alcohol in a self-administration paradigm without impacting alcohol intake (Lardeux and Baunez 2008). In this context, it would be important to examine the motivation for alcohol in an operant paradigm (using a progressive ratio schedule) in PRS animals (Campbell et al. 2009). Finally, despite low BAL, we also observed an enhancement of indices of hepatic damages in PRS rats, whereas no such effects were seen in control rats. A histological analysis of the liver will be necessary to further explore the physiological significance of this interaction.

In conclusion, we showed that prenatal stress did not modulate spontaneous alcohol consumption in male rats. However, induction of *ΔFosB* by the chronic exposure to alcohol was exacerbated in the nucleus accumbens in PRS animals, suggesting that early stress may lead to a higher sensitivity of the brain reward systems to alcohol. It remains to determine whether the exacerbated *ΔFosB* upregulation in PRS rats could be extended to other reinforcing stimuli.

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NEUROSYSTEMS

Selective serotonin reuptake inhibitor antidepressants potentiate methylphenidate (Ritalin)-induced gene regulation in the adolescent striatum

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Keywords: caudate–putamen, immediate-early gene, psychostimulant, rat

Abstract

The psychostimulant methylphenidate (Ritalin) is used in conjunction with selective serotonin reuptake inhibitors (SSRIs) in the treatment of medical conditions such as attention-deficit hyperactivity disorder with anxiety/depression comorbidity and major depression. Co-exposure also occurs in patients on SSRIs who use psychostimulant 'cognitive enhancers'. Methylphenidate is a dopamine/norepinephrine reuptake inhibitor that produces altered gene expression in the forebrain; these effects partly mimic gene regulation by cocaine (dopamine/norepinephrine/serotonin reuptake inhibitor). We investigated whether the addition of SSRIs (fluoxetine or citalopram; 5 mg/kg) modified gene regulation by methylphenidate (2–5 mg/kg) in the striatum and cortex of adolescent rats. Our results show that SSRIs potentiate methylphenidate-induced expression of the transcription factor genes *zif268* and c-*fos* in the striatum, rendering these molecular changes more cocaine-like. Present throughout most of the striatum, this potentiation was most robust in its sensorimotor parts. The methylphenidate + SSRI combination also enhanced behavioral stereotypies, consistent with dysfunction in sensorimotor striatal circuits. In so far as such gene regulation is implicated in psychostimulant addiction, our findings suggest that SSRIs may enhance the addiction potential of methylphenidate.

Introduction

Use of the psychostimulant methylphenidate (Ritalin), both in the treatment of attention-deficit hyperactivity disorder (ADHD) and as a 'cognitive enhancer' in the healthy, has increased considerably over the past decades (Kollins *et al.*, 2001; Swanson & Volkow, 2008; Bogle & Smith, 2009). Although it remains controversial whether the medical use of psychostimulants is completely safe (Kollins, 2008; Wilens *et al.*, 2008), especially in children and adolescents (Carlezon & Konradi, 2004; Andersen, 2005), one aspect of such drug treatments is often overlooked: potential drug interactions. Methylphenidate is frequently administered together with selective serotonin reuptake inhibitors (SSRIs). This combination treatment is used, for example, to treat ADHD with anxiety/depression comorbidity (Safer *et al.*, 2003; Bhatara *et al.*, 2004), as a high percentage of ADHD patients are also diagnosed with major depressive or bipolar disorder (Kollins, 2008). Methylphenidate + SSRI combination treatments are also employed in depression, for example as augmentation therapy in major depressive disorder (e.g. Nelson, 2007; Ishii *et al.*, 2008; Ravindran *et al.*, 2008), as acceleration treatment with SSRIs (e.g. Lavretsky *et al.*, 2003), or to treat sexual dysfunction (e.g. Csoka *et al.*, 2008). In addition to such clinical co-administration, it is presently unknown how much uncontrolled methylphenidate + SSRI co-exposure occurs as a result of 'cognitive enhancer' use (Greely *et al.*, 2008) by patients on SSRIs.

Concerns regarding potential harmful consequences of methylphenidate + SSRI co-exposure are related to the neurochemical effects of these drugs. The mode of action of methylphenidate overlaps with that of other psychostimulants. Like cocaine, methylphenidate blocks the dopamine (and norepinephrine) transporter, thus indirectly producing excessive dopamine receptor stimulation and ensuing changes in gene regulation in dopamine target areas such as the striatum [for a review, see Yano & Steiner (2007)]. Among the many genes affected by methylphenidate (Adriani *et al.*, 2006a,b), those encoding transcription factors [immediate-early genes (IEGs)] such as *zif268* and c-*fos* (Lin *et al.*, 1996; Brandon & Steiner, 2003; Chase *et al.*, 2003; Yano & Steiner, 2005b) are of special interest, as they regulate the expression of effector genes and are thus implicated in the neuroplasticity underlying psychostimulant addiction (Hyman & Nestler, 1996; Berke & Hyman, 2000).

However, methylphenidate differs from cocaine in that it has a much lower affinity for the serotonin transporter and does not produce serotonin overflow (Kuczenski & Segal, 1997; Borycz *et al.*, 2008; for review, see Yano & Steiner, 2007). This may explain why not all of the gene regulation effects of cocaine are mimicked by methylphenidate (Yano & Steiner, 2007). There is evidence indicating that serotonin interacts with dopamine to modify striatal gene regulation by psychostimulants. For example, interruption of serotonin transmission

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by transmitter depletion (Bhat & Baraban, 1993) or receptor deletion (Lucas *et al.*, 1997) reduces IEG induction by cocaine.

We here determied whether concomitant treatment with SSRIs, which elevate extracellular serotonin levels, enhances methylphenidate-induced gene regulation and produces more cocaine-like effects. We investigated, in adolescent rats, the effects of the SSRIs fluoxetine and citalopram on gene regulation in the striatum and cortex. We assessed the expression of the two transcription factors/IEGs *zif268* and c-*fos* to allow for a more sensitive two-marker correlation analysis of drug effects (Yano & Steiner, 2005a,b). Our results show that these SSRIs potentiate methylphenidate-induced gene expression preferentially in the sensorimotor striatum. A partial account of our findings has been presented in a brief report (Steiner *et al.*, 2010).

Materials and methods

Subjects

Male Sprague–Dawley rats (35 days old at the time of the drug treatment; Harlan, Madison, WI, USA) were housed two per cage under standard laboratory conditions (12-h/12-h light/dark cycle; lights on at 07:00 h) with food and water available *ad libitum*. Experiments were performed between 13:00 and 17:00 h. Prior to the drug treatment, the rats were allowed 1 week of acclimation, during which they were repeatedly handled. All procedures met the NIH guidelines for the care and use of laboratory animals, and were approved by the Rosalind Franklin University Animal Care and Use Committee.

Drug treatments

Rats received a single intraperitoneal injection of vehicle (V), methylphenidate HCl [2 mg/kg (MP2) or 5 mg/kg (MP5) in 0.02% ascorbic acid, 1 mL/kg; Sigma, St Louis, MO, USA], fluoxetine HCl (5 mg/kg, FLX; Sigma), or methylphenidate plus fluoxetine (MP2 + FLX or MP5 + FLX) (n = 5–7 each). Other groups were treated with citalopram HBr (5 mg/kg, CIT; Sigma), MP5 + CIT or cocaine HCl (25 mg/kg; Sigma). After the injection, the rat was placed in an open-field apparatus (43 × 43 cm), and locomotion (ambulatory distance) and stereotypy ('stereotypy 2') counts were measured for 40 min with an activity monitoring system (Truscan, Coulbourn Instruments, Allentown, PA, USA). These 'stereotypy' counts reflect local, repetitive movements (e.g. head bobbing and focused sniffing).

Tissue preparation and in situ *hybridization histochemistry*

The rats were killed with CO_2 40 min after the injection. The brain was rapidly removed, frozen in isopentane cooled on dry ice, and then stored at −30°C until cryostat sectioning. Coronal sections (12 μm) were thaw-mounted onto glass slides (Superfrost/Plus, Daigger, Wheeling, IL, USA), dried on a slide warmer, and stored at −30°C. In preparation for the *in situ* hybridization histochemistry, the sections were fixed in 4% paraformaldehyde/0.9% saline for 10 min at room temperature, incubated in a fresh solution of 0.25% acetic anhydride in 0.1 M triethanolamine/0.9% saline (pH 8.0) for 10 min, dehydrated, defatted for 2 × 5 min in chloroform, rehydrated, and air-dried. The slides were then stored at −30°C until hybridization.

Oligonucleotide probes (48-mers; Invitrogen, Rockville, MD, USA) were labeled with [^{35}S]dATP as described previously (Steiner & Kitai, 2000). The probes had the following sequence: *zif268*, complementary to bases 352–399, GenBank accession number M18416; c-*fos*, bases 207–254, ×06769. One hundred microliters of hybridization buffer containing labeled probe (~3 × 10^6 c.p.m.) was added to each slide. The sections were coverslipped and incubated at 37°C overnight. After incubation, the slides were rinsed in four washes of 1× saline citrate (150 mM sodium chloride, 15 mM sodium citrate), washed three times for 20 min each in 2× saline citrate/50% formamide at 40°C, and then washed twice for 30 min each in 1× saline citrate at room temperature. After a brief water rinse, the sections were air-dried and then apposed to X-ray film (BioMax MR-2; Kodak) for 5–9 days.

Analysis of autoradiograms

Gene expression in the cortex was assessed in sections from four rostrocaudal levels (Fig. 1): frontal, approximately at +2.7 mm relative to bregma (Paxinos & Watson, 1998); rostral, +1.6; middle, +0.4; and caudal, −0.8. Levels of mRNA were measured in a total of 22 cortical regions (from medial to lateral; Paxinos & Watson, 1998):

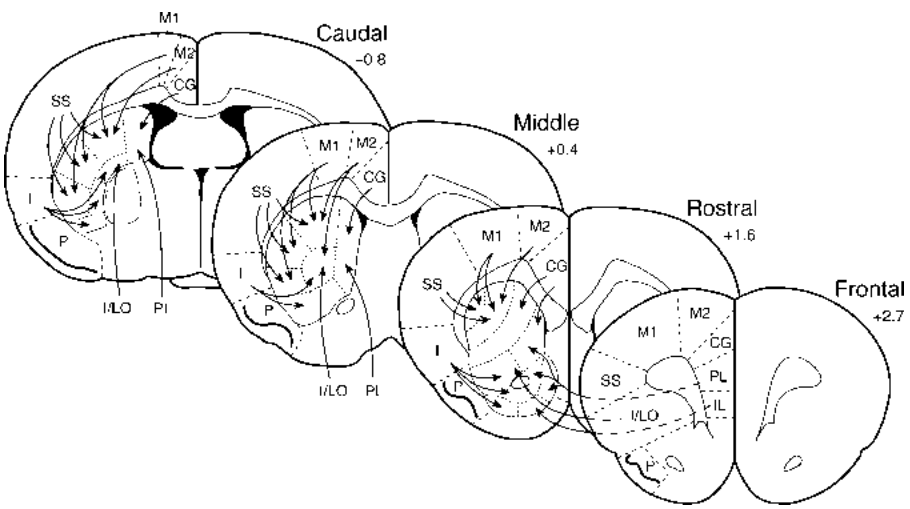

FIG. 1. Schematic illustration of the 23 striatal and 22 cortical regions used to measure gene expression. The predominant cortical inputs to these striatal sectors are indicated by arrows (simplified; see Willuhn *et al.*, 2003, for discussion). Gene expression was assessed at four rostrocaudal levels: frontal, rostral, middle, and caudal (ranging from +2.7 to –0.8 mm relative to bregma; Paxinos & Watson, 1998). Cortical areas (from medial to lateral): CG, cingulate; M2, medial agranular; M1, motor; SS, somatosensory; I, insular; P, piriform; IL, infralimbic; PL, prelimbic; I/LO, insular/lateral orbital. For striatal areas, see Fig. 6.

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cingulate, medial agranular, motor, somatosensory and insular cortex at the frontal to caudal levels, and infralimbic, prelimbic and insular/lateral orbital cortex at the frontal level. Striatal gene expression was determined at the rostral, middle and caudal levels in a total of 23 sectors mostly defined by their predominant cortical inputs (Fig. 1) (see Willuhn *et al.*, 2003). Eighteen of these sectors represented the caudate–putamen (medial, dorsomedial, dorsal, dorsolateral, ventrolateral, ventral, central, dorsal central, and ventral central) and five the nucleus accumbens (medial core, lateral core, medial shell, ventral shell and lateral shell) (Yano & Steiner, 2005a).

Hybridization signals on film autoradiograms were measured by densitometry (NIH Image; Wayne Rasband, NIMH, Bethesda, MD, USA). The films were captured using a light table (Northern Light; Imaging Research, St Catharines, Ontario, Canada) and a Sony CCD camera (Imaging Research). The 'mean density' value of a region of interest was measured by placing a template over the captured image. Mean densities were corrected for background by subtracting mean density values measured over white matter (corpus callosum). Values from corresponding regions in the two hemispheres were then averaged. The illustrations of film autoradiograms displayed in Figs 3, 5 and 10 are computer-generated images, and are contrast-enhanced where necessary. The maximal hybridization signal is in black.

Statistics

Treatment effects were determined by two-factor and three-factor ANOVA with methylphenidate (0, 2 and 5 mg/kg) and fluoxetine or citalopram (0 and 5 mg/kg) as between-subject variables. Newman–Keuls *post hoc* tests were used to describe differences between individual groups (Statistica; StatSoft, Tulsa, OK, USA). For illustrations of topographies (maps), the change in gene expression in a given region was expressed as the percentage of the maximal change in the striatum observed for a particular probe (% maximum). It was of interest to determine whether the regional distribution in the striatum of the changes in gene expression was similar for c-*fos* and *zif268.* Thus, these changes in the 23 striatal sectors were compared by Pearson correlations. Also, our previous studies showed that such two-marker correlation analyses are more sensitive than ANOVAs in measuring threshold drug effects, because trends contribute to correlations (Yano & Steiner, 2005a,b). For these analyses, the values were normalized relative to the maximal change observed for the MP5 treatment.

Results

Effects of fluoxetine on methylphenidate-induced open-field behavior

Administration of methylphenidate increased ambulation (MP2 vs. V, $P < 0.01$; MP5 vs. V, $P < 0.001$) and stereotypy counts (MP2 vs. V, $P < 0.001$; MP5 vs. V, $P < 0.01$) in the first half (0–20 min) and second half (20–40 min) of the test (Fig. 2). Fluoxetine alone had no effect on these two parameters (FLX vs. V, $P > 0.05$). The methylphenidate–fluoxetine combinations increased ambulation counts in a manner similar to methylphenidate alone (MP2 + FLX vs. MP2 and MP5 + FLX vs. MP5, $P > 0.05$) and more than fluoxetine alone (MP2 + FLX vs. FLX and MP5 + FLX vs. FLX, $P < 0.001$). Like methylphenidate alone, the methylphenidate–fluoxetine combinations induced stereotypies (MP2 + FLX vs. V or FLX, and MP5 + FLX vs. V or FLX, $P < 0.001$). This increase in stereotypy counts was similar in the MP2 and MP2 + FLX groups ($P > 0.05$). However, the MP5 + FLX group showed higher stereotypy counts than the MP5-alone group ($P < 0.001$; Fig. 2) early and late in the test, demonstrating that fluoxetine potentiated methylphenidate-induced stereotypies for this higher methylphenidate dose.

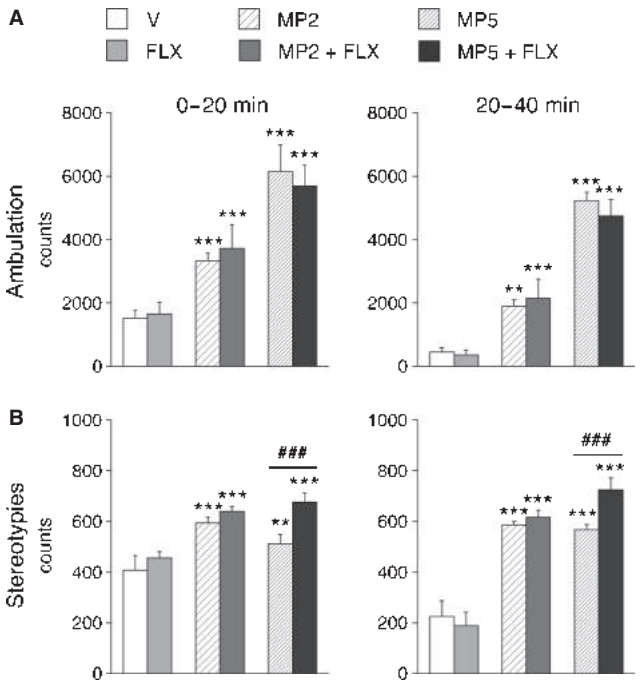

FIG. 2. Drug effects on open-field behavior. Ambulation (A) and stereotypy counts (B) are shown for animals that received a systemic injection of vehicle (V), fluoxetine (5 mg/kg; FLX), methylphenidate (2 or 5 mg/kg; MP2 and MP5), or methylphenidate + fluoxetine combinations (MP2 + FLX and MP5 + FLX) (n = 5–7) and were tested for 40 min in a novel open field. Fluoxetine selectively potentiated MP5-induced stereotypies. $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. respective control group (V or FLX). $^{###}P < 0.001$, MP5 + FLX vs. MP5 (potentiation).

Effects of fluoxetine on methylphenidate-induced gene expression in the striatum

Administration of methylphenidate alone induced a dose-dependent increase in *zif268* and c-*fos* expression in the striatum at all three rostrocaudal levels (Figs 3–6 and Tables 1 and 2), consistent with our previous findings (Brandon & Steiner, 2003; Yano & Steiner, 2005a,b). For *zif268*, a significant increase in expression was observed in five (MP2) and 17 (MP5) of the 23 striatal sectors, and for c-*fos* in three (MP2) and 11 (MP5) sectors ($P < 0.05$ vs. V) (Fig. 6). Gene regulation varied considerably between different striatal regions. For both *zif268* and c-*fos*, the most robust increase was observed at the middle and caudal striatal levels, in dorsal/central and medial sectors (Figs 3–6) that receive sensorimotor and cingulate cortical inputs (Fig. 1). In contrast, the nucleus accumbens displayed more modest drug effects. No statistically significant changes in gene expression were seen with MP2 alone ($P > 0.05$ vs. V). The 5 mg/kg dose significantly increased *zif268* expression in the lateral shell only (MP5 vs. V, $P < 0.001$; Figs 4 and 6). In order to compare the regional patterns of methylphenidate-induced *zif268* and c-*fos* expression across the 23 striatal sectors, we performed a correlation analysis. This analysis confirmed that the regional distribution of increases (vs. vehicle-treated controls) was highly correlated between *zif268* and c-*fos* expression (*zif268* × c-*fos*: MP2, $r = 0.844$, $P < 0.001$; MP5, $r = 0.931$, $P < 0.001$; not shown).

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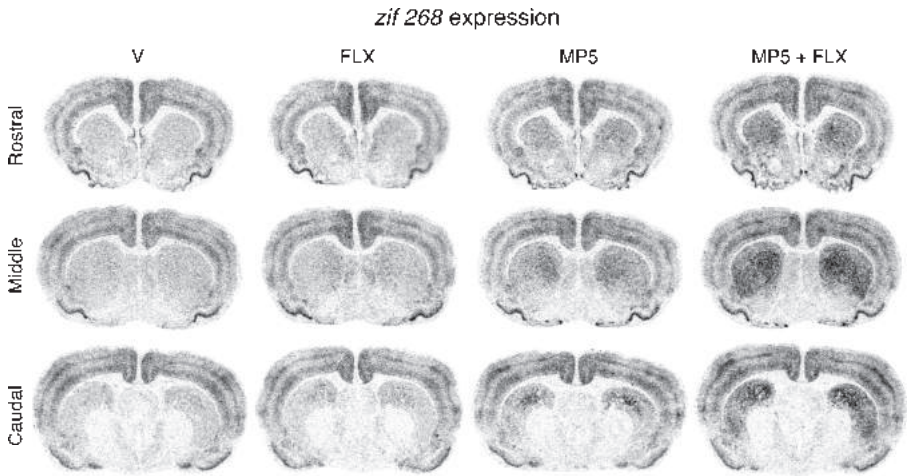

FIG. 3. Fluoxetine potentiates methylphenidate-induced *zif268* expression in the striatum. Illustrations of film autoradiograms depict *zif268* expression in coronal sections from the rostral, middle and caudal striatum in rats treated with vehicle (V), fluoxetine (5 mg/kg; FLX), methylphenidate (5 mg/kg; MP5), or methylphenidate + fluoxetine (MP5 + FLX). The maximal hybridization signal is in black.

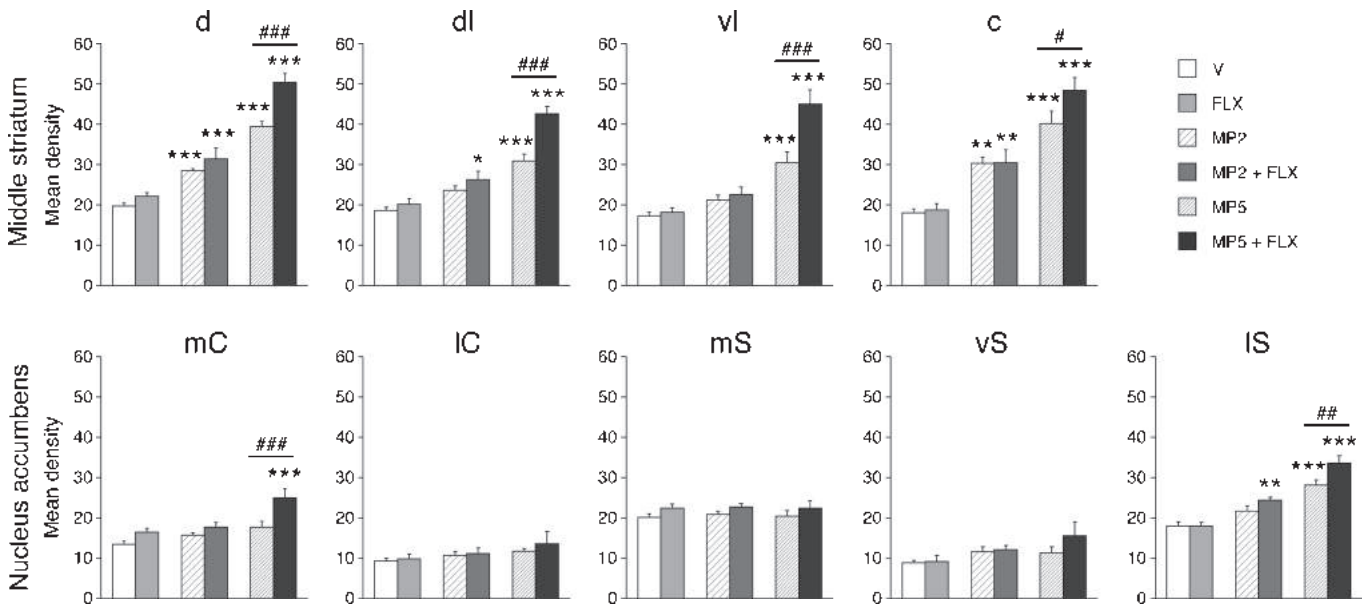

FIG. 4. Fluoxetine potentiation of methylphenidate-induced *zif268* expression in specific striatal sectors. Mean density values (mean ± standard error of the mean) for *zif268* expression in rats that received an injection of vehicle (V), fluoxetine (5 mg/kg; FLX), methylphenidate (2 or 5 mg/kg; MP2 and MP5), or methylphenidate + fluoxetine combinations (MP2 + FLX and MP5 + FLX) (n = 5–7) are depicted for four middle striatal sectors (top) and the five sectors of the nucleus accumbens (bottom). Caudate–putamen: d, dorsal; dl, dorsolateral; vl, ventrolateral; c, central. Nucleus accumbens: mC, medial core; lC, lateral core; mS, medial shell; vS, ventral shell; lS, lateral shell. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ vs. respective control group (V or FLX). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$, MP + FLX vs. MP (potentiation).

In contrast to methylphenidate, FLX alone did not modify gene expression, either in the caudate–putamen or in the nucleus accumbens (Figs 3–6). None of the 23 sectors showed significant changes in *zif268* or c-*fos* expression ($P > 0.05$ vs. V, Figs 4 and 6, Tables 1 and 2).

However, when given in conjunction with methylphenidate, fluoxetine potentiated methylphenidate-induced IEG expression in the striatum. Correlation analyses showed that the regional distribution of this potentiation (i.e. the difference between methylphenidate + fluoxetine and methylphenidate) was similar for *zif268* and c-*fos* expression [*zif268* × c-*fos*: potentiation of MP2 effects (POT2), $r = 0.775$, $P < 0.001$; potentiation of MP5 effects (POT5), $r = 0.840$, $P < 0.001$; Fig. 7]. Moreover, despite relatively modest potentiation for MP2, mainly the same sectors were affected as for MP5 (POT2 × POT5: *zif268*, $r = 0.659$, $P < 0.05$; c-*fos*, $r = 0.759$, $P < 0.01$; not shown).

The fluoxetine potentiation was reflected in a higher proportion of the 23 striatal sectors displaying significantly increased *zif268* and c-*fos* expression after the methylphenidate + fluoxetine treatment as compared with methylphenidate alone (*zif268*: MP2 + FLX vs. MP2, 10 sectors vs. five sectors; MP5 + FLX vs. MP5, 19 sectors vs. 17 sectors) (c-*fos*: MP2 + FLX vs. MP2, six sectors vs. three sectors; MP5 + FLX vs. MP5, 16 sectors vs. 11 sectors; Fig. 6). Direct statistical comparisons showed that, for MP2, c-*fos* induction was significantly more robust in the MP2 + FLX group than in the MP2 group in one sector (middle level, dorsal sector; Fig. 6, POT2). For MP5, the potentiation (MP5 + FLX vs. MP5, POT5) was statistically

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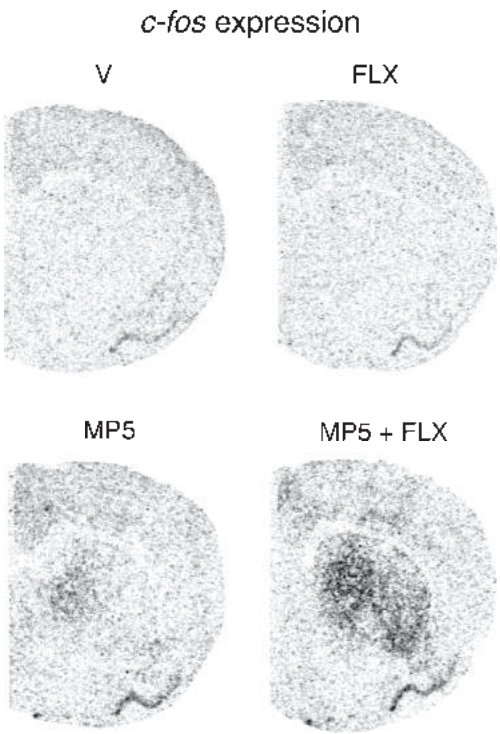

FIG. 5. Fluoxetine potentiates methylphenidate-induced c-*fos* expression in the striatum. Illustrations of film autoradiograms depict c-*fos* expression in coronal sections from the middle striatum in rats that received vehicle (V), fluoxetine (5 mg/kg; FLX), methylphenidate (5 mg/kg; MP5), or methylphenidate + fluoxetine (MP5 + FLX). The maximal hybridization signal is in black.

significant in 15 and 13 of the 23 striatal sectors, for *zif268* and c-*fos*, respectively. Further analysis showed that the magnitude of the fluoxetine potentiation was principally related to the magnitude of gene induction produced by methylphenidate alone (MP5 × POT5: *zif268*, $r = 0.638$, $P < 001$; c-*fos*, $r = 0.814$, $P < 001$; Fig. 8) (MP2: $P > 0.05$; not shown). However, more pronounced potentiation than predicted by the methylphenidate response was seen in the dorsolateral and ventrolateral (sensorimotor) sectors of the middle striatum for both *zif268* and c-*fos* expression (Fig. 8). This finding confirms that the lateral (sensorimotor) striatum displayed more pronounced fluoxetine potentiation of gene regulation than the medial (associative) striatum; this is also apparent in the potentiation maps (Fig. 6).

Fluoxetine also potentiated methylphenidate-induced gene expression in selective regions of the nucleus accumbens, predominantly in the lateral part of the shell (Figs 4 and 6). After treatment with MP2 + FLX, but not with MP2 alone, *zif268* expression was significantly increased in the lateral shell, as was c-*fos* expression after treatment with MP5 + FLX, but not with MP5 alone. For *zif268*, significant potentiation in MP5 + FLX vs. MP5 animals (POT5) was seen in the lateral shell as well as in the medial core (Fig. 6).

Effects of fluoxetine on methylphenidate-induced zif268 *expression in the cortex*

Administration of methylphenidate alone induced a dose-dependent upregulation of *zif268* expression in the cortex at all four rostrocaudal levels (Fig. 9, Table 3). A statistically significant increase in *zif268* mRNA levels was observed in seven (MP2) and 11 (MP5) of the 22 cortical areas ($P < 0.05$ vs. V). However, this effect was restricted to dorsomedial cortical regions, including the cingulate, medial agranular and motor cortex (mainly on the rostral to caudal levels), as well as the prelimbic and insular/lateral orbital cortex (frontal level). These are mostly limbic and associative areas. In contrast, the somatosensory cortex and insular cortex (except for the frontal level) were not affected by methylphenidate on any rostrocaudal levels (MP2 or MP5 vs. V, $P > 0.05$).

Fluoxetine given alone tended to increase *zif268* mRNA levels in many cortical areas, but this effect was statistically significant only in the cingulate (frontal level) and motor cortex (caudal level) (FLX vs. V, $P < 0.05$ and $P < 0.01$, respectively; Fig. 9 and Table 3). After the methylphenidate + fluoxetine treatment, significantly enhanced *zif268* expression was found in eight (MP2 + FLX) and 14 (MP5 + FLX) of the 22 cortical areas (as compared to seven and 11, respectively, for methylphenidate only; see above). However, the methylphenidate + fluoxetine treatment did not produce significantly higher *zif268* mRNA levels than methylphenidate alone in any cortical area (methylphenidate + FLX vs. MP; $P > 0.05$), except the infralimbic cortex at the frontal level for MP2 (Fig. 9; MP2 + FLX vs. MP2, $P < 0.01$; MP5 + FLX vs. MP5, $P > 0.05$). Therefore, in contrast to what was seen in the striatum, this dose of fluoxetine did not robustly potentiate cortical gene regulation by methylphenidate.

Our earlier findings (Yano & Steiner, 2005a; Cotterly *et al.*, 2007) showed that there is a positive correlation between psychostimulant-induced gene expression in cortical areas and gene induction in the striatal sectors targeted by these cortical areas, indicating coordinated molecular changes in cortical and striatal nodes of corticostriatal circuits. We assessed whether such a relationship existed in the present study and whether it was affected by the present SSRI treatments. Thus, drug-induced increases in *zif268* expression in cortical areas were compared with those in their respective striatal target sectors (see Fig. 1). When a striatal sector received input from more than one cortical area, the values of these cortical areas were averaged. Our results show that, overall, methylphenidate-induced *zif268* expression in the cortical areas was positively correlated with that in their connected 23 striatal sectors (MP2, $r = 0.493$, $P < 0.05$; MP5, $r = 0.436$, $P < 0.05$), confirming our earlier findings (Yano & Steiner, 2005a; Cotterly *et al.*, 2007). This effect was more robust when only the 18 sectors of the caudate–putamen were included (MP2, $r = 0.556$, $P < 0.05$; MP5, $r = 0.676$, $P < 0.01$). The addition of fluoxetine to methylphenidate weakened this correlation (23 sectors: MP2 + FLX, $r = 0.326$, $P > 0.05$; MP5 + FLX, $r = 0.307$, $P > 0.05$) (18 sectors: MP2 + FLX, $r = 0.503$, $P < 0.05$; MP5 + FLX, $r = 0.567$, $P < 0.05$).

Effects of citalopram on methylphenidate-induced IEG expression in the striatum

We also assessed whether the potentiation of methylphenidate-induced gene regulation could be generalized to other SSRIs. Our results demonstrate that this is the case. Administration of the SSRI citalopram (5 mg/kg) together with MP5 potentiated methylphenidate-induced expression of *zif268* (Fig. 10) and c-*fos* (not shown) in the striatum. The regional distribution of this potentiation was similar to that produced by fluoxetine.

Discussion

In these studies, we demonstrate that concomitant administration of SSRIs (fluoxetine or citalopram) robustly potentiates gene regulation

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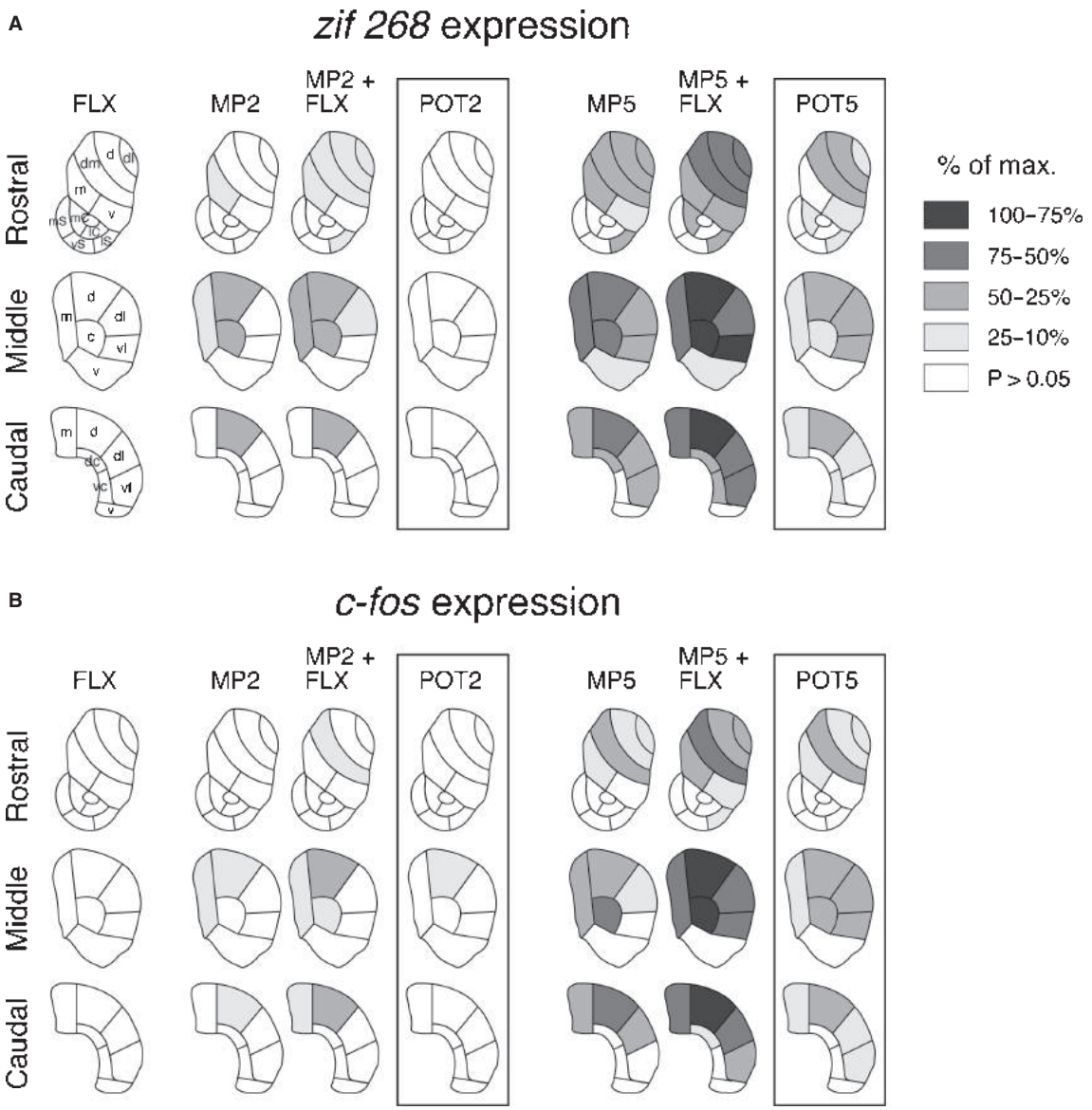

FIG. 6. Topography of fluoxetine-potentiated gene regulation by methylphenidate. Maps depict the distribution of *zif268* (A) and c-*fos* expression (B) in the rostral, middle and caudal striatum after an injection of fluoxetine (5 mg/kg; FLX), methylphenidate (2 or 5 mg/kg; MP2 and MP5), or methylphenidate + fluoxetine combinations (MP2 + FLX and MP5 + FLX). The potentiation (POT) denotes the difference between methylphenidate + fluoxetine and methylphenidate alone. The data are normalized relative to the maximal increase observed in the striatum (% of maximum). Sectors with significant differences vs. vehicle-treated controls ($P < 0.05$) are shaded as indicated. Sectors without significant effects are in white. Caudate–putamen: c, central; d, dorsal; dc, dorsal central; dl, dorsolateral; dm, dorsomedial; m, medial; v, ventral; vc, ventral central; vl, ventrolateral. Nucleus accumbens: mC, medial core; lC, lateral core; mS, medial shell; vS, ventral shell; lS, lateral shell.

by the psychostimulant and dopamine reuptake blocker methylphenidate, consistent with the notion that serotonin facilitates dopamine-mediated gene regulation (see Yano & Steiner, 2007). Our findings show enhanced induction of IEG transcription factors (*zif268* and c-*fos*) in the striatum, with the most robust effects occurring in sensorimotor parts, which mediate motor learning/habit formation and are implicated in compulsive aspects of drug taking (see below), and more modest effects in the nucleus accumbens, which participates in reward processes. These molecular changes were associated with selective potentiation of motor stereotypies, which are thought to reflect dysfunction in sensorimotor striatal circuits and may be related to compulsive behavior.

SSRIs potentiate methylphenidate-induced IEG expression in the striatum and nucleus accumbens

Activation of transcription factors by psychostimulants regulates the expression of effector genes, and is thus critical for many forms of long-term neuroplasticity related to addiction. The effects of methylphenidate on *zif268* and c-*fos* expression emerged with 2 mg/kg (i.p.) and were more pronounced with 5 mg/kg, consistent with previous findings (e.g. Brandon & Steiner, 2003; Yano & Steiner, 2005b; see also Yano & Steiner, 2007). Our present results show that 5 mg/kg fluoxetine, which by itself had no effect on gene expression, potentiated gene regulation by both 2 and 5 mg/kg methylphenidate, with more robust potentiation being seen for the

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TABLE 1. Effects of fluoxetine, methylphenidate and methylphenidate + fluoxetine on *zif268* expression in the striatum

	V	FLX	MP2	MP2 + FLX	MP5	MP5 + FLX
Rostral						
dl	18.9 ± 0.8	20.4 ± 1.1	22.8 ± 1.0	24.4 ± 1.6	29.0 ± 0.9***	36.8 ± 2.2***,###
d	19.9 ± 0.8	23.3 ± 1.4	24.5 ± 0.9	27.5 ± 1.2	31.2 ± 0.8***	39.9 ± 2.6***,###
dm	20.4 ± 0.7	23.5 ± 0.9	24.8 ± 0.5	28.5 ± 1.3	34.5 ± 0.9***	43.9 ± 3.1***,###
m	17.9 ± 0.5	20.7 ± 1.3	22.7 ± 0.9*	25.4 ± 0.7*	31.6 ± 1.8***	34.3 ± 1.8***
v	16.2 ± 0.9	17.7 ± 1.4	18.8 ± 0.7	20.4 ± 0.8	21.9 ± 1.3*	27.7 ± 1.8***,##
mC	13.4 ± 0.9	16.4 ± 1.0	15.7 ± 0.7	17.7 ± 1.1	17.8 ± 1.4	25.0 ± 2.4***,###
lC	9.3 ± 0.7	9.9 ± 1.1	10.7 ± 0.9	11.2 ± 1.4	11.8 ± 0.7	13.6 ± 3.0
mS	20.2 ± 0.8	22.5 ± 1.0	20.9 ± 0.8	22.7 ± 0.9	20.5 ± 1.5	22.5 ± 1.8
vS	8.8 ± 0.7	9.2 ± 1.4	11.6 ± 1.2	12.1 ± 1.0	11.3 ± 1.5	15.6 ± 3.4
lS	18.0 ± 1.0	18.1 ± 0.8	21.7 ± 1.3	24.5 ± 0.7**	28.2 ± 1.2***	33.6 ± 1.9***,##
Middle						
m	19.0 ± 1.1	20.9 ± 0.9	26.4 ± 1.2**	27.8 ± 2.3**	36.3 ± 1.7***	42.6 ± 1.2***,##
d	19.8 ± 0.7	22.2 ± 0.9	28.4 ± 0.6***	31.4 ± 2.7***	39.5 ± 1.3***	50.4 ± 2.1***,###
dl	18.6 ± 0.9	20.2 ± 1.4	23.6 ± 1.1	26.3 ± 2.1*	30.9 ± 1.6***	42.6 ± 1.9***,###
vl	17.3 ± 0.9	18.1 ± 1.2	21.2 ± 1.2	22.6 ± 1.8	30.4 ± 2.7***	45.0 ± 3.5***,###
v	13.1 ± 0.6	13.6 ± 0.9	14.9 ± 1.0	14.7 ± 1.2	19.1 ± 1.7**	20.6 ± 1.4**
c	18.0 ± 1.1	18.7 ± 1.6	30.3 ± 1.6**	30.4 ± 3.3**	40.1 ± 3.2***	48.4 ± 3.1***,#
Caudal						
m	15.5 ± 1.2	15.8 ± 1.3	19.8 ± 1.5	20.0 ± 1.4	25.3 ± 2.5**	33.4 ± 2.5***,##
d	16.3 ± 0.9	18.0 ± 1.1	26.3 ± 1.0***	28.3 ± 2.1***	37.4 ± 1.1***	49.9 ± 2.3***,###
dl	13.6 ± 1.4	15.6 ± 1.4	19.1 ± 1.4	20.2 ± 1.4	28.4 ± 1.2***	34.5 ± 2.9***,#
vl	13.2 ± 1.1	15.7 ± 1.6	14.9 ± 1.1	16.1 ± 1.2	26.3 ± 3.5**	32.0 ± 4.0***
v	11.6 ± 1.1	13.5 ± 0.8	11.6 ± 0.9	10.9 ± 0.9	13.2 ± 1.6	15.5 ± 1.5
vc	8.6 ± 0.9	10.5 ± 0.9	10.4 ± 0.8	10.4 ± 0.9	15.2 ± 2.8	21.6 ± 3.5***,#
dc	9.8 ± 1.3	11.6 ± 0.7	12.0 ± 1.3	11.2 ± 1.6	20.4 ± 2.4**	25.3 ± 3.2***

Mean density values (mean ± standard error of the mean) measured in different striatal sectors at the rostral, middle and caudal levels for rats that received an injection of vehicle (V), fluoxetine (5 mg/kg; FLX), methylphenidate (2 or 5 mg/kg; MP2 and MP5), or methylphenidate + fluoxetine combinations (MP2 + FLX and MP5 + FLX). Nucleus accumbens: lC, lateral core; mC, medial core; lS, lateral shell; mS, medial shell; vS, ventral shell. Caudate–putamen: c, central; d, dorsal; dc, dorsal central; dl, dorsolateral; dm, dorsomedial; m, medial; v, ventral; vc, ventral central; vl, ventrolateral. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ vs. respective control group (V or FLX). #$P < 0.05$, ##$P < 0.01$ and ###$P < 0.001$, MP + FLX vs. MP (potentiation).

TABLE 2. Effects of fluoxetine, methylphenidate and methylphenidate + fluoxetine on c-*fos* expression in the striatum

	V	FLX	MP2	MP2 + FLX	MP5	MP5 + FLX
Rostral						
dl	2.2 ± 0.6	3.4 ± 0.7	4.3 ± 0.7	5.2 ± 0.8	8.7 ± 0.6***	13.0 ± 1.9***,##
d	2.8 ± 0.4	3.6 ± 0.4	5.2 ± 0.6	6.1 ± 1.2	8.8 ± 0.4**	14.8 ± 2.0***,###
dm	3.2 ± 0.6	3.9 ± 0.3	5.8 ± 0.5	7.5 ± 1.1*	11.0 ± 0.6***	19.1 ± 2.1***,###
m	3.7 ± 0.5	4.8 ± 0.6	6.1 ± 0.8	7.1 ± 0.7	8.0 ± 1.2*	12.4 ± 1.5***,##
v	2.3 ± 0.6	2.1 ± 0.5	4.2 ± 0.8	2.7 ± 0.4	4.2 ± 0.8	7.2 ± 1.7**
mC	5.1 ± 1.1	3.7 ± 0.7	5.7 ± 0.6	5.5 ± 1.3	4.3 ± 1.4	4.8 ± 1.7
lC	2.1 ± 0.6	1.7 ± 0.8	3.7 ± 1.4	2.5 ± 1.4	1.3 ± 0.8	2.3 ± 2.4
mS	5.2 ± 0.9	4.3 ± 0.9	6.2 ± 1.0	7.4 ± 1.1	4.5 ± 1.1	5.4 ± 1.3
vS	2.1 ± 1.0	1.4 ± 0.8	3.3 ± 1.6	1.4 ± 1.2	2.0 ± 1.1	1.9 ± 2.2
lS	3.7 ± 0.8	2.7 ± 0.6	5.8 ± 0.9	6.1 ± 0.8	8.2 ± 1.6	11.3 ± 2.0***
Middle						
m	4.1 ± 0.5	3.5 ± 1.1	7.3 ± 1.0*	8.3 ± 1.2*	13.3 ± 1.1***	20.9 ± 1.0***,###
d	3.1 ± 0.3	2.9 ± 0.9	7.0 ± 0.6*	11.0 ± 2.4***,#	17.1 ± 0.6***	32.3 ± 2.2***,###
dl	2.5 ± 0.6	1.6 ± 0.8	3.8 ± 0.4	4.7 ± 1.1	8.7 ± 0.8**	20.2 ± 1.9***,###
vl	3.4 ± 1.1	2.0 ± 0.9	3.9 ± 0.6	4.1 ± 1.6	8.5 ± 1.0	22.5 ± 3.4***,###
v	4.2 ± 1.1	2.6 ± 1.0	4.8 ± 0.7	2.9 ± 1.4	5.6 ± 1.8	8.5 ± 1.2*
c	2.9 ± 0.7	1.3 ± 0.7	10.3 ± 1.1	9.8 ± 2.4*	18.9 ± 1.5***	27.9 ± 4.6***,##
Caudal						
m	3.2 ± 0.5	3.9 ± 0.6	7.9 ± 1.0	8.8 ± 1.5	12.5 ± 1.9***	20.2 ± 2.5***,###
d	3.8 ± 0.3	4.2 ± 0.5	11.1 ± 1.3**	11.9 ± 2.0**	20.2 ± 1.5***	34.9 ± 2.7***,###
dl	2.4 ± 0.4	1.6 ± 0.6	6.3 ± 1.3	5.9 ± 0.7	10.9 ± 1.6***	18.3 ± 2.6***,###
vl	1.8 ± 0.7	1.7 ± 0.7	3.2 ± 1.2	3.4 ± 0.9	6.4 ± 2.6	13.1 ± 2.5***,##
v	2.6 ± 0.6	1.8 ± 0.6	3.7 ± 1.2	1.9 ± 1.0	1.8 ± 1.6	4.7 ± 1.3
vc	2.1 ± 0.7	1.3 ± 0.7	3.1 ± 1.2	1.4 ± 0.9	2.3 ± 2.1	6.0 ± 1.2
dc	1.5 ± 0.5	2.2 ± 0.5	5.0 ± 1.3	2.0 ± 0.8	4.5 ± 3.0	8.4 ± 1.9*

Mean density values (mean ± standard error of the mean) measured in different striatal sectors at the rostral, middle and caudal levels for rats that received an injection of vehicle (V), fluoxetine (5 mg/kg; FLX), methylphenidate (2 or 5 mg/kg; MP2 and MP5), or methylphenidate + fluoxetine combinations (MP2 + FLX and MP5 +FLX). Nucleus accumbens: lC, lateral core; mC, medial core; lS, lateral shell; mS, medial shell; vS, ventral shell. Caudate–putamen: c, central; d, dorsal; dc, dorsal central; dl, dorsolateral; dm, dorsomedial; m, medial; v, ventral; vc, ventral central; vl, ventrolateral. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ vs. respective control group (V or FLX). #$P < 0.05$, ##$P < 0.01$ and ###$P < 0.001$, MP + FLX vs. MP (potentiation).

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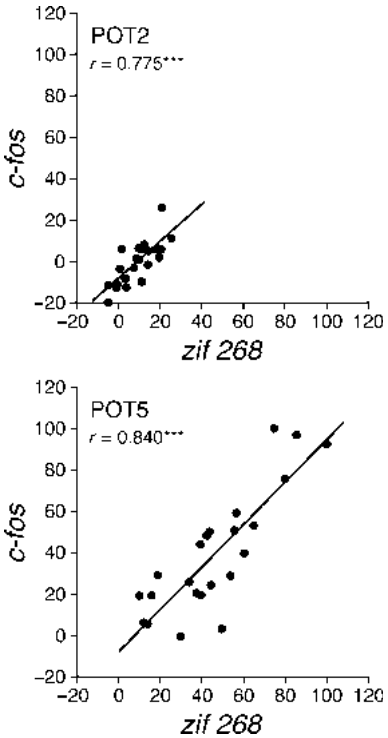

FIG. 7. The potentiation of gene induction displays a similar regional distribution in the striatum for *zif268* and c-*fos*. Scatterplots show the correlations between *zif268* and c-*fos* potentiation for 2 mg/kg ($r = 0.775$, POT2, top) and 5 mg/kg ($r = 0.840$, POT5, bottom) of methylphenidate in the 23 striatal sectors. The values are expressed as the percentages of the maximal value in the 5 mg/kg group. ***$P < 0.001$.

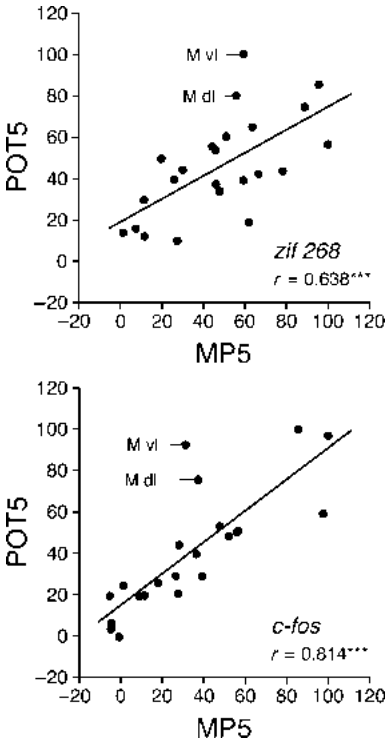

FIG. 8. Relationship between gene induction by methylphenidate alone and fluoxetine potentiation in the different striatal sectors. Scatterplots depict the correlations between gene induction by 5 mg/kg methylphenidate (MP5) and fluoxetine potentiation (POT5) in the 23 sectors, for *zif268* ($r = 0.638$, top) and c-*fos* ($r = 0.814$, bottom). The values are expressed as the percentages of the maximal value in each group. M vl, middle-level ventrolateral sector; M dl, middle-level dorsolateral sector. ***$P < 0.001$.

higher methylphenidate dose. We further confirmed this SSRI potentiation of methylphenidate-induced gene regulation with a second SSRI, citalopram (5 mg/kg). These findings highlight the ability of SSRIs to potentiate psychostimulant-induced molecular changes.

Acute IEG induction by psychostimulants is predictive of altered gene regulation after repeated psychostimulant treatments (Brandon & Steiner, 2003; Willuhn *et al.*, 2003; Cotterly *et al.*, 2007; Unal *et al.*, 2009), and thus serves as a marker for the identification of brain regions prone to such neuroplasticity induced by repeated treatments. In so far as such molecular changes underlie addiction (Hyman & Nestler, 1996; Berke & Hyman, 2000; Nestler, 2001), it is of interest to compare the propensity of methylphenidate to alter gene regulation with that of psychostimulants such as cocaine (Yano & Steiner, 2007). Although they are similar to cocaine effects in many ways, methylphenidate effects after acute and repeated treatment also display distinct differences (for review, see Yano & Steiner, 2007). For example, previous mapping studies revealed, and the present results confirmed, that methylphenidate-induced *zif268* and c-*fos* expression is most pronounced at middle-to-caudal striatal levels (Brandon & Steiner, 2003; Yano & Steiner, 2005a,b; Cotterly *et al.*, 2007), whereas cocaine-induced gene regulation peaks at more caudal levels (Willuhn *et al.*, 2003; Unal *et al.*, 2009). Regarding the medial–lateral distribution, methylphenidate-induced gene regulation is most robust in medial and central striatal regions (associative striatum) (see above), again confirmed here, whereas cocaine prominently involves the lateral (sensorimotor) striatum as well (Willuhn *et al.*, 2003; Unal *et al.*, 2009).

Our present findings demonstrate that the SSRI potentiation of methylphenidate-induced gene regulation occurs at all rostrocaudal levels of the striatum and, generally, is directly related to the magnitude of gene induction by methylphenidate alone. Thus, the addition of the SSRI did not produce a shift in the rostrocaudal distribution of gene regulation. However, this was not the case for the medial–lateral distribution. Our correlation analysis shows that two striatal sectors, the dorsolateral and ventrolateral (sensorimotor) sectors at the middle level, displayed more pronounced potentiation than predicted by the gene response to methylphenidate alone. This preferential potentiation in these two sensorimotor sectors shifted the regional distribution to also include the lateral striatum. In this respect, SSRI-potentiated gene regulation by methylphenidate has a greater resemblance to gene regulation induced by cocaine (Willuhn *et al.*, 2003; Unal *et al.*, 2009). As these preferentially affected lateral striatal regions subserve habit formation (Packard & Knowlton, 2002), it will be important to determine whether such concomitant SSRI + methylphenidate treatment facilitates drug-taking habits/addiction, similarly to cocaine (see below).

Our earlier studies showed that, in addition to the dorsal striatum, methylphenidate-induced gene regulation also occurs in the nucleus accumbens, although to a more modest extent (Brandon & Steiner, 2003; Yano & Steiner, 2005a,b; Cotterly *et al.*, 2007). Consistent with these earlier findings, the most robust gene regulation in the nucleus accumbens in the present study was seen in the lateral part of the shell. Fluoxetine also produced a statistically significant potentiation of methylphenidate-induced *zif268* expression in the lateral shell (as well as in the medial core), but had no effect on c-*fos* expression. These regional effects also mimic those of cocaine (Unal *et al.*, 2009). The functional consequences of these changes in specific nucleus accumbens subregions remain to be determined.

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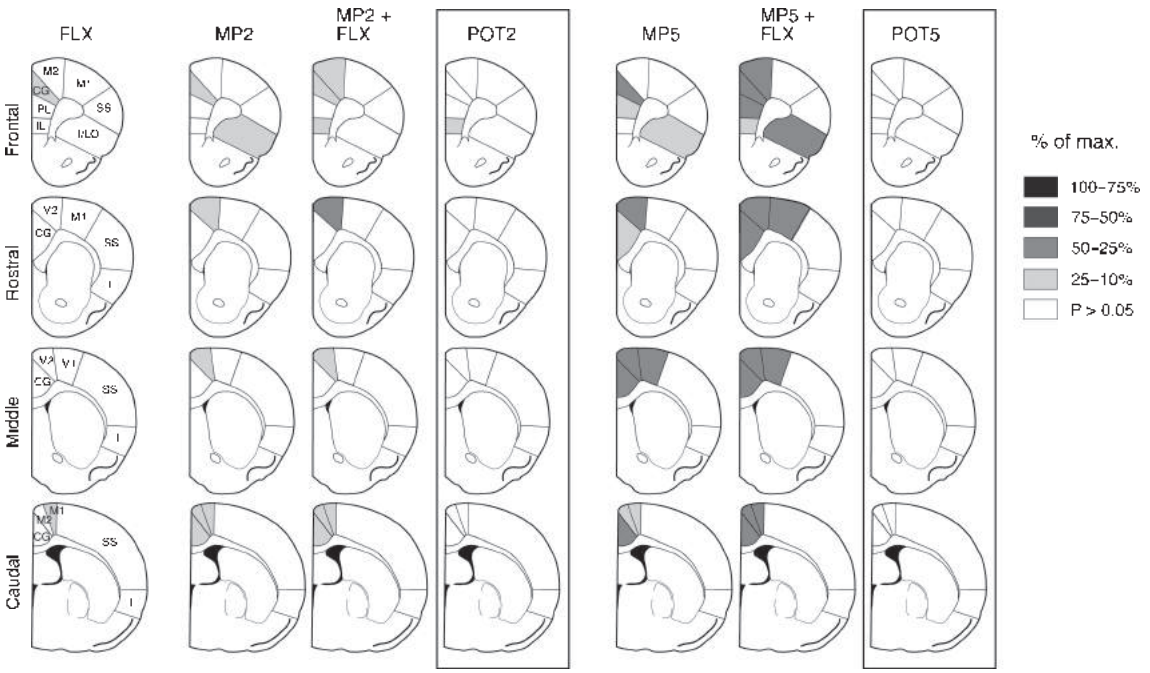

FIG. 9. Effects of methylphenidate + fluoxetine combination treatment on *zif268* expression in the cortex. Maps show the distribution of *zif268* expression in the cortex at the frontal, rostral, middle and caudal levels after an injection of fluoxetine (5 mg/kg; FLX), methylphenidate (2 or 5 mg/kg; MP2 and MP5), or methylphenidate + fluoxetine combinations (MP2 + FLX and MP5 + FLX). The potentiation (POT) denotes the difference between methylphenidate + fluoxetine and methylphenidate alone. The data are normalized relative to the maximal increase observed in the striatum (% of maximum). Sectors with significant differences vs. vehicle-treated controls ($P < 0.05$) are shaded as indicated. Sectors without significant effects are in white. CG, cingulate; M2, medial agranular; M1, motor; SS, somatosensory; I, insular; IL, infralimbic; PL, prelimbic; I/LO, insular/lateral orbital.

Mechanisms that may mediate the SSRI potentiation

A number of mechanisms may account for the SSRI potentiation of methylphenidate-induced gene regulation as described in the present study. It is unlikely that metabolic interactions between methylphenidate and SSRIs contributed to the observed effects. The principal metabolic pathway for methylphenidate is de-esterification by carboxylesterases (e.g. Sun *et al.*, 2004; Zhu *et al.*, 2008), and, to our knowledge, there is no evidence for an inhibition of carboxylesterases by SSRIs. Conversely, the metabolism of SSRIs involves mostly demethylation by liver enzymes (cytochrome P450 system) (Sandson *et al.*, 2005). Although there is some evidence that methylphenidate can inhibit such liver enzymes (Le Nedelec & Rosengren, 2002), it is unclear whether the specific P450 isozymes that metabolize fluoxetine and citalopram (Sandson *et al.*, 2005) are affected. Also, a recent study failed to find altered pharmacokinetics for methylphenidate (10 mg/kg) after the addition of citalopram (5 mg/kg), at time points when this drug combination facilitated dopamine overflow in the prefrontal cortex (Weikop *et al.*, 2007). Furthermore, it would be difficult to envision how systemic drug interactions could result in a potentiation of gene regulation with the distinct regional variations observed here.

More likely, this potentiation is mediated by system-level interactions between dopamine and serotonin neurotransmission (e.g. Bhat & Baraban, 1993; Gardier *et al.*, 2000). The effects of psychostimulants (including methylphenidate; Yano *et al.*, 2006) on gene expression in the striatum are principally mediated by the activation of dopamine receptors (for reviews, see Steiner & Gerfen, 1998; Yano & Steiner, 2007), but are also dependent on cortical (glutamate) inputs (e.g. Wang & McGinty, 1996; Steiner, 2010). Both glutamate and dopamine inputs are modulated by serotonin. For example, serotonin and agonists are well known to enhance activity of the mesostriatal and mesolimbic/cortical dopamine pathways, by complex interactions in both the dopamine terminal regions (e.g. Benloucif & Galloway, 1991; Benloucif *et al.*, 1993; Balcioglu & Wurtman, 1998; Bubar *et al.*, 2003) and the somatodendritic areas in the midbrain (for reviews, see Muller & Huston, 2006; Weikop *et al.*, 2007; Bubar & Cunningham, 2008). Consistent with these findings, a facilitatory role for serotonin in dopamine/glutamate-mediated gene regulation in the striatum has been shown before (Bhat & Baraban, 1993; Torres & Rivier, 1993; Guerra *et al.*, 1998; Wirtshafter & Cook, 1998; Gardier *et al.*, 2000; Horner *et al.*, 2005). Therefore, the present SSRI potentiation of methylphenidate-induced gene regulation in the striatum could reflect increased cortical input to the striatum and/or potentiated dopamine action that occurs with enhanced serotonin activity in the striatum and/or other brain areas.

As drugs were administered systemically, we do not know which of the above local mechanisms played a role in this SSRI potentiation. However, our further analysis suggests that enhanced cortical input is not a main determinant. Our previous work showed that acute administration of methylphenidate produces coordinated IEG induction in cortical neurons and their striatal targets (Yano & Steiner, 2005a; Cotterly *et al.*, 2007), which suggests enhanced activity in specific corticostriatal circuits. In order to assess a possible contribution of enhanced cortical activity, we thus compared *zif268* induction between specific cortical areas and their striatal target sectors (see Willuhn *et al.*, 2003). Our results confirmed coordinated upregulation of *zif268* expression between cortical areas and functionally related striatal sectors for both doses of methylphenidate used. However, the co-administration of fluoxetine with methylphenidate disrupted this coordinated response, mainly because fluoxetine robustly potentiated

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TABLE 3. Effects of fluoxetine, methylphenidate and methylphenidate + fluoxetine on *zif268* expression in the cortex

	V	FLX	MP5	MP5 + FLX
Frontal				
IL	24.9 ± 1.2	28.1 ± 1.2	29.9 ± 2.0	33.2 ± 1.2
PL	38.2 ± 2.3	41.9 ± 1.7	46.3 ± 2.9*	48.4 ± 1.2
CG	39.4 ± 1.8	45.5 ± 2.3*	51.5 ± 2.5***	50.5 ± 1.1
M2	26.1 ± 1.9	32.0 ± 2.7	33.4 ± 1.8	36.8 ± 0.7
M1	18.5 ± 2.5	23.0 ± 3.4	19.6 ± 2.1	23.9 ± 2.0
SS	18.6 ± 1.9	22.6 ± 3.5	20.0 ± 2.0	23.2 ± 2.2
I/LO	33.4 ± 0.9	36.4 ± 1.8	40.3 ± 1.5**	39.8 ± 0.8
Rostral				
CG	42.3 ± 1.6	43.6 ± 2.9	50.4 ± 1.6*	50.8 ± 1.4
M2	37.3 ± 1.9	39.7 ± 2.1	46.1 ± 1.6**	51.2 ± 1.4***
M1	25.9 ± 1.9	28.6 ± 2.6	32.7 ± 1.6	37.8 ± 1.3*
SS	23.3 ± 2.5	26.1 ± 4.4	25.8 ± 2.2	31.0 ± 2.6
I	22.9 ± 0.7	26.1 ± 2.3	27.7 ± 2.1	30.0 ± 2.0
Middle				
CG	38.0 ± 1.7	41.1 ± 2.8	46.6 ± 2.0*	46.8 ± 1.7
M2	33.4 ± 1.7	40.4 ± 2.8	46.3 ± 2.1**	45.3 ± 1.2
M1	28.1 ± 1.5	31.6 ± 1.7	36.6 ± 1.8**	35.8 ± 1.9
SS	25.4 ± 2.1	28.0 ± 3.5	28.2 ± 1.7	31.0 ± 2.6
I	20.3 ± 1.1	23.2 ± 2.5	22.7 ± 2.6	24.0 ± 1.9
Caudal				
CG	41.9 ± 2.0	43.9 ± 2.7	52.6 ± 1.5**	52.5 ± 0.7*
M2	37.5 ± 1.5	40.6 ± 1.6	45.2 ± 1.7**	47.1 ± 1.6*
M1	32.5 ± 1.3	38.6 ± 0.8**	40.4 ± 1.6***	42.8 ± 0.8
SS	28.3 ± 2.2	31.1 ± 2.9	33.2 ± 2.2	36.2 ± 1.8
I	18.0 ± 1.1	17.5 ± 1.7	17.6 ± 1.3	18.0 ± 1.1

Mean density values (mean ± standard error of the mean) measured in different cortical areas at the frontal, rostral, middle and caudal levels for rats that received an injection of vehicle (V), fluoxetine (5 mg/kg; FLX), methylphenidate (5 mg/kg; MP5), or methylphenidate + fluoxetine (MP5 + FLX). CG, cingulate; I, insular; IL, infralimbic; LO, lateral orbital; M1, motor; M2, medial agranular; PL, prelimbic; SS, somatosensory. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ vs. respective control group (V or FLX).

gene induction in the striatum, but not in the cortex. This dissociation suggests that the potentiated gene regulation in the striatum is not likely to be a consequence of enhanced activity in corticostriatal projections.

A host of serotonin receptor subtypes are known to mediate serotonin/dopamine interactions (for reviews, see Muller & Huston, 2006; Bubar & Cunningham, 2008) and to regulate striatal gene expression (Keefe & Horner, 2010), and could thus conceivably contribute to SSRI potentiation. Interestingly, a recent study provided evidence for a role of the 5-HT1B receptor subtype in fluoxetine-potentiated methylphenidate effects (Borycz *et al.*, 2008). This study showed that adjunct treatment with fluoxetine facilitated the stimulatory effects of methylphenidate on locomotor activity (Borycz *et al.*, 2008). Moreover, this behavioral potentiation by fluoxetine was inhibited by a selective 5-HT1B receptor antagonist and mimicked by a 5-HT1B receptor agonist (Borycz *et al.*, 2008). 5-HT1B receptors have previously also been shown to induce IEG expression (Wirtshafter & Cook, 1998) and facilitate cocaine-induced gene regulation (Lucas *et al.*, 1997; Castanon *et al.*, 2000) in the striatum. Therefore, the 5-HT1B serotonin receptor subtype may be one of the mediators of the SSRI potentiation of striatal gene regulation, and could thus be a potential target for prevention of this effect. Future studies with local administration of selective serotonin receptor agents will need to determine the relevant serotonin receptors in the striatum and/or other brain regions.

Stereotypies: behavioral correlates of the SSRI-potentiated striatal gene regulation

In our study, the fluoxetine potentiation of methylphenidate-induced gene regulation in the striatum was accompanied by potentiation of behavioral stereotypies. Like the gene regulation effects, this behavioral potentiation was dose-dependent; it emerged with the lower methylphenidate dose (2 mg/kg; statistically not significant) and was very robust with the higher dose (5 mg/kg). Although this behavioral effect is principally consistent with the above findings of Borycz *et al.* (2008), we did not see potentiated locomotor activity (ambulation) in our study. Different drug doses for fluoxetine or methylphenidate (10 mg/kg each in Borycz *et al.*, 2008) and/or other experimental variables (e.g. non-habituated animals in our study vs. habituated animals in the study by Borycz *et al.*) may account for these differences.

Previous work has related behavioral stereotypies to drug-induced dysfunction and molecular changes in sensorimotor striatal circuits

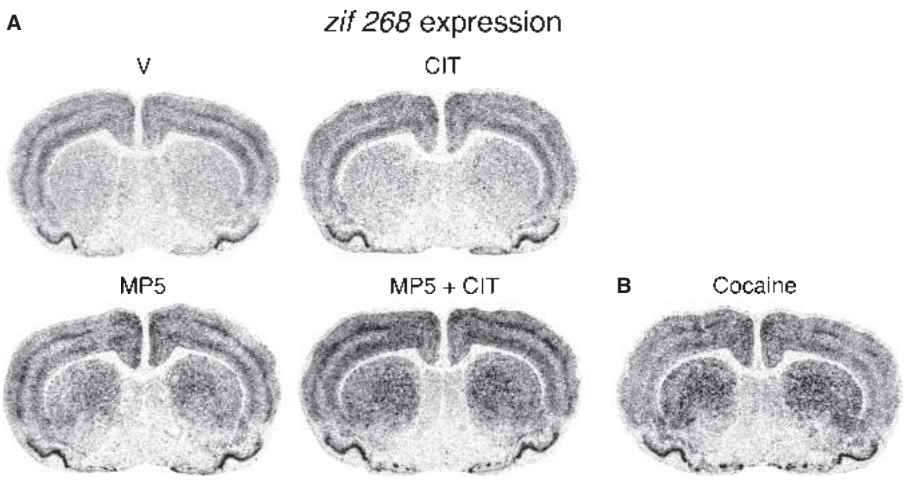

FIG. 10. Citalopram potentiates methylphenidate-induced *zif268* expression in the striatum. (A) Illustrations of film autoradiograms depict *zif268* expression in coronal sections from the middle striatum in rats treated with vehicle (V), citalopram (5 mg/kg; CIT), methylphenidate (5 mg/kg; MP5), or methylphenidate + citalopram (MP5 + CIT). (B) For comparison, *zif268* induction by cocaine (25 mg/kg) is also shown. The maximal hybridization signal is in black.

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(for reviews, see Graybiel *et al.*, 2000; Steiner, 2010). 'Stereotypies' denotes a compulsive repetition of specific behavioral elements without apparent purpose (Randrup & Munkvad, 1967; Ellinwood & Balster, 1974). Sensorimotor striatal circuits are critical for selection and switching of motor actions (and thoughts) (e.g. Mink, 1996; Redgrave *et al.*, 1999), and behavioral stereotypies have been interpreted as a switching dysfunction (Redgrave & Gurney, 2006). It has also been pointed out that such stereotypies share both phenomenological characteristics (Randrup & Munkvad, 1967) and neuronal substrates (Graybiel *et al.*, 2000) with motor compulsions in humans. It is presently unclear whether stereotypies are mechanistically related to compulsions; however, stereotypies may, to some degree, reflect similar basal ganglia output deficiencies (see Steiner, 2010). Our findings thus suggest that one of the functional consequences of concomitant methylphenidate and SSRI treatment is an enhanced propensity for compulsive-like behavior.

Functional implications of SSRI-potentiated gene regulation by psychostimulants

A wealth of literature demonstrates that the most robust molecular changes induced by psychostimulants such as cocaine (Willuhn *et al.*, 2003; Unal *et al.*, 2009), amphetamine (Badiani *et al.*, 1998; Uslaner *et al.*, 2003) and methylphenidate (Yano & Steiner, 2005b; Cotterly *et al.*, 2007) occur in the dorsal/lateral striatum that includes sensorimotor circuits (for reviews, see Berke & Hyman, 2000; Steiner, 2010). Our present findings show that the SSRI potentiation of methylphenidate-induced gene regulation preferentially occurs in the sensorimotor striatum. The sensorimotor striatum is known to mediate stimulus–response learning/habit formation (Graybiel, 1995; Packard & Knowlton, 2002), and it has been proposed that such drug-induced molecular changes may contribute to aberrant habit formation and compulsive aspects of drug taking (Berke & Hyman, 2000; Everitt & Robbins, 2005). Indeed, previous studies showed that the sensorimotor striatum is critical for relapse to cocaine seeking in the self-administration paradigm in animals (Vanderschuren *et al.*, 2005; Fuchs *et al.*, 2006; See *et al.*, 2007).

Methylphenidate is also self-administered (Kollins *et al.*, 2001), and pretreatment with this psychostimulant facilitates subsequent self-administration of cocaine (Brandon *et al.*, 2001) in animal models of addiction, both signifying a certain addiction liability for methylphenidate. Given that the SSRI potentiation of striatal gene regulation is paralleled by more pronounced compulsive-like behavior, future studies will need to determine whether methylphenidate + SSRI combinations enhance drug-seeking/taking tendencies and/or facilitate relapse.

Conclusions

Our study demonstrates that SSRIs potentiate gene regulation effects of methylphenidate in the striatum, with the most robust effects in sensorimotor parts. Such molecular changes are implicated in psychostimulant addiction. These findings thus suggest that such concomitant drug exposure, for example during medical treatments or in patients on SSRIs who also use methylphenidate as a cognitive enhancer or for recreational purposes, may increase the liability for drug abuse disorder.

Acknowledgements

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Abbreviations

ADHD, attention-deficit hyperactivity disorder; CIT, citalopram HBr 5 mg/kg; FLX, fluoxetine HCl 5 mg/kg; IEG, immediate-early gene; MP2, methylphenidate HCl 2 mg/kg; MP5, methylphenidate HCl 5 mg/kg; POT2, potentiation of effects of methylphenidate HCl 2 mg/kg; POT5, potentiation of effects of methylphenidate HCl 5 mg/kg; SSRI, selective serotonin reuptake inhibitor; V, vehicle.

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Addiction-related gene regulation: Risks of exposure to cognitive enhancers *vs.* other psychostimulants

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ABSTRACT

The psychostimulants methylphenidate (Ritalin, Concerta), amphetamine (Adderall), and modafinil (Provigil) are widely used in the treatment of medical conditions such as attention-deficit hyperactivity disorder and narcolepsy and, increasingly, as "cognitive enhancers" by healthy people. The long-term neuronal effects of these drugs, however, are poorly understood. A substantial amount of research over the past two decades has investigated the effects of psychostimulants such as cocaine and amphetamines on gene regulation in the brain because these molecular changes are considered critical for psychostimulant addiction. This work has determined in some detail the neurochemical and cellular mechanisms that mediate psychostimulant-induced gene regulation and has also identified the neuronal systems altered by these drugs. Among the most affected brain systems are corticostriatal circuits, which are part of cortico-basal ganglia-cortical loops that mediate motivated behavior. The neurotransmitters critical for such gene regulation are dopamine in interaction with glutamate, while other neurotransmitters (e.g., serotonin) play modulatory roles. This review presents (1) an overview of the main findings on cocaine- and amphetamine-induced gene regulation in corticostriatal circuits in an effort to provide a cellular framework for (2) an assessment of the molecular changes produced by methylphenidate, medical amphetamine (Adderall), and modafinil. The findings lead to the conclusion that protracted exposure to these cognitive enhancers can induce gene regulation effects in corticostriatal circuits that are qualitatively similar to those of cocaine and other amphetamines. These neuronal changes may contribute to the addiction liability of the psychostimulant cognitive enhancers.

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Abbreviations: ADHD, attention-deficit hyperactivity disorder; IEG, immediate-early gene; M2, medial agranular cortex; mRNA, messenger RNA; SUD, substance use disorder.

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1. Introduction

Cognitive enhancers, sometimes called "smart drugs" or "memory enhancers," are substances taken with the expectation that they increase mental functions such as attention, concentration, alertness, memory, motivation, planning, and decision making (Svetlov et al., 2007; Lanni et al., 2008; Husain and Mehta, 2011). The most widely used cognitive enhancers include the psychostimulant medications methylphenidate (Ritalin, Concerta), amphetamine (Adderall), and modafinil (Provigil). The oldest of these drugs is amphetamine, which was first synthesized in 1887 and has been used in the clinic since the 1930s (Berman et al., 2009). Methylphenidate, first produced in 1944, has also been used as a medication for many decades (Leonard et al., 2004), whereas modafinil was introduced only in the early 1990s (Minzenberg and Carter, 2008).

1.1. Medical and nonprescription uses of psychostimulants

These psychostimulant medications are valued and widely prescribed for their efficacy in controlling symptoms of attention-deficit hyperactivity disorder (ADHD) (methylphenidate, amphetamine) or excessive daytime sleepiness associated with narcolepsy and other sleep disorders (modafinil, amphetamine, methylphenidate) (Leonard et al., 2004; Minzenberg and Carter, 2008; Berman et al., 2009). Furthermore, there is a rationale for the use of methylphenidate and modafinil in the treatment of behavioral deficits associated with psychostimulant addiction (e.g., Goldstein et al., 2010; Volkow et al., 2010; Loland et al., 2012; Reichel and See, 2012; for reviews, see Brady et al., 2011; Sofuoglu et al., 2013). But these medications are also recognized by the US Drug Enforcement Administration (DEA) for their abuse potential and are therefore classified as Schedule II (amphetamine, methylphenidate) or Schedule IV (modafinil) controlled substances.

ADHD is among the most common neurobehavioral disorders and, in the United States, affects approximately 7.8% of children aged 4–17 and 4.4% of adults (Kollins, 2008). It is arguably the dramatic increase in diagnosis and pharmacological treatment of ADHD over the past two decades that has led to a parallel increase in production of these psychostimulants (Biederman et al., 2007; Kollins, 2008; Swanson and Volkow, 2008; Berman et al., 2009). For example, the number of prescriptions for amphetamine increased 16-fold during the 1990s, and in 2000 the US annual production of amphetamine reached 30,000 kg (Berman et al., 2009). Likewise, the DEA aggregate production quota for methylphenidate increased from 5000 kg in 1993 to 15,000 kg in 2000 to 50,000 kg in 2009.[2]

Not surprisingly, with increasing availability came increasing diversion and use of psychostimulant medications as cognitive enhancers or party drugs (Kollins et al., 2001; Svetlov et al., 2007; Kollins, 2008; Swanson and Volkow, 2008; Wilens et al., 2008; Mache et al., 2012). It is difficult to accurately estimate the amounts and extent of their use as cognitive enhancers, but surveys indicate that students are frequent consumers of these drugs (Svetlov et al., 2007). Thus, studies of the misuse and diversion of prescription ADHD medications found that rates of self-reported past-year use range from 4% to 30% among college students (Kollins et al., 2001; Kollins, 2008; Wilens et al., 2008; Berman et al., 2009) and 5% to 9% in grade school- and high school-aged children (Wilens et al., 2008). The most often reported motives for illicit use among college students are to increase attention, concentration, or alertness (to help study), and to a lesser extent to get "high" (Babcock and Byrne, 2000; Teter et al., 2006; White et al., 2006). Furthermore, the trend for using cognitive enhancers is growing not only among students (Greely et al., 2008): in a recent poll of 1400 academics (*Nature* readers), 20% indicated that they had used methylphenidate (62%) or modafinil (44%) to combat jet lag, to improve general concentration, or to assist them in a particular task (Maher, 2008).

1.2. Neurobehavioral and molecular impacts of psychostimulant use

It remains controversial whether the medical use of psychostimulants is completely safe (Kollins, 2008; Wilens et al., 2008; see Section 8), especially in children and adolescents (Carlezon and Konradi, 2004; Andersen, 2005; Berman et al., 2009). Even less clear are the potential long-term effects of cognitive enhancer use in the healthy, in part because widespread use is a relatively new phenomenon and adverse effects of early drug exposure may

[2] Data available at the DEA website, www.deadiversion.usdoj.gov/fed_regs/quotas/2009/fr10212.htm (accessed August 2, 2012).

appear only late in life (e.g., Bolanos et al., 2003; Tropea et al., 2008; Warren et al., 2011). There is concern especially that long-term exposure to psychostimulants during the sensitive period of brain development may increase the risk for maladaptive neurobehavioral changes that may facilitate drug addiction and other neuropsychiatric disorders (Bolanos et al., 2003; Warren et al., 2011; for reviews, see Carlezon and Konradi, 2004; Andersen, 2005; Carrey and Wilkinson, 2011; Marco et al., 2011).

There is little doubt that changes in gene regulation produced by illicit psychostimulants such as cocaine play a critical role in addiction (Hyman and Nestler, 1996; Nestler, 2001) because only these molecular changes endure long enough to mediate behavioral pathologies that can last a lifetime such as addiction (Renthal and Nestler, 2008). Therefore, the addiction liability of medical psychostimulants most likely also rests on their propensity to induce altered gene regulation.

In this article, we review changes in gene regulation produced by medical amphetamine (Adderall), methylphenidate, and modafinil as determined in animal models. We discuss these findings in the context of the known molecular changes that are produced by illicit psychostimulants (e.g., cocaine) and are considered part of the molecular basis for drug addiction.

2. Neurochemical effects of psychostimulant cognitive enhancers

2.1. Changes in monoamine transmission

Psychostimulants cause, among other effects, amplification of monoamine neurotransmission by promoting release and/or blocking reuptake of monoamines and thus prolonging their actions (Natarajan and Yamamoto, 2011; Sulzer, 2011). Psychostimulant-induced potentiation of the dopamine transmission (Di Chiara and Imperato, 1988) is considered critical to the addiction process, whereas serotonin and norepinephrine play modulatory roles (Berke and Hyman, 2000; Nestler, 2001).

Adderall is a mixture of D- and L-amphetamine salts (Berman et al., 2009) and, like cocaine, amphetamines produce elevated extracellular levels of the monoamines dopamine, norepinephrine, and serotonin (Di Chiara and Imperato, 1988; Hurd and Ungerstedt, 1989; Ritz et al., 1990; Kuczenski and Segal, 1997, 2001). Modafinil seems to primarily inhibit dopamine and norepinephrine reuptake (Madras et al., 2006; Volkow et al., 2009; Schmitt and Reith, 2011; Loland et al., 2012) but, probably indirectly, affects other neurotransmitters as well (e.g., histamine, orexin and serotonin; see Minzenberg and Carter, 2008). Better established are the effects of methylphenidate. Methylphenidate binds to and blocks the dopamine and norepinephrine transporters (Schweri et al., 1985; Gatley et al., 1996) and thus produces overflow of these two monoamines (Hurd and Ungerstedt, 1989; Kuczenski and Segal, 1997; Volkow et al., 1998; Gerasimov et al., 2000; Bymaster et al., 2002; Berridge et al., 2006). In contrast, methylphenidate has low affinity for the serotonin transporter (Pan et al., 1994; Wall et al., 1995; Gatley et al., 1996; Bymaster et al., 2002) and produces minimal or no effects on serotonin levels, even with high doses (30 mg/kg, i.p.) (Kuczenski and Segal, 1997; Segal and Kuczenski, 1999; Kankaanpaa et al., 2002).

In vivo microdialysis studies demonstrate that these psychostimulant-induced neurochemical effects are very robust in the prefrontal cortex and in parts of the basal ganglia (Fig. 1), especially the striatum (dorsal striatum/caudate–putamen, ventral striatum/nucleus accumbens) (Di Chiara and Imperato, 1988; Hurd and Ungerstedt, 1989; Kuczenski and Segal, 1997, 2001; Gerasimov et al., 2000; Berridge et al., 2006). Low, clinically relevant doses of cognitive enhancers seem to preferentially boost extracellular levels of dopamine and norepinephrine in the prefrontal cortex (Berridge and Devilbiss, 2011, but see Volkow et al., 2001). Higher doses (presumably associated with abuse) predominantly affect dopamine in the striatum due to orders of magnitude higher levels of dopamine tissue content in the striatum.

2.2. Other effects

In addition to the direct neurochemical effects described above, all of these drugs have a number of other acute effects that can, directly or indirectly, further modify monoamine (and other) transmission (see Yano and Steiner, 2007). The following examples pertain to methylphenidate: (1) Recent studies showed that acute methylphenidate administration alters the distribution and function of the vesicular monoamine transporter-2 (VMAT-2) in the striatum (Sandoval et al., 2002, 2003), similar to cocaine (Fleckenstein et al., 2009). (2) Methylphenidate produces enhanced phosphorylation of glutamate receptors (GluR1) in the prefrontal cortex, similar to amphetamine (Pascoli et al., 2005). (3) Methylphenidate affects second messenger cascades that mediate dopamine signaling. Thus, acute methylphenidate was found to increase and decrease phosphorylation of DARPP-32 at Thr34 and Thr75, respectively, in striatal slices from adult mice, an effect that was dependent on D_1 dopamine receptor stimulation (Fukui et al., 2003). These findings demonstrate that there are several independent mechanisms by which cognitive enhancers can affect addiction-related neurotransmission.

Chronic perturbation of neurotransmission by psychostimulants often elicits compensatory (homeostatic) neuroadaptations, which are considered critical for addiction and dependence (Hyman and Nestler, 1996). Thus, repeated treatment with such drugs produces neuronal changes ranging from altered cell signaling (Yano and Steiner, 2007; McGinty et al., 2008) to structural modifications (e.g., in dendritic spine density; Robinson and Kolb, 1997; Jedynak et al., 2007; Kim et al., 2009), and the longevity of these alterations likely requires adaptations in gene expression (Renthal and Nestler, 2008).

Many excellent reviews have surveyed the effects of psychostimulants (primarily amphetamines and cocaine) on gene regulation and their role in addiction in general (e.g., Hyman and Nestler, 1996; Harlan and Garcia, 1998; Torres and Horowitz, 1999; Berke and Hyman, 2000; Nestler, 2001; Kelley, 2004; Hyman, 2005; McGinty et al., 2008; Renthal and Nestler, 2008). In this review we first summarize the molecular changes produced by amphetamine[3] and cocaine to provide context for a discussion of findings on the effects of medical amphetamine (Adderall), methylphenidate, and the little that is known about modafinil. Most of these findings were obtained in rat and mouse models.

3. Gene regulation by amphetamine and cocaine in corticostriatal circuits

Most studies on the molecular effects of psychostimulants have focused on gene regulation in dopamine target areas, especially the striatum, which displays particularly robust changes in gene regulation after treatments with amphetamine, cocaine, and other abused drugs (Harlan and Garcia, 1998; Berke and Hyman, 2000).

The striatum, the main input nucleus of the basal ganglia, is an important component of cortico-basal ganglia-cortical circuits (Gerfen and Bolam, 2010;Fig. 1), which play a critical role in

[3] We do not specifically address the molecular effects of methamphetamine and related compounds, which are also approved by the US Food and Drug Administration for the treatment of ADHD and other conditions (Berman et al., 2009). Many methamphetamine effects on gene regulation are similar to those of amphetamine; we refer the interested reader to a recent review by Keefe and Horner (2010) on this topic.

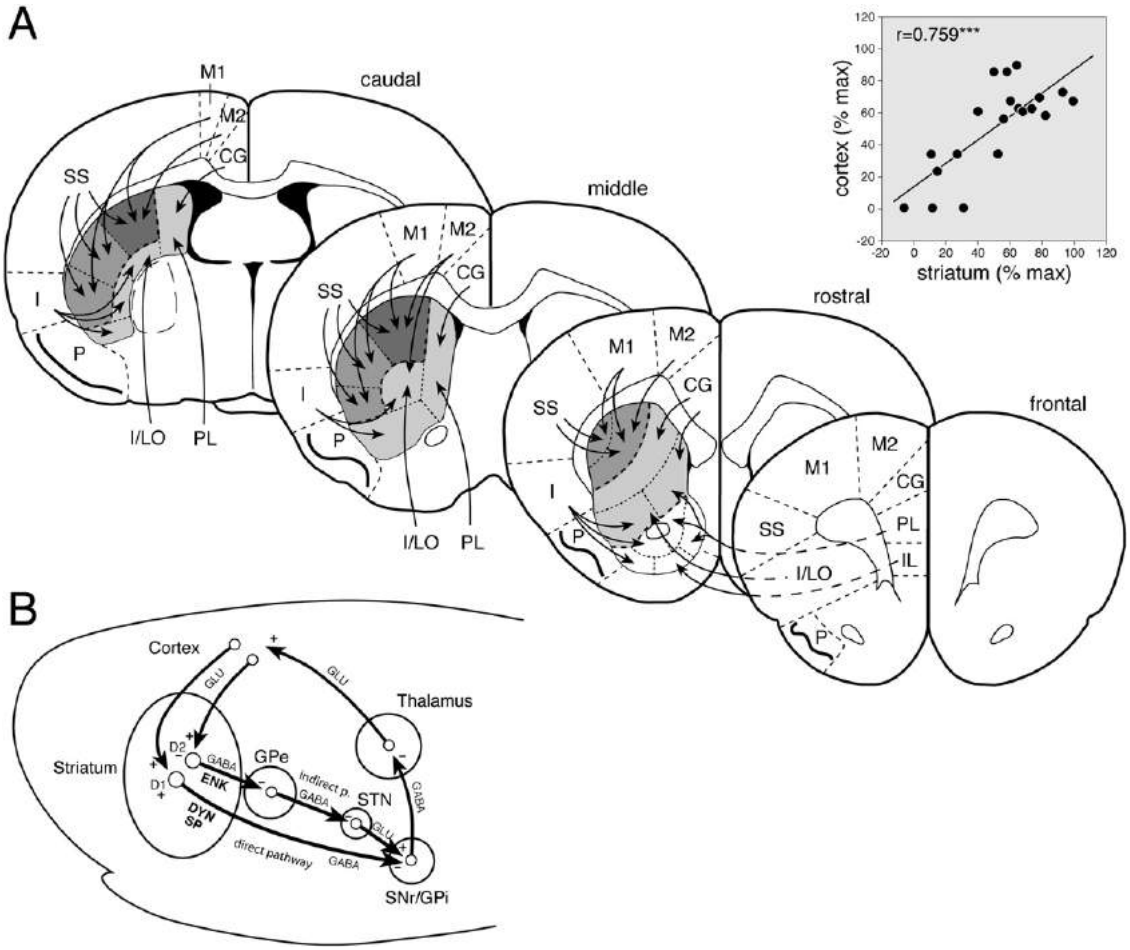

Fig. 1. Schematic illustrations of cortico-basal ganglia-thalamocortical circuits. (A) Striatal sectors used for mapping gene expression and their main cortical inputs (arrows) are shown for frontal, rostral, middle, and caudal levels of the rat forebrain (for details, see Willuhn et al., 2003; Yano and Steiner, 2005a,b). Psychostimulant-induced gene regulation is maximal in the dorsal sensorimotor sectors of the middle and caudal striatum (darkest shading), which receive inputs from the medial agranular (M2), primary motor (M1), and somatosensory (SS) cortex (see Section 3.1.2). Limbic (white), associative (light grey), and sensorimotor sectors (darker grey) are indicated. The scatterplot (inset upper right) displays the association between methylphenidate-induced *Zif268* expression in individual striatal sectors and *Zif268* expression in their indicated cortical input regions (values averaged if more than one input). Values are differences in gene expression between animals sacrificed 40 min after methylphenidate administration (5 mg/kg, i.p.) and controls sacrificed immediately after drug injection, and are expressed as the percentage of maximal increase in the striatum (see Yano and Steiner, 2005a). CG, cingulate; I, insular; IL, infralimbic; I/LO, insular/lateral orbital; P, piriform; PL, prelimbic. $^{***}p < 0.001$. (B) Direct and indirect striatal output pathways in the cortico-basal ganglia-thalamocortical circuits. Direct pathway (striatonigral) neurons contain mainly D_1 dopamine receptors and the neuropeptides substance P (SP) and dynorphin (DYN), whereas neurons that give rise to the indirect pathway (striatopallidal neurons) express mostly D_2 receptors and the peptide enkephalin (ENK), in addition to their main neurotransmitter γ-aminobutyric acid (GABA). (+) and (−) denote facilitatory and inhibitory, respectively. GLU, glutamate; GPe, globus pallidus external segment; GPi, globus pallidus internal segment; SNr, substantia nigra pars reticulata; STN, subthalamic nucleus.

motivational, executive, and motor aspects of all goal-directed behavior and thus in addiction (Steiner, 2010). Psychostimulant-induced molecular changes in these circuits through the striatum are important for various aspects of addiction, including abnormal reward processing, habit formation, and compulsive behavior (Robbins and Everitt, 1999; Berke and Hyman, 2000; Hyman and Malenka, 2001; Gerdeman et al., 2003; Everitt and Robbins, 2005; Belin and Everitt, 2010). However, some of the most affected (dorsal) striatal circuits (Willuhn et al., 2003; Yano and Steiner, 2005a,b; Unal et al., 2009) also participate in frontostriatal attentional networks and may thus be a therapeutic target in ADHD (Robbins et al., 1998; Solanto, 2002). We therefore focus on psychostimulant-induced gene regulation in corticostriatal circuits.

Microarray investigations indicate that hundreds of genes are affected by dopamine and psychostimulants in these circuits (Berke et al., 1998; McClung and Nestler, 2003; Konradi et al., 2004; Yuferov et al., 2005; Adriani et al., 2006a,b; Black et al., 2006; Yano and Steiner, 2007; Heiman et al., 2008). However, the vast majority of studies have assessed effects on the expression of neuropeptide transmitters and immediate-early genes (IEGs). Neuropeptides are often selectively contained in specific neuronal subtypes and thus serve as cell type markers (see Section 3.1.3), but they also modulate basal ganglia functions on several levels (e.g., Steiner, 2010). IEGs are useful as markers for cell activation due to their rapid and transient induction by neuronal activity and drug treatments (Sharp et al., 1993; Chaudhuri, 1997; Harlan and Garcia, 1998). They are thus frequently used to map drug effects in the brain.

Immediate-early genes are also of interest because of their direct involvement in neuroplasticity. Many IEGs encode transcription factors that regulate the expression of other genes (e.g., *c-Fos*, *Zif268*; Knapska and Kaczmarek, 2004). Others (e.g., *Homer 1a*) code for members of a family of scaffolding proteins that anchor receptors to the postsynaptic density and play a role in receptor trafficking, dendritic spine formation, and other processes of synaptic plasticity (Xiao et al., 2000; Thomas, 2002). These latter processes may be involved in the abnormal spine formation in striatal neurons produced by psychostimulant treatment (Robinson and Kolb, 1997; Ferrario et al., 2005; Jedynak et al., 2007; Kim et al., 2009).

In the following sections, we first describe the functional domains of the striatum and then present studies that illustrate psychostimulant-induced effects on gene regulation in these domains. The findings reveal which cortico-basal ganglia-cortical circuits (functional domains, cell types) are affected by these drugs and establish a cellular framework for evaluating and understanding the effects of cognitive enhancers such as methylphenidate and modafinil. We then provide examples of molecular changes induced by repeated psychostimulant treatment and discuss their potential functional significance.

3.1. Corticostriatal circuits affected

3.1.1. Functional domains of the striatum

The functional domains of the striatum are defined by their cortical inputs (Fig. 1). According to current models of basal ganglia function, the basal ganglia and cortex are interconnected by several parallel anatomical circuits/loops that arise in the cortex and project in a topographical manner to the striatum and from there via the basal ganglia output nuclei and thalamus back to the cortex (Alexander et al., 1986, 1990; Albin et al., 1989; Groenewegen et al., 1990; Haber, 2003; Joel and Weiner, 1994; Redgrave et al., 2010). Functionally, these circuits can be roughly categorized as limbic, associative, and sensorimotor, arising, respectively, in the limbic, associative, and sensory and motor regions of the cortex and projecting to their associated domains in all basal ganglia nuclei. The behavioral consequences of psychostimulant-induced molecular changes (or indeed of any pathological changes) in the basal ganglia are therefore dependent on the particular circuits affected. Thus there is interest in determining which circuits/functional domains in the striatum are altered by these drugs.

3.1.2. Topography of psychostimulant-induced gene regulation

Early descriptions of the regional distribution of psychostimulant-induced gene regulation in the striatum were in relatively vague terms anatomically (e.g., dorsolateral quadrant, dorsal *vs.* ventral). Nevertheless, the findings were clear and consistent between laboratories that these effects differ considerably between the different striatal regions. This variability is characteristic of the effects of amphetamine, cocaine (Fig. 2A), and methylphenidate (Fig. 2B).

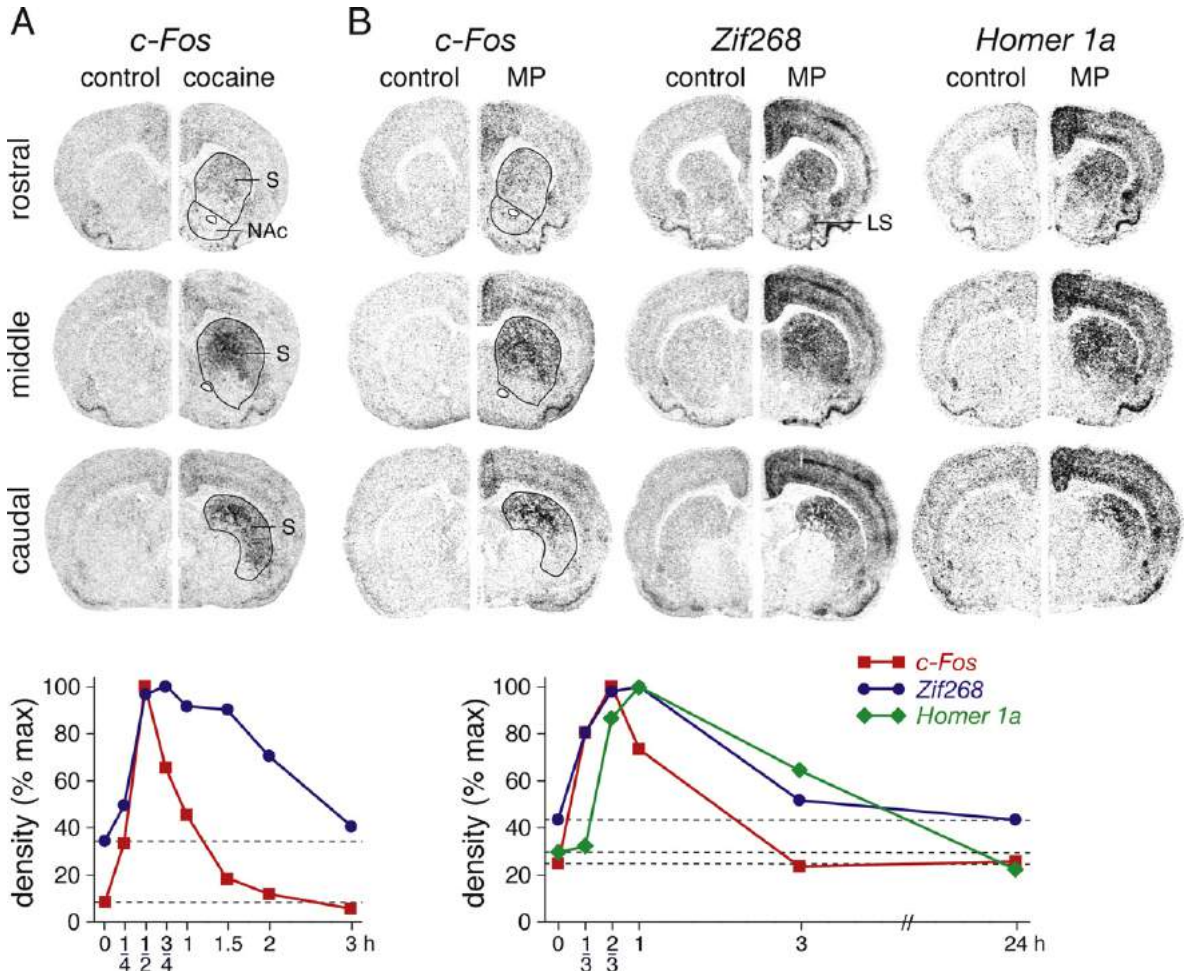

Fig. 2. Psychostimulant-induced immediate-early gene expression in corticostriatal circuits as determined by *in situ* hybridization histochemistry. (A) Cocaine-induced *c-Fos* expression. *Top*: Film autoradiograms depict *c-Fos* expression in coronal sections from rostral (top), middle (center), and caudal striatal levels (bottom) in rats that received a vehicle injection (control, left halfbrain) or a cocaine injection (25 mg/kg; right halfbrain) and were sacrificed 30 min later (Brandon and Steiner, 2003). Striatum (S) and nucleus accumbens (NAc) are outlined in the cocaine-treated animals. Note the considerable regional differences in the *c-Fos* response. *Bottom*: Time course of cocaine (30 mg/kg)-induced *c-Fos* and *Zif268* expression in the dorsal striatum on the middle level (mean density, expressed as percentage of maximal induction) (Steiner and Gerfen, 1998). Basal expression is indicated by broken lines. (B) Methylphenidate-induced gene expression. *Top*: Film autoradiograms show *c-Fos* (left), *Zif268* (middle), and *Homer 1a* expression (right) at 0 min (control, left halfbrain) and 40 min (*c-Fos*) or 1 h (*Zif268*, *Homer 1a*) after methylphenidate injection (MP, 5 mg/kg, i.p.; right halfbrain) (Yano and Steiner, 2005a,b). *Bottom*: Time course of methylphenidate (5 mg/kg)-induced expression of *c-Fos*, *Zif268*, and *Homer 1a* for the dorsal striatal sector on the middle level (Yano and Steiner, 2005a,b). LS, lateral shell of nucleus accumbens.

Graybiel et al. (1990) first showed that *c-Fos* induction by acute cocaine treatment, although widespread in the striatum, had a distinctive topography. It was most pronounced in the dorsal central portion of the sensorimotor striatum and was fairly limited (or absent) in parts of the ventral (limbic) striatum, including the nucleus accumbens (Fig. 2A). Other early studies confirmed this general pattern for *c-Fos* and other genes (e.g., Young et al., 1991; Hope et al., 1992; Moratalla et al., 1992; Bhat and Baraban, 1993; Steiner and Gerfen, 1993; Johansson et al., 1994; see Harlan and Garcia, 1998 for a review of the early work).

Many investigators found an overall similar (dorsal-ventral) distribution for amphetamine effects (Graybiel et al., 1990; Moratalla et al., 1992; Wang et al., 1994a; Badiani et al., 1998; Adams et al., 2001), but there are also differences between amphetamine and cocaine effects, for example, in their distribution across the striatal patch/matrix compartments (Harlan and Garcia, 1998). In contrast to the rather uniform gene induction by cocaine in terms of patch/matrix distribution, the IEG response to amphetamine appears reduced in the matrix relative to that in the patches (striosomes) (Graybiel et al., 1990, 2000). This finding was confirmed by many (e.g., Moratalla et al., 1992; Nguyen et al., 1992; Wang et al., 1995) but not all (Johansson et al., 1994; Wang et al., 1994b; Jaber et al., 1995) subsequent studies.

To better relate psychostimulant-induced molecular changes to specific corticostriatal circuits/functional domains in the rat, we mapped striatal gene regulation using 23 sampling areas (sectors)—based largely on their predominant cortical inputs—on three rostrocaudal levels (Fig. 1A) (Willuhn et al., 2003; Yano and Steiner, 2005a,b; Cotterly et al., 2007; Unal et al., 2009). These studies revealed the following patterns (see Steiner, 2010 for review):

(1) The most robust cocaine-induced changes in gene regulation occur in sensorimotor sectors of the middle and caudal striatum (Fig. 2A) (e.g., Steiner and Gerfen, 1993; Willuhn et al., 2003; Unal et al., 2009). A similar regional distribution has been shown for amphetamine (e.g., Badiani et al., 1998).
(2) Within the sensorimotor striatum, maximal changes occur in the dorsal sectors (approximately the dorsal third) (Fig. 2A). These sectors are unique in that they receive the densest input from the medial agranular cortex (M2; Fig. 1A) (Reep et al., 2003) in addition to convergent inputs from the somatosensory (or visual) and primary motor cortex (cf. Willuhn et al., 2003). Surrounding tissue that is, to a lesser extent, also targeted by medial agranular projections also shows robust changes in gene expression. The rat medial agranular cortex has mixed prefrontal/premotor features (Reep et al., 1987; Passingham et al., 1988; Preuss, 1995; Reep et al., 2003; Uylings et al., 2003) and can therefore be considered a prefrontal/motor interface. Our findings thus indicate that sensorimotor striatal circuits under the influence of medial agranular (prefrontal/premotor) input are particularly prone to psychostimulant-induced neuroplasticity.
(3) Medial and rostral striatal sectors (associative sectors) were affected to a lesser degree (Fig. 2A). These sectors receive inputs from prefrontal regions including the cingulate, prelimbic, and orbital cortex (Fig. 1A) (e.g., Berendse et al., 1992).
(4) On all three rostrocaudal levels, minimal or no changes in gene regulation were seen in ventral striatal sectors (Fig. 2A) that receive inputs mostly from the dorsal agranular insular cortex (Fig. 1A) (e.g., Berendse et al., 1992).
(5) Psychostimulant-induced molecular changes in the nucleus accumbens are well appreciated in the addiction literature (e.g., Graybiel et al., 1990; Hope et al., 1992, 1994; for reviews, see Berke and Hyman, 2000; Nestler, 2001) because they are implicated in motivational (reward) processes (Pierce and Kalivas, 1997). However, consistent with the earlier literature (see above), our studies show that gene regulation effects of cocaine in the nucleus accumbens (Fig. 2A) are modest compared with those in the sensorimotor striatum (Steiner and Gerfen, 1993; Willuhn et al., 2003; Unal et al., 2009). This effect reflects the finding that cocaine strongly activates only a small proportion of sparsely distributed neurons in the nucleus accumbens (as well as in the most rostral striatum) (Mattson et al., 2008). The nucleus accumbens shell appears more affected than the core, and the most robust effects were seen in the lateral part of the shell (Unal et al., 2009), which also receives medial agranular input (Reep et al., 1987) in addition to inputs from the ventral agranular insular cortex (Berendse et al., 1992) and other limbic areas (e.g., McGeorge and Faull, 1989; Brog et al., 1993; Wright and Groenewegen, 1996). The functional significance of these lateral shell effects is not known, but it is of interest to note that the insular cortex, one of the input regions of that part of the nucleus accumbens, is associated with craving in drug addiction (Naqvi et al., 2007), which often drives relapse.

In summary, amphetamine and cocaine produce changes in gene regulation in limbic striatal regions, but these are relatively modest. These changes are likely involved in altered reward processing in addiction (e.g., Belin and Everitt, 2010). Studies that compared effects in different striatal regions demonstrate that more robust drug-induced changes in gene regulation occur in the sensorimotor striatum. These molecular changes probably mediate the functional changes seen in these regions as the addiction disorder progresses (Porrino et al., 2007). Behaviorally, sensorimotor striatal changes may be responsible for habitual and compulsive aspects of drug taking (Berke and Hyman, 2000; Gerdeman et al., 2003; Everitt and Robbins, 2005; Belin and Everitt, 2010), and are likely also important for relapse to drug seeking after abstinence (Vanderschuren et al., 2005; Fuchs et al., 2006; See et al., 2007).

3.1.3. Striatal cell types

The main cell type of the striatum is the medium-sized spiny projection neuron ("medium spiny neuron"); in the rat, interneurons account for less than 3% of striatal neurons (Oorschot, 2010, in press). Colocalization studies indicate that psychostimulants affect gene regulation in projection neurons but have minimal or no effect in interneurons (Berretta et al., 1992). For example, cholinergic interneurons showed cocaine-induced IEG expression only in the ventromedial striatum and the medial shell of the nucleus accumbens but not in the core or in the dorsolateral striatum (Berlanga et al., 2003).

Striatal projection neurons are divided into two subtypes that are intermingled and approximately equal in number and that give rise to two different striatal output pathways (Fig. 1B). The "direct pathway" (striatonigral neurons) connects the striatum directly to the basal ganglia output nuclei (substantia nigra pars reticulata, entopeduncular nucleus/internal pallidum); the "indirect pathway" begins with the striatopallidal neurons and projects to the output nuclei indirectly via the globus pallidus (external pallidum) and subthalamic nucleus (Gerfen and Bolam, 2010).

Both subtypes of striatal projection neurons use γ-aminobutyric acid as their main neurotransmitter, but they differ in a number of receptors and neuropeptides they express (Steiner and Gerfen, 1998; Heiman et al., 2008). Striatonigral neurons contain predominantly the D_1 receptor subtype and the neuropeptides substance P and dynorphin (Fig. 1B), whereas striatopallidal neurons mostly express the D_2 receptor and the neuropeptide enkephalin. (Because of this differential receptor/neuropeptide distribution, these neurons are sometimes referred to as D_1 or D_2

neurons, respectively, and these neuropeptides often serve as markers to differentiate effects of drug treatments between these striatal output pathways.)

These two striatal output pathways have opposite effects on basal ganglia output and motor control. According to current models of basal ganglia function (Fig. 1B), activity in the direct pathway inhibits basal ganglia output, thus disinhibiting thalamocortical (and brainstem) activity (Chevalier and Deniau, 1990) and facilitating behavior, whereas activity in the indirect pathway (i.e., in striatopallidal neurons) results in disinhibition of basal ganglia output, thus arresting behavior (Albin et al., 1989; DeLong, 1990; Redgrave et al., 2010). It is thought that activity in the direct pathway (the "Go pathway") functions to initiate (or facilitate selection of) motor programs, whereas activity in the indirect pathway (the "Stop pathway") interrupts motor programs and/or suppresses unwanted (or incompatible) movement. (For an elegant recent demonstration of this oppositional movement control, see Kravitz et al., 2010.) Although these concepts were initially derived from anatomical findings in the dorsal/sensorimotor striatum (Alexander et al., 1986; Albin et al., 1989), recent results confirmed such antagonistic functions of these two pathways for cocaine-induced behavior mediated by the dorsal striatum (Ferguson et al., 2011) and reward processes mediated by the limbic/ventral striatum (Lobo et al., 2010).

Given the differential functional roles of the two subtypes of striatal projection neurons, it was of considerable interest to determine whether psychostimulants alter gene regulation in both subtypes or whether one is preferentially affected. Early clues were obtained from drug effects on the expression of the neuropeptides that are differentially localized in these neurons and thus serve as cell type markers (Steiner and Gerfen, 1998). Many studies showed that amphetamine and cocaine robustly induce expression of substance P and dynorphin (e.g., Hanson et al., 1987; Sivam, 1989; Hurd and Herkenham, 1992, 1993; Steiner and Gerfen, 1993; Daunais and McGinty, 1994; Wang and McGinty, 1995a; Drago et al., 1996; Adams et al., 2001; Frankel et al., 2008), which are contained in striatonigral neurons. In contrast, enkephalin expression (in striatopallidal neurons) is only modestly affected by psychostimulants (Steiner and Gerfen, 1993; Jaber et al., 1995; Wang and McGinty, 1996a; Spangler et al., 1997; Mathieu-Kia and Besson, 1998). (It should be noted, however, that enkephalin expression is readily induced by glutamate receptor stimulation or D_2 receptor blockade [e.g., Steiner and Gerfen, 1999].)

Colocalization studies using neuropeptide messenger RNAs (mRNAs) or tract tracers as markers confirmed this differential gene regulation for IEGs as well. Amphetamine and cocaine induce IEGs predominantly in striatonigral neurons (Berretta et al., 1992; Cenci et al., 1992; Johansson et al., 1994; Jaber et al., 1995; Kosofsky et al., 1995; Badiani et al., 1999). However, depending on the treatment conditions (i.e., with enough cortical activation/glutamate input; see Steiner, 2010), some IEG induction also occurs in striatopallidal neurons (e.g., Jaber et al., 1995; Badiani et al., 1999; Uslaner et al., 2001; Ferguson and Robinson, 2004).

3.1.4. Dopamine receptor subtypes

The differential effects on striatonigral *vs.* striatopallidal neurons are likely based on the differential distribution of dopamine receptor subtypes between the two projection neuron subtypes (Fig. 1B): As mentioned above, D_1 receptors are predominantly expressed in striatonigral neurons, and D_2 receptors mostly in striatopallidal neurons (Gerfen et al., 1990; Le Moine et al., 1990, 1991; Curran and Watson, 1995; Le Moine and Bloch, 1995). Numerous studies show that D_1 receptor stimulation and resulting activation of second messenger signaling cascades (Bronson and Konradi, 2010; Caboche et al., 2010) are critical for psychostimulant-induced gene regulation in striatal neurons. Thus, IEG expression induced by amphetamine and cocaine is eliminated either by systemic or intrastriatal administration of D_1 receptor antagonists (Graybiel et al., 1990; Young et al., 1991; Moratalla et al., 1992; Cole et al., 1992; Steiner and Gerfen, 1995) or by targeted deletion of the D_1 receptor (D_1 receptor knockouts) (Drago et al., 1996; Moratalla et al., 1996b; Zhang et al., 2004).

D_2 receptors also affect gene regulation in striatal neurons. In contrast to D_1 receptors, however, stimulation of D_2 receptors *inhibits* gene expression in striatopallidal neurons (e.g., Gerfen et al., 1990; Le Moine et al., 1997; Pinna et al., 1997), whereas *blockade* of D_2 receptors (e.g., by antipsychotic drugs) increases gene expression in these neurons (e.g., Steiner and Gerfen, 1998). This difference in effect presumably reflects the fact that D_2 receptors inhibit second messenger signaling, as opposed to the stimulatory action of D_1 receptors (Bronson and Konradi, 2010). However, stimulation of D_2 plus D_1 receptors potentiates D_1 receptor–mediated gene regulation in striatonigral neurons (D_1–D_2 receptor synergy; e.g., Paul et al., 1992; LaHoste et al., 1993; Gerfen et al., 1995). Consistent with this observation, a full gene response to psychostimulants requires combined stimulation of D_1 and D_2 receptors (Ruskin and Marshall, 1994). This interaction between D_1 and D_2 receptors is thought to be mediated by cholinergic interneurons (Wang and McGinty, 1996b; Pisani et al., 2007)—for example, via a D_2 receptor-mediated inhibition of inhibitory cholinergic input to striatonigral neurons (Wang and McGinty, 1996b).

In addition, D_3 receptors modify such molecular effects. These receptors are predominantly present in ventral striatal regions where they are partly coexpressed with D_1 receptors in striatonigral neurons (Le Moine and Bloch, 1996; Schwartz et al., 1998). Because they also exert opposite (inhibitory) effects on second messenger signaling (Zhang et al., 2004), D_3 receptors dampen gene induction by D_1 receptor stimulation (Carta et al., 2000; Zhang et al., 2004).

In summary, these findings demonstrate that (1) amphetamine- and cocaine-induced changes in gene regulation in the striatum occur preferentially (but not exclusively) in direct pathway (striatonigral) neurons (see also Lobo and Nestler, 2011), and (2) D_1 receptors (and their downstream signaling cascades; Caboche et al., 2010) are critical for these molecular changes.

3.2. Relationship between gene regulation in striatum and cortex

Imaging studies in humans and other primates show that exposure to psychostimulants such as cocaine and amphetamine produces functional changes also in various regions of the cortex (e.g., London et al., 1990; Breiter et al., 1997; Beveridge et al., 2006; Porrino et al., 2007). Similarly, systemic administration of cocaine, amphetamine, and other dopamine agonists causes increases in gene expression in the cortex (Fig. 2) (e.g., Graybiel et al., 1990; Paul et al., 1992; Dilts et al., 1993; Johansson et al., 1994; Steiner and Gerfen, 1994; Wang and McGinty, 1995a; LaHoste et al., 1996; Badiani et al., 1998). These cortical effects are widespread (Harlan and Garcia, 1998), but a recent detailed mapping study showed that acute and repeated cocaine treatments produce the most robust changes in IEG regulation in sensory and motor regions of the cortex (Unal et al., 2009), thus mirroring the distribution of such molecular changes across striatal functional domains. Other studies have also revealed preferential gene regulation in the sensorimotor cortex for cocaine (e.g., Daunais and McGinty, 1994; Johansson et al., 1994) and amphetamine (e.g., Wang et al., 1994a, 1995; Curran et al., 1996; Badiani et al., 1998; Uslaner et al., 2001).

Some of these cortical effects may be a consequence of drug action directly in the cortex. However, consistent with the models of cortico-basal ganglia-cortical circuits (Fig. 1B), many of the cortical changes are caused by drug-induced alterations in basal

ganglia output as a consequence of changed activity in the D_1 receptor-regulated direct striatal output pathway (for review, see Steiner, 2007). Thus, stimulation of striatal D_1 receptors produces widespread increases in gene expression throughout the cortex (Steiner and Kitai, 2000; Gross and Marshall, 2009; see Steiner, 2007).

True to the loop architecture of these circuits, reentrant activity from the cortex (or thalamus; Cotterly et al., 2007) to the striatum is also important for psychostimulant-induced gene regulation in the striatum. Studies demonstrated that blockade of glutamate (*N*-methyl-D-aspartate) receptors (e.g., Johnson et al., 1991; Torres and Rivier, 1993; Wang et al., 1994a; Hanson et al., 1995) or elimination of corticostriatal afferents (Cenci and Björklund, 1993; Vargo and Marshall, 1995; Ferguson and Robinson, 2004) attenuates psychostimulant-induced gene expression in striatal neurons. Therefore, striatal effects of psychostimulants are a consequence of drug-induced overstimulation of striatal D_1 receptors in interaction with cortical (glutamate) input (Hyman et al., 1996; Wang and McGinty, 1996b).

Other findings demonstrate that psychostimulants engage cortical and striatal nodes of corticostriatal circuits in a coordinated manner; gene induction in cortical areas and in their associated functional domains in the striatum is correlated (Fig. 1A; Cotterly et al., 2007; Yano and Steiner, 2005a). Given their role in neuroplasticity, these gene regulation effects indicate coordinated neuroplastic changes in cortical and functionally related striatal areas.

3.3. Molecular effects of repeated amphetamine and cocaine exposure

Repeated psychostimulant exposure produces a variety of neuroadaptations and other neuronal changes in the basal ganglia (e.g., Hyman and Nestler, 1996; Kuhar and Pilotte, 1996; Berke and Hyman, 2000; Nestler, 2001; Kelley, 2004). In this section, we provide a few examples of such molecular changes for comparison with similar changes induced by cognitive enhancers, presented later. These examples involve the same IEG and neuropeptide markers as discussed in the previous sections.

As would be expected for molecular adaptations, changes after repeated treatments occur in the same striatal regions and neurons that display the acute drug effects and are directly correlated in magnitude with the acute effects (Steiner and Gerfen, 1993; Willuhn et al., 2003; Unal et al., 2009).

3.3.1. Blunted gene inducibility

One of the best-established molecular consequences of repeated psychostimulant treatment is blunting (repression) of gene inducibility in the striatum. Thus, after repeated treatments, genes are still inducible by a drug challenge, but this induction is typically attenuated compared with acute induction. Such blunting was first demonstrated after repeated amphetamine and cocaine treatment for several transcription factor IEGs (e.g., *c-Fos* and *Zif268*; Hope et al., 1992, 1994; Persico et al., 1993; Steiner and Gerfen, 1993; Daunais and McGinty, 1994; Moratalla et al., 1996a). Other genes are similarly affected—for example, the effector IEG *Homer 1a* (Unal et al., 2009) and the neuropeptide substance P (Steiner and Gerfen, 1993; Jaber et al., 1995).

Blunting of gene induction is long-lasting. A recent study showed marked attenuation in *Zif268* and *Homer 1a* inducibility even 3 weeks after a 5-day repeated cocaine treatment (Unal et al., 2009).

Consistent with a compensatory neuroadaptation, the degree of blunting is directly related to the magnitude of the initial (acute) gene induction in a given striatal region—the greater the induction after the first drug administration, the more blunted the induction after chronic treatment (Willuhn et al., 2003; Unal et al., 2009). Mapping studies showed that repeated cocaine treatment produces the most robust blunting in the dorsal/lateral (sensorimotor) striatum at middle to caudal striatal levels (Willuhn et al., 2003; Unal et al., 2009). [It should be noted, however, that gene induction is not universally blunted in all striatal areas after repeated psychostimulant treatments; in parts of the nucleus accumbens, increased rather than reduced gene induction has been demonstrated in several studies (Crombag et al., 2002; Todtenkopf et al., 2002; Brandon and Steiner, 2003; Cotterly et al., 2007; Damez-Werno et al., 2012).]

Various mechanisms may contribute to blunting of gene induction after repeated drug treatment, some shorter-lasting, some long-lasting. Investigators have proposed systems-level neuroadaptations as well as intracellular (epigenetic) adaptations. Examples are:

(1) Given the importance of excitatory inputs for striatal gene regulation, blunted gene induction may partly reflect dampened inputs from the cortex (and/or thalamus), perhaps involving long-term depression-like synapse modifications (see Graybiel et al., 2000; Unal et al., 2009, for discussion).
(2) Neuropeptides such as dynorphin modulate dopamine and glutamate input to striatal neurons and thus indirectly also affect gene regulation (Steiner, 2010). For example, acute IEG induction by cocaine and D_1 receptor agonist treatment is inhibited by stimulation of dynorphin (kappa opioid) receptors in the striatum (Steiner and Gerfen, 1995, 1996). Blunting of gene induction may thus at least in part reflect increased dynorphin function and resulting inhibition of dopamine or glutamate action after repeated psychostimulant treatment (as we discuss below).
(3) Epigenetic regulation of gene expression involving chromatin modifications (e.g., histone acetylation and methylation) may best explain the endurance of gene blunting (for reviews see Renthal and Nestler, 2008; Caboche et al., 2010). For example, chromatin modification has been shown to contribute to blunting of *c-Fos* expression after repeated amphetamine treatment (e.g., Renthal et al., 2008) and to blunting/priming of *FosB* expression after repeated cocaine treatment (Damez-Werno et al., 2012).

The exact consequences of blunted gene induction for basal ganglia function are unknown. However, the functional integrity of neurons depends on balanced regulation of gene expression because cellular components have limited half-lives and must be replenished. It is assumed that disruption of such homeostatic regulation by psychostimulants results in deficient neuronal function that contributes to behavioral manifestations of psychostimulant addiction (e.g., Hyman and Nestler, 1996; Nestler, 2001).

3.3.2. Alternative splicing: accumulation of deltaFosB

Another often described molecular change caused by psychostimulants is accumulation of the transcription factor deltaFosB in striatonigral neurons (McClung et al., 2004). DeltaFosB is induced by many manipulations that involve excessive neuronal activation (McClung et al., 2004). DeltaFosB is a truncated isoform of FosB (member of the AP-1 family of transcription factors) that is produced by alternative splicing (Nakabeppu and Nathans, 1991). The truncation renders the molecule highly stable. With repeated drug treatments, deltaFosB accumulates in cells and displaces other members of the AP-1 family from the AP-1 transcriptional complex, thus altering the function of this complex (Nakabeppu and Nathans, 1991; McClung et al., 2004).

DeltaFosB accumulation is well established for repeated amphetamine and cocaine treatments (Hope et al., 1994; Nye

et al., 1995; Renthal et al., 2008). A recent mapping study showed increased deltaFosB levels in many striatal regions, with maximal increases in the dorsal/lateral striatum, after repeated cocaine treatment (Sato et al., 2011).

Findings indicate that many genes (e.g., those with AP-1 and CRE binding sites in their promoter) are affected by this abnormal transcription factor; some are activated and some are repressed, depending in part also on the length of the drug treatment (McClung and Nestler, 2003; McClung et al., 2004). For example, deltaFosB action appears to upregulate dynorphin expression (Andersson et al., 1999; but see McClung et al., 2004), while playing a role in blunting of *c-Fos* induction after repeated amphetamine treatment (Renthal et al., 2008).

3.3.3. Increased dynorphin expression

A third widely demonstrated consequence of repeated psychostimulant treatment is increased dynorphin expression in the striatonigral (direct) pathway (Steiner, 2010; Butelman et al., 2012; Yoo et al., 2012). Many laboratories have reported elevated dynorphin mRNA (e.g., Hurd and Herkenham, 1992; Spangler et al., 1993; Steiner and Gerfen, 1993; Daunais and McGinty, 1994; Wang et al., 1994a; Adams et al., 2003; Willuhn et al., 2003) or peptide levels (e.g., Hanson et al., 1987; Li et al., 1988; Sivam, 1989; Smiley et al., 1990) after repeated amphetamine and cocaine treatment. Notably, increased dynorphin expression has also been found in human cocaine addicts (Hurd and Herkenham, 1993; Frankel et al., 2008).

After a single psychostimulant administration, elevated dynorphin mRNA levels persist in the rat for at least 18–30 h (Smith and McGinty, 1994; Wang and McGinty, 1995a,b). Thus, this mRNA accumulates with daily drug treatments. Indeed, after repeated treatment with a dopamine agonist, elevated dynorphin mRNA levels in the striatum lasted several weeks past cessation of the treatment (Andersson et al., 2003). Again, repeated cocaine treatment produces maximally increased dynorphin expression in the dorsal/lateral (sensorimotor) sectors of the middle to caudal striatum (Steiner and Gerfen, 1993; Willuhn et al., 2003).

What is the functional significance of increased dynorphin expression in the striatum? Findings indicate that opioid peptides such as dynorphin (striatonigral neurons) and enkephalin (striatopallidal neurons) act, at least in part, as negative feedback mechanisms (Steiner and Gerfen, 1998) to limit dopamine and glutamate input to these neurons (Steiner, 2010). Repeated excessive activation of these neurons by pharmacological treatments (or other experimental manipulations) is thought to trigger compensatory upregulation of opioid peptide function to counteract the activation (i.e., to act as a "brake") and maintain systems homeostasis (Hyman and Nestler, 1996).

In the case of upregulated dynorphin function after repeated psychostimulant exposure, it is thus to be expected that during early withdrawal from drug use the "brake" is still on for some time given the relatively long half-life of changes in dynorphin expression. The increased dynorphin signaling would then excessively inhibit inputs to striatal neurons (Hyman and Nestler, 1996; Steiner and Gerfen, 1998; Shippenberg et al., 2007). There is good evidence that increased dynorphin function in this manner contributes to somatic signs of withdrawal such as dysphoria, anxiety, anhedonia, and depression after discontinuation of drug use (Nestler and Carlezon, 2006; Shippenberg et al., 2007; Butelman et al., 2012; Yoo et al., 2012). These effects are thought to contribute to maintenance of drug use or relapse during abstinence.

4. Gene regulation by oral Adderall

The above reviewed effects of amphetamine in animal studies were mostly obtained with intraperitoneal (i.p.) or subcutaneous (s.c.) administration of relatively high doses (~3 to 10 mg/kg). How relevant are these findings for therapeutic use of Adderall, which involves lower doses and predominantly oral administration?

Psychostimulant effects on gene regulation are dose-dependent. Higher doses produce greater increases in gene expression across a wide range of doses (e.g., Steiner and Gerfen, 1993; Wang and McGinty, 1995b, 1997; Brandon and Steiner, 2003; Chase et al., 2003; Chase et al., 2005a; Yano and Steiner, 2005b). [Occasionally, very high doses have been found to result in attenuated expression (Wang and McGinty, 1995b, 1997), similar to other neuronal effects (e.g., Hanson et al., 2002), possibly due to receptor inactivation (internalization) by the high dose or other mechanisms.]

Gene regulation is also under the control of the drug delivery rate. For example, fast intravenous (i.v.) delivery of a certain cocaine dose (2 mg/kg) produced greater *c-Fos* induction in the striatum than slower delivery of the same dose (Samaha et al., 2004; Samaha and Robinson, 2005). Similarly, with repeated treatment (self-administration model), faster drug delivery produced more robust blunting of *c-Fos* inducibility (Wakabayashi et al., 2010). These enhanced neuronal changes were associated with indices of a greater addiction liability (greater escalation of drug intake and propensity to relapse; Wakabayashi et al., 2010).

Conversely, oral (or intragastric) administration of drugs produces slower (and lower) uptake (Swanson and Volkow, 2003; Kuczenski and Segal, 2005; Yano and Steiner, 2007), which would thus be expected to produce less molecular changes. Few studies have investigated the molecular effects of oral amphetamine administration in a therapeutic dose range. Researchers recently used a model with prepubertal rats to assess whether a low dose (1.6 mg/kg) of orally (p.o.) administered Adderall (mix of D- and L-amphetamine), which resulted in amphetamine levels in the blood close to those of children treated with amphetamine, caused changes in *c-Fos* expression in corticostriatal circuits (Allen et al., 2010). The results showed that despite the low dose and oral route, acute Adderall administration produced significant *c-Fos* induction in the striatum and cortex (Allen et al., 2010). Moreover, repeated treatment (1.6 mg/kg, p.o., once daily for 14 days) resulted in blunting of *c-Fos* inducibility in these brain regions (Allen et al., 2010). An earlier study in adult cats reported that 1 mg/kg (p.o.) of amphetamine induced *c-Fos* in the cortex and striatum (Lin et al., 1996). Consistent with these findings, another study in young rats showed that repeated treatment with a low dose of amphetamine (0.5 mg/kg, s.c., twice daily for 13 days), which resulted in amphetamine plasma concentrations corresponding to the clinical range used in the treatment of ADHD, produced altered dendritic architecture in the prefrontal cortex (Diaz Heijtz et al., 2003).

In summary, the findings obtained with faster administration and higher doses of amphetamine (and cocaine) may be more relevant for abuse of psychostimulants. But the results described above indicate that therapeutically relevant amphetamine doses and routes of administration can produce qualitatively similar molecular changes in neurons of corticostriatal circuits (Carrey and Wilkinson, 2011).

5. Gene regulation by methylphenidate

Methylphenidate, widely used in the treatment of ADHD and other mental disorders, is also popular as a cognitive enhancer (see Introduction). Although methylphenidate has been effective in the clinic for several decades, assessment of its molecular impacts began only about 10 years ago (Yano and Steiner, 2007). Because both clinical and recreational exposure to methylphenidate occurs predominantly in children and adolescents, preclinical studies often focus on the effects in prepubertal/adolescent

animals (Yano and Steiner, 2007; Carrey and Wilkinson, 2011; Marco et al., 2011).

Early microarray studies in adolescent rats showed that acute and repeated treatment with 2 mg/kg (i.p.) of methylphenidate altered the expression of more than 2000 genes in the striatum (Adriani et al., 2006a,b). Similar to other psychostimulants (Section 3), methylphenidate affected genes that encode transcription factors, neurotransmitter receptors, ion channels, postsynaptic density proteins, and other signaling-related molecules as well as many other classes (e.g., molecules involved in cell migration, survival, maturation, and other forms of neuroplasticity) (Adriani et al., 2006a,b; see also Yano and Steiner, 2007; Carrey and Wilkinson, 2011; Marco et al., 2011). Some of the molecular changes persisted well past the termination of the drug treatment, into the adulthood of the animals (Adriani et al., 2006a,b; for similarly long-lasting changes, see Chase et al., 2007; Warren et al., 2011).

Most of these wide-ranging effects will have to be confirmed in follow-up studies; but the effects on the expression of transcription factors/IEGs and neuropeptides in corticostriatal circuits are well established. We summarize these findings here for comparison with the effects of amphetamine and cocaine described above.

5.1. Regulation of immediate-early genes and neuropeptides

The first demonstration of gene regulation by methylphenidate was through oral administration in adult cats (Lin et al., 1996). This study showed that 2.5 mg/kg (p.o.) induced *c-Fos* expression in many brain areas, including the cortex and striatum. The regional patterns were described as "highly similar" to those produced by 1 mg/kg (p.o.) of amphetamine (these doses were compared because of their similar effects on wakefulness). Importantly, the *c-Fos* expression patterns of both drugs were different from those induced by modafinil (5 mg/kg, p.o.), also chosen for a similar waking effect (Lin et al., 1996). This comparison indicates that these regional patterns reflect the pharmacological targets of these drugs rather than their waking effect. Acute *c-Fos* induction by methylphenidate in the striatum (Fig. 2B) was confirmed by many subsequent studies in both mice (Penner et al., 2002; Trinh et al., 2003; Hawken et al., 2004) and rats (Brandon and Steiner, 2003; Chase et al., 2003, 2005b; Yano and Steiner, 2005b).

Examples of other IEGs induced in the striatum include the transcription factors *Zif268* (Fig. 2B) (Brandon and Steiner, 2003; Yano and Steiner, 2005a) and FosB (Chase et al., 2005a) as well as the effector IEGs *Arc* (Chase et al., 2007; Banerjee et al., 2009) and *Homer 1a* (Fig. 2B) (Yano and Steiner, 2005a; Adriani et al., 2006a; Cotterly et al., 2007).

A few studies show that methylphenidate also affects neuropeptide markers in striatal output neurons. These studies indicate that methylphenidate increases substance P expression (striatonigral neurons) (Fig. 3) in a manner similar to other psychostimulants, whereas the opioid peptides dynorphin (striatonigral neurons) and enkephalin (striatopallidal neurons) appear to be less affected (Yano and Steiner, 2007).

We directly compared methylphenidate effects on the expression of these genes by monitoring their mRNA levels between 20 min and 24 h after acute injection of methylphenidate (2–10 mg/kg, i.p., adult rats; Yano and Steiner, 2005b) (Fig. 3). Similar to the effects of cocaine/amphetamine (see Section 3.1.3), we found that substance P expression increased in many striatal sectors in a dose-dependent and very robust manner, with elevated mRNA levels present within 20 min and lasting for more than 3 h. Conversely, for dynorphin mRNA, we detected a statistically significant, if modest, increase only in two sectors at 1 h (Fig. 3) (Yano and Steiner, 2005b). This latter finding contrasts with studies on amphetamine and cocaine effects, which showed

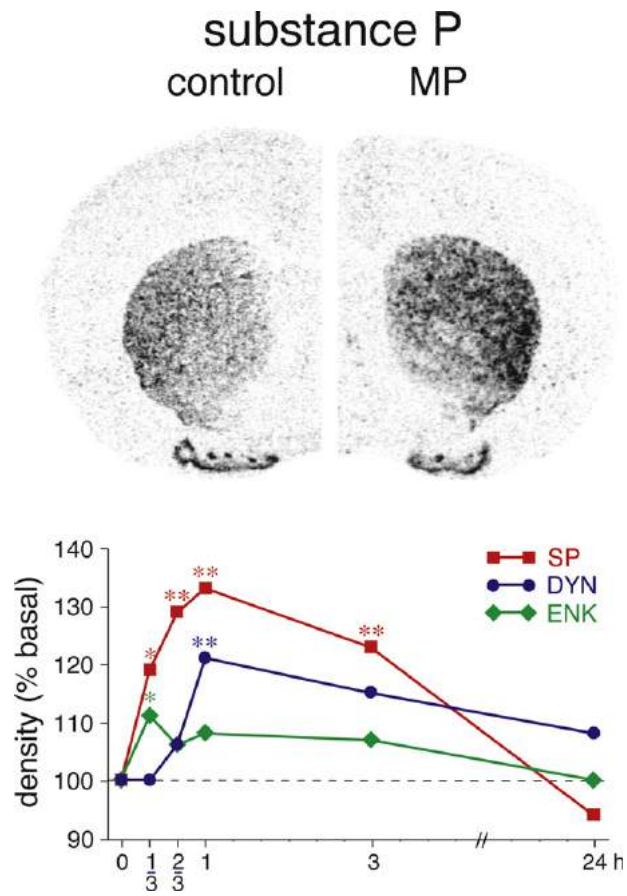

Fig. 3. Methylphenidate-induced neuropeptide expression. *Top*: Film autoradiograms depict substance P expression in the middle striatum at 0 min (control, left halfbrain) and 1 h after injection of methylphenidate (MP, 5 mg/kg, i.p.; right halfbrain). *Bottom*: Time course of methylphenidate (5 mg/kg)-induced expression of substance P (SP), dynorphin (DYN), and enkephalin (ENK) (in percentage of basal expression) for the dorsal striatal sector on the middle level (Yano and Steiner, 2005b). Note that substance P expression increased in 13 of the 23 striatal sectors, whereas dynorphin and enkephalin expression significantly increased in only two and one sector, respectively (values in area of maximal increase are shown here; see Yano and Steiner, 2005b, for details). $^{**}p < 0.01$, $^{*}p < 0.05$ *vs.* 0 min.

that significantly increased dynorphin mRNA levels are present within 30 min (Willuhn et al., 2003), are prominent at 2–3 h (Hurd and Herkenham, 1992; Smith and McGinty, 1994), and last 18–30 h (Smith and McGinty, 1994; Wang et al., 1995) after acute drug administration.

Enkephalin, which is strongly induced, for example, by D_2 receptor antagonists (Steiner and Gerfen, 1998), is only moderately affected by acute cocaine and amphetamine treatments (Hurd and Herkenham, 1992; Steiner and Gerfen, 1993; Wang and McGinty, 1995a,b, 1996a). Acute methylphenidate did not produce consistent effects on enkephalin expression (Yano and Steiner, 2005b).

Neurotensin is another neuropeptide expressed in striatal output pathways; it is contained in both types of projection neurons, and its expression is also regulated by D_1, D_2 and glutamate receptors (see Hanson et al., 1992; Alburges et al., 2011). Its apparent interactions with the mesolimbic and mesostriatal dopamine systems suggest that neurotensin may influence the addictive properties of psychostimulants (cf. Alburges et al., 2011). Both cocaine and amphetamine treatments produce increased neurotensin expression (e.g., Letter et al., 1987; Hanson et al., 1989; Gygi et al., 1994), and a recent study shows that methylphenidate increases neurotensin expression as well (Alburges et al., 2011).

Overall, the molecular effects of methylphenidate described above typically emerged with doses of ≥2 mg/kg (i.p. or s.c.), and juvenile/adolescent rodents tended to be more sensitive than adults, as is true with other psychostimulants (Yano and Steiner, 2007; Carrey and Wilkinson, 2011).

5.2. Corticostriatal circuits affected

5.2.1. Functional domains of the striatum

To determine which corticostriatal circuits/functional domains are affected by methylphenidate treatment, we first mapped gene regulation throughout the striatum (Yano and Steiner, 2005a,b; Cotterly et al., 2007). We assessed the same 23 striatal sectors (Fig. 1A), reflecting specific corticostriatal circuits from the rostral to the caudal striatum, as in our cocaine studies (Willuhn et al., 2003; Unal et al., 2009) to allow direct comparisons. Our findings show that, overall, methylphenidate- and cocaine-induced gene regulation in the striatum display a similar but not identical topography (Fig. 2), as follows:

(1) Similar to cocaine, methylphenidate produces the most robust changes in gene expression in sensorimotor sectors of the middle and caudal striatum. Maximal effects are present in the dorsal sectors (Fig. 2B) that receive the densest input from the medial agranular cortex. However, unlike cocaine-induced gene regulation, which peaks in the postcommissural caudal striatum (corresponding to the middle-to-caudal putamen) (Willuhn et al., 2003; Unal et al., 2009), methylphenidate-induced gene regulation peaks in somewhat more rostral parts of the sensorimotor striatum (Fig. 2B) (Yano and Steiner, 2005a,b; Cotterly et al., 2007).
(2) For both drugs, medial and rostral (associative) sectors are also affected to some extent, although they appear to be more changed by methylphenidate than by cocaine (Fig. 2).
(3) Similar to cocaine (Willuhn et al., 2003; Unal et al., 2009), small or no effects are seen in ventral striatal sectors on all rostrocaudal levels (Fig. 2B). In the nucleus accumbens, methylphenidate-induced gene regulation appears even less robust than that induced by cocaine; however, the most prominent effects were again found in the lateral part of the shell (Fig. 2B) (Brandon and Steiner, 2003; Yano and Steiner, 2005a,b; Cotterly et al., 2007). Such differential gene regulation between sensorimotor striatum and nucleus accumbens, with pronounced effects in the former and minor or no effects in the latter, was also found by others (neurotensin, Alburges et al., 2011; IEGs, e.g., Lin et al., 1996; Trinh et al., 2003; Chase et al., 2005b).

5.2.2. Striatal cell types and dopamine receptors

The striatal output pathways affected by methylphenidate require confirmation by double-labeling studies, but the robust effects on substance P expression (Fig. 3) (Brandon and Steiner, 2003; Yano and Steiner, 2005b) strongly indicate that, in line with other psychostimulants, methylphenidate alters gene expression in neurons of the D_1 receptor-regulated direct pathway (Fig. 1B). This conclusion is supported by dopamine receptor antagonist studies showing that blockade of D_1 receptors in the striatum eliminates methylphenidate-induced IEG (Yano et al., 2006) and neuropeptide expression (Alburges et al., 2011). Again similar to other psychostimulants (Ruskin and Marshall, 1994; see Section 3.1.4), D_2 receptor stimulation also appears to facilitate such gene regulation (Alburges et al., 2011).

Consistent with the above findings, a recent study in bacterial artificial chromosome-transgenic D_1- or D_2-EGFP-expressing[4] mice found that repeated methylphenidate treatment increased *FosB* expression in neurons of the direct pathway (D_1) but not the indirect pathway (D_2) (Kim et al., 2009). However, dendritic spine densities in the nucleus accumbens were increased in both subtypes of projection neurons (Kim et al., 2009).

Together with the dearth of effects on enkephalin expression (indirect pathway) (Brandon and Steiner, 2003; Yano and Steiner, 2005b; Van Waes et al., 2012a), these findings indicate that methylphenidate may more selectively affect direct pathway neurons than do amphetamine and cocaine (for possible mechanisms, see Van Waes et al., 2012a).

5.3. Relationship between gene regulation in striatum and cortex

As mentioned above, and as with other psychostimulants, methylphenidate produces IEG induction also in other brain areas, particularly the cortex (Fig. 2B) (Lin et al., 1996; Chase et al., 2005b; Yano and Steiner, 2005a; Banerjee et al., 2009).

We mapped methylphenidate-induced IEG expression throughout the major functional subdivisions of the rat cortex (22 areas on four rostrocaudal levels; Fig. 1A) (Yano and Steiner, 2005a; Cotterly et al., 2007). Our results show that acute methylphenidate induces IEG expression most robustly in the medial agranular (M2; premotor) and cingulate cortex (Fig. 2B), followed closely by motor and somatosensory areas, with minor effects in the insular cortex (Yano and Steiner, 2005a). Although the overall topography of these methylphenidate-induced cortical changes was thus similar to that of cocaine, the methylphenidate effects tended to spread more into rostral and medial cortical areas (Yano and Steiner, 2005a; Cotterly et al., 2007) than the effects of cocaine (Unal et al., 2009).

Our results indicate that cortical gene regulation by methylphenidate occurs in similar functional domains as IEG regulation in the striatum (Fig. 2B). Indeed, our regional analysis determined that these IEG responses were positively correlated between cortical areas and their striatal target sectors, confirming that specific corticostriatal projections are affected (Fig. 1A) (Yano and Steiner, 2005a; Cotterly et al., 2007). Cortical IEG regulation was also correlated with striatal substance P and dynorphin induction (striatonigral neurons) but not with enkephalin expression (striatopallidal neurons) (Yano and Steiner, 2005a). These findings thus indicate coordinated methylphenidate-induced neuroplasticity between the cortex and neurons of the direct (but not indirect) striatal output pathway.

5.4. Effects of repeated methylphenidate treatment

5.4.1. Blunted gene inducibility

As discussed in the section on amphetamine and cocaine effects, a well-established neuroadaptation that occurs during repeated psychostimulant treatment is blunting (repression) of gene inducibility. Repeated methylphenidate treatment produces a similar effect. Methylphenidate-induced blunting of gene induction in the striatum has been demonstrated, for example, for *c-Fos*, *Zif268*, *Arc*, and substance P (Brandon and Steiner, 2003; Chase et al., 2003, 2007; Hawken et al., 2004; Cotterly et al., 2007) and can last more than 4 weeks (Chase et al., 2005a). As with repeated cocaine treatment (Unal et al., 2009), the degree of blunting after repeated methylphenidate treatment is directly related to the strength of the acute gene response in a particular striatal region (Cotterly et al., 2007).

Most often gene blunting after repeated methylphenidate treatment has been demonstrated by the (reduced) response to a subsequent methylphenidate challenge. However, given that these drugs share some of their neurochemical effects (see Section 2.1), it is not surprising that repeated methylphenidate pretreatment also

[4] EGFP, enhanced green fluorescent protein.

results in blunted gene induction by a cocaine challenge (Brandon and Steiner, 2003).

The mechanisms underlying gene blunting by these two drugs, however, may not be identical. For example, repeated cocaine treatment blunted striatal *Zif268* and *Homer 1a* induction to a similar extent (Unal et al., 2009), while repeated methylphenidate treatment (10 mg/kg, i.p., 7 days) produced significant blunting of striatal *Zif268* induction but minimal changes in *Homer 1a* induction (Cotterly et al., 2007).

5.4.2. Alternative splicing: accumulation of deltaFosB

DeltaFosB accumulation in striatal neurons after repeated amphetamine and cocaine treatment is well established (e.g., Hope et al., 1994; Nye et al., 1995; McClung et al., 2004). Repeated methylphenidate treatment also increases levels of FosB immunoreactivity in the striatum and cortex (Chase et al., 2005a,b; Kim et al., 2009). In the striatum, the increased FosB signal was selectively present in striatonigral (D_1) neurons (Kim et al., 2009). This immunoreactivity is thought to reflect deltaFosB (Kim et al., 2009), but this remains to be confirmed.

5.4.3. Increased dynorphin expression

As mentioned above, in contrast to cocaine and amphetamine, a single methylphenidate injection caused only a modest increase in dynorphin expression in the striatum (Yano and Steiner, 2005b). Consistent with this finding, recent studies indicate that repeated methylphenidate treatment also produces more modest upregulation of dynorphin expression compared with cocaine and amphetamine. For example, a study using reverse-transcription polymerase chain reaction to measure gene expression failed to find altered striatal dynorphin expression after daily methylphenidate treatment with a low dose (2 mg/kg, i.p.; adolescent rats) for 16 days (Adriani et al., 2006a). Another investigation demonstrated that methylphenidate treatment with a high dose (10 mg/kg, i.p.; adolescent rats) once daily for 7 days, which produced robust blunting of IEG and substance P induction, resulted in a significant but more limited (compared with cocaine and amphetamine effects) increase in dynorphin expression (Brandon and Steiner, 2003). A more aggressive methylphenidate treatment (four injections of 10 mg/kg, s.c., over 6 h) produced increased dynorphin peptide levels (immunoreactivity) in the striatum and substantia nigra 18 h later (Alburges et al., 2011). This treatment also enhanced neurotensin expression in striatal output pathways (Alburges et al., 2011).

The findings above, together with the unchanged *Homer 1a* regulation (Cotterly et al., 2007), indicate that some genes are less affected by methylphenidate than by cocaine or amphetamine. Below (Section 7) we discuss potential mechanisms underlying these differential effects and possible clinical relevance.

5.5. Gene regulation by oral methylphenidate treatment

Since the first demonstration of gene regulation by methylphenidate through oral administration (Lin et al., 1996), few studies have assessed oral effects (Carrey and Wilkinson, 2011). Given that therapeutic use of methylphenidate typically involves oral administration, a recent study investigated whether oral treatment (in freely moving prepubertal rats) with doses that produced clinically relevant methylphenidate blood levels would alter gene regulation in the striatum (Chase et al., 2007). The results showed that acute administration of 7.5–10 mg/kg (p.o.), but not 2.5–5 mg/kg, induced robust IEG expression (*Arc*) in the striatum. However, although repeated s.c. injections of 7.5 mg/kg of methylphenidate did cause blunting of *Arc* induction, 14 days of daily oral treatment with this threshold dose did not attenuate *Arc* inducibility (Chase et al., 2007). The investigators did not examine higher doses (or other genes).

Future studies will have to determine whether this effect reflected a qualitatively different potential for neuroadaptations by oral treatment compared with injected methylphenidate, or was, more likely, simply a consequence of slower uptake and too low methylphenidate plasma levels after oral administration of this threshold dose.

5.6. Methylphenidate effects: conclusions

The reviewed findings indicate that methylphenidate, even in therapeutically relevant doses, can produce changes in gene regulation in cortical and striatal neurons that are qualitatively similar to those of cocaine and amphetamine, although some of the investigated genes seem to be less affected. Methylphenidate also appears to alter the same corticostriatal circuits/functional domains; these are mostly sensorimotor and to some degree associative domains. Overall, these findings are consistent with an addiction liability for methylphenidate, if reduced compared with cocaine and amphetamine (Svetlov et al., 2007).

6. Gene regulation by modafinil

Modafinil is a relatively novel agent that promotes wakefulness and is thus widely used to treat excessive daytime sleepiness associated with narcolepsy and other sleep disorders, but it is also gaining popularity as a cognitive enhancer (see Introduction). Its effects on gene regulation have been described in only a handful of studies.

To our knowledge, the first study to indicate such molecular effects showed increased expression of glutamine synthetase, an enzyme involved in brain metabolism, after a single injection of modafinil in rats (Touret et al., 1994). More recently, a gene microarray study identified several molecule classes affected by modafinil, including transcription factors such as *c-Fos* (Hasan et al., 2009), and thus suggested that modafinil exposure may alter various neuronal processes. However, most studies to date have assessed only *c-Fos* expression (Fos immunoreactivity) as a marker to identify neuronal systems involved in the regulation of sleep and wakefulness.

The 1996 study by Lin and colleagues showed that in adult cats modafinil (5 mg/kg, p.o.) produced minor *c-Fos* induction in striatum and cortex (i.e., considerably less than induced by methylphenidate [2.5 mg/kg, p.o.] and amphetamine [1 mg/kg, p.o.], despite causing similar wakefulness), but induced pronounced *c-Fos* expression in the hypothalamus and other brain regions (Lin et al., 1996). A study in rats (300 mg/kg, i.p.; Engber et al., 1998) confirmed these effects.

More recent work found *c-Fos* induction by modafinil (75–300 mg/kg, i.p.) in various nuclei from the hypothalamus to the brainstem, but also described considerable induction in the cortex and striatum in mice (Willie et al., 2005; Hasan et al., 2009) and rats (Scammell et al., 2000; Fiocchi et al., 2009). Based on findings with other psychostimulants, which typically show correlated regulation of several genes (Steiner and Gerfen, 1993; Willuhn et al., 2003; Yano and Steiner, 2005a,b; Unal et al., 2009), it is likely that modafinil alters the expression of various genes in concert in these brain regions.

Little is known about regional variations in the cortex and striatum or about the cell types and receptors involved. Given that less than 3% of striatal neurons are interneurons (Oorschot, 2010), it is clear that striatal *c-Fos* induction in these studies also predominantly occurred in projection neurons. In most studies, *c-Fos* induction in the dorsal striatum was abundant, while the nucleus accumbens showed only modest (Willie et al., 2005) or no

induction (Scammell et al., 2000; Fiocchi et al., 2009). A recent mapping study in rats used a modafinil dose (10 mg/kg, i.v.) that produced clinically relevant plasma levels and found the most robust increase in *c-Fos* expression in the dorsomedial striatum, with a significant *c-Fos* response also in the nucleus accumbens shell (but not core) and the cingulate cortex (Gozzi et al., 2012).

Based on the mechanisms underlying gene regulation by other dopamine-enhancing drugs, it can be assumed that dopamine receptors are also important for modafinil-induced gene regulation, although this remains to be determined. However, in support of this notion, recent studies showed that D_1 (and D_2) receptors are important for modafinil-induced increases in motivation and arousal (Qu et al., 2008; Young and Geyer, 2010). Future studies will have to elucidate which corticostriatal circuits are affected and identify the mechanisms underlying these molecular changes.

In summary, knowledge on the molecular effects of modafinil in corticostriatal circuits is currently limited, but early findings suggest that this psychostimulant may have the potential to produce effects that are qualitatively similar to those of cocaine, amphetamine, and methylphenidate.

7. Drug interactions: SSRI antidepressants potentiate methylphenidate-induced gene regulation

As discussed, drug-induced gene regulation in neurons critically depends on the neurochemical effects of the drug. In this section we address an aspect of drug treatments that is often overlooked in the assessment of addiction liability: drug interactions based on the neurochemical effects.

If a combination drug treatment results in altered net neurochemical effects, modified gene regulation and thus presumably addiction liability should be expected. Such drug interactions in gene regulation have recently been shown for methylphenidate and certain prescription medications that modify serotonin transmission. Methylphenidate alone increases dopamine overflow but does not affect serotonin (e.g., Kuczenski and Segal, 1997; Borycz et al., 2008; see Section 2.1) and appears to have a reduced propensity to produce neuroadaptations compared with cocaine and amphetamine. In contrast to methylphenidate, cocaine and amphetamine elevate extracellular serotonin levels as well (Yano and Steiner, 2007). Would a combination treatment of methylphenidate with a drug that enhances serotonin action therefore produce more cocaine-/amphetamine-like gene regulation?

A host of findings support this possibility. For example, studies have shown that serotonin contributes significantly to various behavioral effects of cocaine (for reviews, see Filip et al., 2005; Muller and Huston, 2006; Carey et al., 2008). Similarly, whereas dopamine is critical for cocaine-induced gene regulation in the striatum (see Section 3.1.4), serotonin facilitates such effects (Bhat and Baraban, 1993). Thus, attenuation of the serotonin transmission by transmitter depletion (Bhat and Baraban, 1993), receptor antagonism (Lucas et al., 1997; Castanon et al., 2000), or receptor deletion (Lucas et al., 1997) reduces IEG induction by cocaine in the striatum. Conversely, direct and indirect serotonin receptor agonists increase the expression of IEGs (Li and Rowland, 1993; Torres and Rivier, 1994; Wirtshafter and Cook, 1998; Gardier et al., 2000) and other genes (e.g., Mijnster et al., 1998; Morris et al., 1988; Walker et al., 1996) in the striatum.

We therefore investigated whether enhancing serotonin transmission by an SSRI (selective serotonin reuptake inhibitor) antidepressant in conjunction with methylphenidate treatment would modify methylphenidate-induced gene regulation. Our results show that this is indeed the case: adding an SSRI (fluoxetine or citalopram) to methylphenidate treatment potentiates acute induction of IEGs (Steiner et al., 2010; Van Waes et al., 2010), and substance P and dynorphin (but not enkephalin) (Van Waes et al., 2012a) in the striatum (Fig. 4). Moreover, repeated treatment with the methylphenidate + SSRI combination produced potentiated blunting of IEG inducibility and increased dynorphin expression (Van Waes et al., 2012b). This SSRI potentiation of methylphenidate-induced gene regulation was present in most striatal sectors (Fig. 4B) but was maximal in the lateral sensorimotor striatum (Van Waes et al., 2010, 2012a), mimicking cocaine effects. Behaviorally, these SSRIs potentiated methylphenidate-induced locomotion (Borycz et al., 2008) and stereotypies (Van Waes et al., 2010), and produced other behavioral changes (e.g., enhanced sensitivity to cocaine reward and stress-eliciting situations; Warren et al., 2011; see Section 8.1).

The potential significance of these findings relates to the medical use of methylphenidate and SSRIs. SSRIs such as fluoxetine are among the first-line treatments for several depressive and anxiety disorders (Petersen et al., 2002) and are given to millions of patients in the United States alone every year. As discussed, methylphenidate is used both in the treatment of conditions such as ADHD (Biederman et al., 2007; Swanson and Volkow, 2008) and as a recreational drug and cognitive enhancer (Greely et al., 2008; Kollins, 2008; Wilens et al., 2008). The rate of accidental

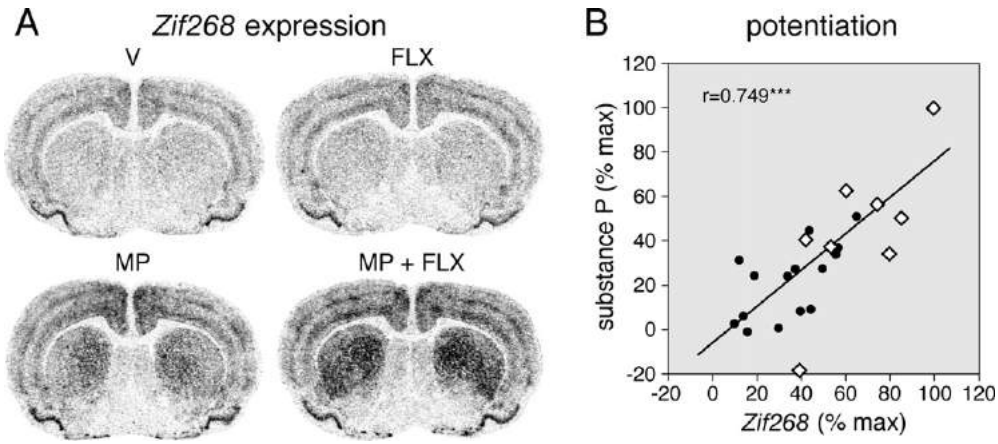

Fig. 4. Fluoxetine potentiates methylphenidate-induced gene regulation. (A) Film autoradiograms depict expression of *Zif268* in the middle striatum for rats that received a single injection of vehicle (V), methylphenidate (MP, 5 mg/kg), fluoxetine (FLX, 5 mg/kg), or a combination of methylphenidate plus fluoxetine (Van Waes et al., 2010). (B) Association between the potentiation of *Zif268* expression (at 40 min; Van Waes et al., 2010) and that of substance P expression (at 90 min after drug injection; Van Waes et al., 2012a) in the 23 striatal sectors (open diamonds, sensorimotor; full circles, non-sensorimotor; data expressed as percentage of maximal increase). Potentiation is the difference between MP + FLX and MP groups. The potentiation was most robust in sectors of the sensorimotor striatum (open diamonds). $^{***}p < 0.001$.

coexposure due to such overlapping drug use/treatments is unclear, but combination therapies of methylphenidate and an SSRI are indicated for several conditions, including ADHD and anxiety/depression comorbidity (Safer et al., 2003; Bhatara et al., 2004; Kollins, 2008). Methylphenidate is also combined with SSRIs as augmentation therapy in major depressive disorder (e.g., Nelson, 2007; Ishii et al., 2008; Ravindran et al., 2008), as acceleration treatment for SSRIs (e.g., Lavretsky et al., 2003), and as treatment for sexual dysfunction related to SSRIs (e.g., Csoka et al., 2008).

Further studies are necessary to determine how much methylphenidate-SSRI coexposure occurs due to clinical administration or as a result of uncontrolled cognitive enhancer use by patients on SSRIs and whether such coexposure enhances the addiction liability of methylphenidate, as the potentiated gene regulation effects might suggest.

8. Behavioral consequences and clinical considerations

While the potential benefits and ethics of cognitive enhancer use are being debated (e.g., Farah et al., 2004; Greely et al., 2008; Chatterjee, 2009; Harris, 2009; Outram, 2010; Hyman, 2011), developmental neurobiologists and addiction researchers warn that the long-term consequences of protracted use of these psychostimulants, especially during brain development, are hardly understood (e.g., Carlezon and Konradi, 2004; Andersen, 2005; Swanson and Volkow, 2008; Berman et al., 2009) (for reviews of neurobehavioral effects of SSRI exposure during development, see, e.g., Oberlander et al., 2009; Olivier et al., 2011). What are the known behavioral consequences of exposure to psychostimulant cognitive enhancers?

8.1. Findings in animal models

Results from animal studies suggest that repeated psychostimulant exposure during preadolescence and adolescence may predispose the individual to substance use or other mental disorders later in life (Brandon et al., 2001; Bolanos et al., 2003; Carlezon et al., 2003; Wiley et al., 2009). It is clear that methylphenidate, for example, produces behavioral changes in animals that mimic those induced by cocaine and amphetamine (for reviews, see Kollins et al., 2001; Carlezon and Konradi, 2004; Kuczenski and Segal, 2005; Yano and Steiner, 2007). Best established is that, similar to cocaine and amphetamine, repeated methylphenidate pretreatment increases locomotor activity/stereotypy levels induced by a subsequent methylphenidate or cocaine/amphetamine challenge ("sensitization") (e.g., Kollins et al., 2001; Yano and Steiner, 2007).

Conditioned place preference (CPP) and drug self-administration in animals are two behavioral models that rank among the most relevant for addiction research. The CPP model determines the conditioned rewarding effects of a drug by assessing whether an animal seeks out/prefers (or avoids) a specific environment in which it previously experienced this drug (Tzschentke, 2007). Psychostimulants typically produce conditioned preference, although high doses can be aversive. Pretreatment with cocaine, for example, either facilitates (e.g., Shippenberg and Heidbreder, 1995) or attenuates (or even produces aversion in) subsequent preference conditioning by cocaine (Carlezon et al., 2003), depending on factors such as the conditioning dose and the age of the animal during the pretreatment. Methylphenidate alone also produces conditioned place preference (e.g., Meririnne et al., 2001; Zhu et al., 2011), and methylphenidate pretreatment in adult rats enhances subsequent preference conditioning by methylphenidate (Meririnne et al., 2001). In contrast, studies have shown that methylphenidate pretreatment in preadolescent rats (postnatal day [PND] 20–35) produces place aversion or attenuates preference conditioning by cocaine (Andersen et al., 2002; Carlezon et al., 2003; Wiley et al., 2009), similar to pretreatment with cocaine (Carlezon et al., 2003).

The latter findings are sometimes interpreted as indicating a protective effect of methylphenidate pretreatment during development against psychostimulant abuse later in life. However, according to recent research (Wiley et al., 2009; Warren et al., 2011), such early-life exposure may result in behavioral abnormalities suggestive of impaired mood functions. These include generally decreased responsiveness to rewarding stimuli (similar to anhedonia; Nestler and Carlezon, 2006) and depression-like states (enhanced sensitivity to anxiety- and stress-inducing situations) (Wiley et al., 2009; Warren et al., 2011). Interestingly, combined treatment with SSRIs appears to enhance some and reverse others of these methylphenidate-induced behavioral deficits (Warren et al., 2011).

Most drugs of abuse are also self-administered by animals, and methylphenidate is no exception (Kollins et al., 2001). Pretreatment with cocaine or amphetamine facilitates the animal's subsequent psychostimulant seeking and self-administration (reviewed in Vezina, 2004). This is also the case for methylphenidate. Thus, repeated methylphenidate pretreatment (i.p.) in preweanling (2 mg/kg, PND 11–20; Crawford et al., 2011), adolescent (2 mg/kg, PND 36–42; Brandon et al., 2001), and adult rats (20 mg/kg; Schenk and Izenwasser, 2002) facilitated subsequent cocaine seeking and self-administration. These findings suggest an enhanced risk for psychostimulant abuse in humans after methylphenidate pretreatment (O'Connor et al., 2011). Use of extended-release formulations to avoid drug spikes associated with immediate-release administration may help reduce molecular changes/neuronal adaptations and related health risks (Gill et al., 2012; but see Thanos et al., 2007).

Future studies will have to clarify whether the apparently contradictory behavioral findings (aversion in the CPP paradigm *vs.* enhanced drug self-administration) are related to age differences (developmental stage) during drug exposure, specifics of drug treatments (e.g., dose), or other experimental variables. Alternatively, given access to cocaine, such rats may be more likely to seek and consume the drug despite a diminished rewarding effect—a characteristic of compulsive drug seeking, which is thought to be mediated by the sensorimotor striatum (Vanderschuren and Everitt, 2005).

8.2. Findings in human studies

Do human studies support an increased risk for drug abuse/addiction (substance use disorder, SUD) after exposure to medical psychostimulants? This question has been investigated in young ADHD patients. Early findings remain equivocal. With the possible exception of an increased risk for smoking, such studies indicated that the risk for SUD was unchanged or even decreased after treatment with psychostimulant medications (e.g., Barkley et al., 2003; Wilens et al., 2003; Kollins, 2008).

However, several issues complicate interpretation of these findings. For one, successful control of symptoms in an ADHD patient likely improves the patient's educational and societal functioning, and resulting socioeconomic advantages may outweigh (and mask) a treatment-inherent biological risk. There are also technical issues. For example, (unmedicated) ADHD patients already show an enhanced risk for SUD (comorbidity; Kollins, 2008), which statistically would be expected to increase the variance, thus favoring the null hypothesis. Another caveat is the often early assessment of outcomes in the clinical studies (a few years after treatment onset in young patients), whereas there is increasing evidence that neurobiological manifestations of early

psychostimulant exposure may appear only later in life (e.g., Bolanos et al., 2003; Tropea et al., 2008; Warren et al., 2011). Thus, conclusions will have to await follow-up studies at an older age (see also Kollins, 2008; Wilens et al., 2008; Berman et al., 2009, for further discussions). Importantly, similar studies in healthy humans who were exposed to psychostimulants, either due to ADHD misdiagnosis or because of cognitive enhancer use, have yet to be conducted.

The reviewed molecular findings indicate that risks may be more serious for abuse of medical psychostimulants. As discussed, the molecular changes induced by psychostimulants are dependent on dose and route of administration (Yano and Steiner, 2007). Proper medical psychostimulant treatment almost always involves oral drug administration, which results in lower drug levels in the brain (Swanson and Volkow, 2003; Kuczenski and Segal, 2005; Carrey and Wilkinson, 2011) and thus a lower risk for neuroadaptations. This contrasts with cognitive enhancer use/abuse. For example, studies show that in recreational settings, intranasal use (snorting of ground-up pills) is not uncommon (e.g., 38.1% prevalence in users among college students; Teter et al., 2006), and intravenous administration also occurs (Parran and Jasinski, 1991; Babcock and Byrne, 2000; Barrett et al., 2005; Teter et al., 2006; White et al., 2006; for a review, see Kollins et al., 2001). These latter routes of administration result in exposure to much faster and higher drug peak levels and are thus expected to have a greater potential for inducing maladaptive neuronal plasticity (Samaha and Robinson, 2005) and enhanced addiction liability. Studies in healthy subjects are needed to evaluate the safety concerns related to cognitive enhancer use and abuse.

9. Conclusions

The findings we have summarized show that psychostimulants such as cocaine and amphetamine produce changes in gene regulation in specific corticostriatal circuits. These effects are most robust in sensorimotor circuits (which are implicated in habit formation and compulsive aspects of drug taking), less pronounced in associative circuits, and more modest in limbic circuits (where they are thought to contribute to altered reward processing in addiction). At the cellular level, psychostimulants alter predominantly neurons of the direct striatal output pathway (''Go pathway''), while the indirect pathway is less affected, and those changes appear to depend on the treatment context (i.e., arousal and associated changes in excitatory striatal input). Overall, these findings support the notion that action selection and initiation are compromised in addiction.

Comparative studies on cognitive enhancers (Adderall, methylphenidate, modafinil) show that they can induce largely similar molecular changes in corticostriatal circuits. Moreover, the same functional domains, cell types, neurotransmitters, and receptors appear to be affected. These effects were mostly explored with high drug doses, but qualitatively similar molecular changes were evident with drug treatments that mimicked medical treatments (oral administration and low drug plasma levels), although it is not clear whether these molecular changes are robust enough to alter behavior. Drug levels associated with cognitive enhancer abuse, however, are likely higher and, with protracted use, likely contribute to molecular changes that increase the addiction liability of these drugs. Further research is necessary to resolve these questions.

Conflicts of interest

There are no conflicts of interest.

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Fluoxetine potentiation of methylphenidate-induced gene regulation in striatal output pathways: Potential role for 5-HT1B receptor

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ABSTRACT

Drug combinations that include the psychostimulant methylphenidate plus a selective serotonin reuptake inhibitor (SSRI) such as fluoxetine are increasingly used in children and adolescents. For example, this combination is indicated in the treatment of attention-deficit/hyperactivity disorder and depression comorbidity and other mental disorders. Such co-exposure also occurs in patients on SSRIs who use methylphenidate as a cognitive enhancer. The neurobiological consequences of these drug combinations are poorly understood. Methylphenidate alone can produce gene regulation effects that mimic addiction-related gene regulation by cocaine, consistent with its moderate addiction liability. We have previously shown that combining SSRIs with methylphenidate potentiates methylphenidate-induced gene regulation in the striatum. The present study investigated which striatal output pathways are affected by the methylphenidate + fluoxetine combination, by assessing effects on pathway-specific neuropeptide markers, and which serotonin receptor subtypes may mediate these effects. Our results demonstrate that a 5-day repeated treatment with fluoxetine (5 mg/kg) potentiates methylphenidate (5 mg/kg)-induced expression of both dynorphin (direct pathway marker) and enkephalin (indirect pathway). These changes were accompanied by correlated increases in the expression of the 5-HT1B, but not 5-HT2C, serotonin receptor in the same striatal regions. A further study showed that the 5-HT1B receptor agonist CP94253 (3–10 mg/kg) mimics the fluoxetine potentiation of methylphenidate-induced gene regulation. These findings suggest a role for the 5-HT1B receptor in the fluoxetine effects on striatal gene regulation. Given that 5-HT1B receptors are known to facilitate addiction-related gene regulation and behavior, our results suggest that SSRIs may enhance the addiction liability of methylphenidate by increasing 5-HT1B receptor signaling.

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1. Introduction

Use of psychotropic medications in children and adolescents is increasing. This is of concern because preclinical studies indicate that such drugs can induce maladaptive neuronal changes suggestive of an increased risk for drug addiction and other neuropsychiatric disorders later in life (for reviews, see Carlezon and Konradi, 2004; Carrey and Wilkinson, 2011; Marco et al., 2011).

The most often used psychotropic drugs in pediatric populations include psychostimulants such as methylphenidate and selective serotonin reuptake inhibitor (SSRI) antidepressants such as fluoxetine. Methylphenidate is widely employed in the treatment of attention-deficit/hyperactivity disorder (ADHD), which is diagnosed in up to 7% of school-age children in the US (DSMMD, 2000; Kollins, 2008). In addition, methylphenidate is increasingly used as a recreational drug or as a so-called cognitive enhancer (Greely et al., 2008) to improve concentration and performance in certain tasks or to study harder (Kollins, 2008; Swanson and Volkow, 2008; Wilens et al., 2008). For example, the 2011 National Survey on Drug Use and Health (NSDUH) reported that approximately 1 million persons age 12 or older in the US admitted current nonmedical use of prescription psychostimulants (SAMHSA, 2012). SSRIs are first-line treatments for major depressive disorder (MDD) and are also helpful to treat anxiety disorders, obsessive compulsive disorder and others. The SSRI fluoxetine is specifically approved for the treatment of pediatric MDD (Iversen, 2006).

While the potential for adverse developmental effects of individual psychotropic drugs is well recognized (see above), possible

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interactions between different drugs have received little attention, despite the fact that co-exposure to more than one drug is quite common. For example, methylphenidate plus SSRI combinations are indicated in the treatment of ADHD/MDD comorbidity (Rushton and Whitmire, 2001; Safer et al., 2003), which occurs in up to 40% of pediatric ADHD cases (Waxmonsky, 2003; Spencer, 2006). Methylphenidate is also added to SSRI treatments, for example, as augmentation therapy in MDD (e.g., Nelson, 2007; Ishii et al., 2008; Ravindran et al., 2008), as acceleration treatment (e.g., Lavretsky et al., 2003), or to treat sexual dysfunction related to SSRIs (e.g., Csoka et al., 2008). It is unknown how much accidental co-exposure occurs in patients on antidepressants who use methylphenidate recreationally or as a cognitive enhancer.

Despite prevalent use, the neurobiological consequences of methylphenidate plus SSRI combination treatments are little understood. The psychostimulant methylphenidate acts by blocking dopamine transporters (Volkow et al., 2002), thus causing dopamine overflow, among other effects, similar to cocaine (Yano and Steiner, 2007). As one consequence of the ensuing dopamine receptor overstimulation (Yano et al., 2006; Alburges et al., 2011), methylphenidate produces altered gene regulation, predominantly in dopamine terminal areas such as the striatum and cortex (Steiner and Van Waes, 2013). However, while methylphenidate has the potential to change the expression of numerous genes (Adriani et al., 2006), other genes that are robustly affected by drugs such as cocaine were minimally or not impacted by methylphenidate treatments (Steiner and Van Waes, 2013). Cocaine, in contrast to methylphenidate (e.g., Kuczenski and Segal, 1997; Segal and Kuczenski, 1999; see Yano and Steiner, 2007), also blocks the serotonin transporter, and serotonin plays a facilitatory role in cocaine-induced gene regulation (e.g., Bhat and Baraban, 1993). Combining methylphenidate (dopamine action) with an SSRI (serotonin action) may thus produce more "cocaine-like" molecular changes than methylphenidate alone.

Our recent series of studies in adolescent rats supports this hypothesis. We showed that adding the SSRIs fluoxetine or citalopram, in doses that by themselves did not affect gene expression, potentiated gene regulation effects of methylphenidate. This potentiation was first shown for acute induction of immediate-early genes (IEGs) such as c-*Fos* and *Zif268* (Steiner et al., 2010; Van Waes et al., 2010), as well as the neuropeptides substance P and dynorphin (Van Waes et al., 2012). However, effects of repeated drug treatments are more relevant for long-term neurobehavioral changes. Repeated treatments with psychostimulants, including methylphenidate, produce several alterations in gene expression, for example, blunting (repression) of IEG induction (Steiner and Van Waes, 2013). We recently showed that IEG blunting is also potentiated by SSRIs. Thus, fluoxetine given in conjunction with methylphenidate for 5 days in adolescent rats potentiated blunting of *Zif268* and *Homer1a* induction by a subsequent cocaine challenge (Van Waes et al., 2013).

In the present study, we assessed the impact of the same repeated treatment on another well-established effect of repeated exposure to psychostimulants, increases in the expression of the opioid peptides dynorphin and enkephalin in the striatum (Steiner and Gerfen, 1998). These neuropeptides are useful cell-type markers due to their differential expression in striatal projection neurons. Neurons of the direct (striatonigral) pathway express dynorphin, whereas neurons of the indirect (striatopallidal) pathway contain enkephalin (Steiner and Gerfen, 1998). Our previous study showed that fluoxetine potentiated acute methylphenidate-induced expression of dynorphin, but not enkephalin (Van Waes et al., 2012), thus suggesting that gene regulation by this drug combination may be restricted to direct pathway neurons. Our present results of repeated combination treatment demonstrate that fluoxetine potentiates gene regulation for both neuropeptides, indicating that indeed both pathways are affected. Moreover, we also addressed the potential underlying mechanisms by investigating associated changes in the expression of serotonin (5-HT) receptor subtypes in the striatum that may mediate these effects. Research shows that 5-HT1B receptor signaling regulates various behavioral responses to cocaine including self-administration (e.g., Parsons et al., 1998; Neumaier et al., 2002; Przegaliński et al., 2004, 2008; Pentkowski et al., 2012; see Neisewander et al., 2014, for review), as well as cocaine-induced gene regulation (e.g., Lucas et al., 1997; Castanon et al., 2000). For comparison, we assessed treatment effects on the 5-HT2C receptor, which also modifies cocaine effects in several ways (Bubar and Cunningham, 2008; Devroye et al., 2013). Our results show increased expression of 5-HT1B by repeated methylphenidate treatment, an effect that is also potentiated by co-treatment with fluoxetine. Furthermore, a role for 5-HT1B in such gene regulation is suggested by our finding that stimulation of 5-HT1B receptors mimics the fluoxetine potentiation of acute *Zif268* induction by methylphenidate.

2. Materials and methods

2.1. Subjects

Male Sprague–Dawley rats (35 days old at the beginning of the drug treatment; Harlan, Madison, WI, USA) were housed 2–3 per cage under standard laboratory conditions (12:12 h light/dark cycle; lights on at 07:00 h) with food and water available ad libitum. Experiments were performed between 13:00 and 17:00 h. Prior to the drug treatment, the rats were allowed one week of acclimation during which they were repeatedly handled. All procedures met the NIH guidelines for the care and use of laboratory animals and were approved by the Rosalind Franklin University Animal Care and Use Committee.

2.2. Drug treatment

In experiment 1, rats received 5 daily injections of vehicle (V, i.p.), methylphenidate HCl (MP, 5 mg/kg; in 0.02% ascorbic acid, 1 ml/kg; Sigma, St. Louis, MO, USA), fluoxetine HCl (FLX, 5 mg/kg; Sigma), or methylphenidate plus fluoxetine (MP + FLX) in their home cage ($n = 6–9$). These rats were killed with CO_2 2 h after the last injection. In experiment 2, rats received an injection of vehicle, or the 5-HT1B receptor agonist CP94253 (CP, 3 or 10 mg/kg; Tocris/R&D Systems, Minneapolis, MN, USA) (Borycz et al., 2008; Przegaliński et al., 2008), followed 15 min later by an injection of vehicle, methylphenidate (5 mg/kg), or methylphenidate plus fluoxetine (5 mg/kg) ($n = 5–9$ each) and were killed 40 min later.

2.3. Tissue preparation and in situ hybridization histochemistry

The brain was rapidly removed, frozen in isopentane cooled on dry ice and then stored at −30 °C until cryostat sectioning. Coronal sections (12 μm) were thaw-mounted onto glass slides (Superfrost/Plus, Daigger, Wheeling, IL, USA), dried on a slide warmer and stored at −30 °C. In preparation for the in situ hybridization histochemistry, the sections were fixed in 4% paraformaldehyde/0.9% saline for 10 min at room temperature, incubated in a fresh solution of 0.25% acetic anhydride in 0.1 M triethanolamine/0.9% saline (pH 8.0) for 10 min, dehydrated, defatted for 2 × 5 min in chloroform, rehydrated, and air-dried. The slides were then stored at −30 °C until hybridization.

Oligonucleotide probes (48-mers; Invitrogen, Rockville, MD, USA) were labeled with [33P]-dATP as described earlier (Steiner and Kitai, 2000). The probes had the following sequence: dynorphin, complementary to bases 862–909, GenBank accession number M10088; enkephalin, bases 436–483, M28263; 5-HT1B (*Htr1b*), bases 62–109, NM022225; 5-HT2C (*Htr2c*), bases 363–410, NM012765; *Zif268* (*Egr1*), bases 352–399, M18416.

One hundred μl of hybridization buffer containing labeled probe (~3×10^6 cpm) was added to each slide. The sections were coverslipped and incubated at 37 °C overnight. After incubation, the slides were first rinsed in four washes of 1× saline citrate (150 mM sodium chloride, 15 mM sodium citrate), and then washed 3 times 20 min each in 2× saline citrate/50% formamide at 40 °C, followed by 2 washes of 30 min each in 1× saline citrate at room temperature. After a brief water rinse, the sections were air-dried and then apposed to X-ray film (BioMax MR-2, Kodak) for 3–14 days.

2.4. Analysis of autoradiograms

Striatal gene expression was assessed in sections from three rostrocaudal levels, rostral (approximately +1.6 mm relative to bregma, Paxinos and Watson, 1998), middle (+0.4) and caudal (−0.8), in a total of 23 sectors (Fig. 1) that are mostly

defined by their predominant cortical inputs (see Willuhn et al., 2003; Yano and Steiner, 2005b). Eighteen of these sectors represent the caudate-putamen and 5 the nucleus accumbens (Fig. 1).

Hybridization signals on film autoradiograms were measured by densitometry (NIH Image; Wayne Rasband, NIMH, Bethesda, MD, USA). The films were captured using a light table (Northern Light, Imaging Research, St. Catharines, Ontario, Canada) and a Sony CCD camera (Imaging Research). The "mean density" value of a region of interest was measured by placing a template over the captured image. Mean densities were corrected for background by subtracting mean density values measured over white matter (corpus callosum). Values from corresponding regions in the two hemispheres were then averaged. The illustrations of film autoradiograms are computer-generated images and are contrast-enhanced. Maximal hybridization signal is black.

2.5. Statistics

Treatment effects were determined by two-factor (experiment 1) or one-factor ANOVAs (experiment 2). Newman–Keuls post hoc tests were used to describe differences between individual groups (Statistica, StatSoft, Tulsa, OK, USA). For the maps illustrating the distribution of changes in gene expression, the difference in signals between a drug treatment group and the vehicle controls was expressed relative to the maximal difference observed for that probe (% max.), for each sector. For the maps showing basal expression of receptors, the signal in each sector was expressed relative to the maximal signal. Changes in neuropeptide expression (dynorphin, enkephalin) across these 23 striatal sectors were compared with those in 5-HT receptor expression (present study) and changes in IEG expression [*Zif268*; as reported before (Van Waes et al., 2010; Van Waes et al., 2013)], by Pearson correlations.

3. Results

3.1. Repeated treatment with methylphenidate + fluoxetine produces increased expression of dynorphin and enkephalin in the striatum

The 5-day repeated treatment (experiment 1) with the moderate dose of methylphenidate (5 mg/kg) alone did not induce changes in dynorphin expression in any of the 23 striatal sectors ($P > 0.05$ for all sectors; Figs. 1A and 2A). Similarly, repeated treatment with fluoxetine (5 mg/kg) alone had no statistically significant effects on the expression of this neuropeptide. In contrast, the drug combination of methylphenidate + fluoxetine produced a widespread increase in the expression of dynorphin (vs. vehicle controls, $P < 0.05$ in 9 of the 18 sectors of the caudate-putamen; Figs. 1A and 2A). This effect occurred on all three rostrocaudal levels, but was most robust in the middle to caudal striatum. On all levels, dynorphin expression was increased in dorsal and lateral (sensorimotor) sectors. On the middle level, a significant increase was also found in the medial and central sectors (Fig. 2A). No effect was seen in the 5 sectors of the nucleus accumbens (Fig. 1A).

Similar but less widespread effects were found for enkephalin expression (Figs. 1B and 2B). Again, neither methylphenidate alone nor fluoxetine alone produced significant changes in expression in the striatum. In contrast, the combination treatment increased enkephalin expression in 3 sectors (medial, dorsal, central) on the middle level (Fig. 2B). Compared to the distribution of the changes in dynorphin expression, the changes in enkephalin expression were thus more centered in the middle and medial striatum (Fig. 1), and there was no significant correlation between dynorphin and enkephalin for "potentiation" (i.e., difference MP + FLX minus MP) in the 23 sectors ($r = 0.148$, $P > 0.05$).

We also compared the distribution of the present changes in dynorphin expression with other neuronal changes induced by acute and repeated methylphenidate + fluoxetine treatment. For example, there was a positive correlation between the potentiation of dynorphin expression after repeated treatment (present results) and the previously reported fluoxetine potentiation of acute IEG (*Zif268*) induction (Van Waes et al., 2010) across the 23 striatal sectors ($r = 0.493$, $P < 0.02$). Moreover, our correlation analysis (Fig. 3) demonstrates that the potentiation of *Zif268* blunting found

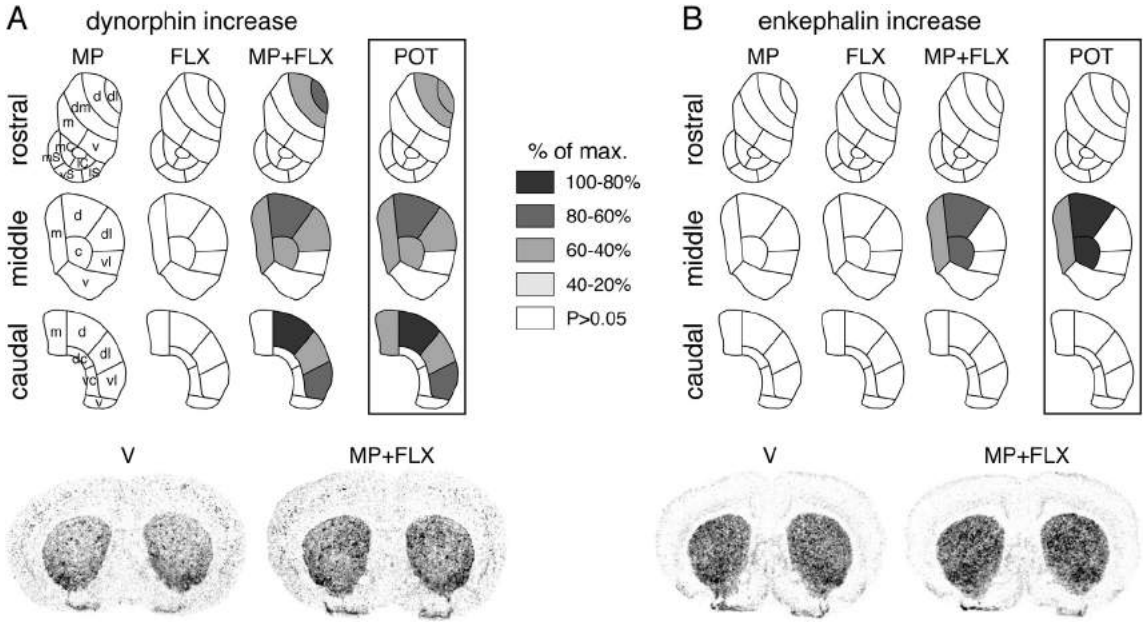

Fig. 1. Topography of potentiated neuropeptide expression in the striatum after repeated methylphenidate plus fluoxetine treatment. Maps depict the distribution of the increases (vs. V) in dynorphin (A) and enkephalin expression (B) in the rostral, middle and caudal striatum after 5 daily injections of methylphenidate (5 mg/kg, i.p.; MP), fluoxetine (5 mg/kg; FLX) or methylphenidate + fluoxetine (5 mg/kg each; MP + FLX). Potentiation (POT) denotes the difference between methylphenidate + fluoxetine and methylphenidate groups. The increases are expressed relative to the maximal increase for each neuropeptide (% of max.). Sectors with significant differences [vs. vehicle (V) controls, or methylphenidate + fluoxetine vs. methylphenidate (POT)] ($P < 0.05$) are coded as indicated. Sectors without significant effects are in white. Illustrations of film autoradiograms depicting the expression of dynorphin (left) and enkephalin (right) in coronal sections from the middle striatum after repeated treatment with vehicle (V) or methylphenidate + fluoxetine (MP + FLX) are shown below the maps. Abbreviations: caudate-putamen: c, central; d, dorsal*; dc, dorsal central; dl, dorsolateral*; dm, dorsomedial; m, medial; v, ventral; vc, ventral central; vl, ventrolateral*; nucleus accumbens: mC, medial core; lC, lateral core; mS, medial shell; vS, ventral shell; lS, lateral shell; *sensorimotor sectors (see Yano and Steiner, 2005a).

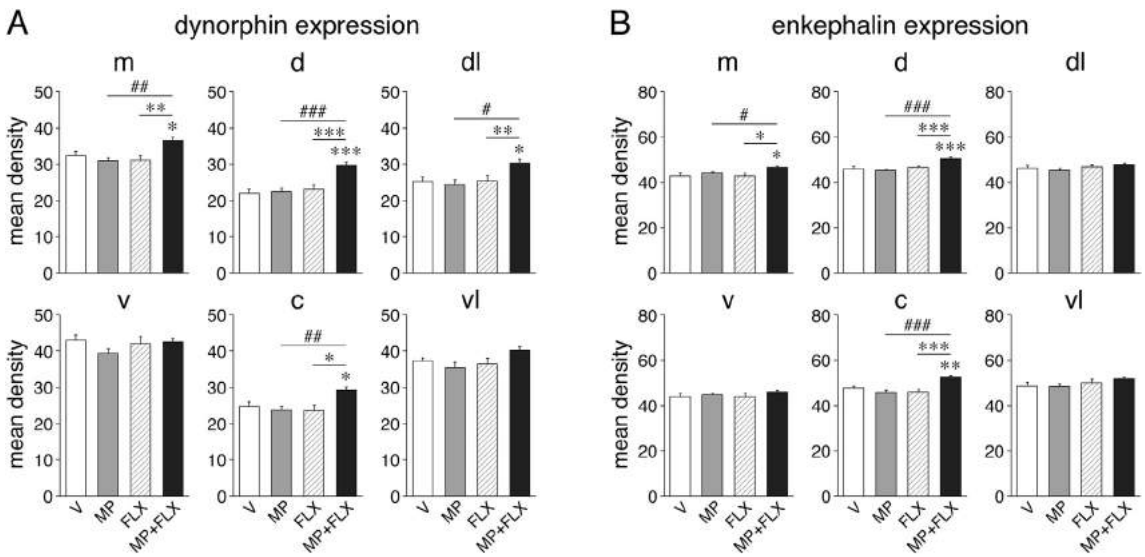

Fig. 2. Potentiation of striatal neuropeptide expression after repeated methylphenidate plus fluoxetine treatment. The changes in dynorphin (A) and enkephalin expression (B) in the 6 sectors of the middle striatum are shown. Mean density values (mean ± SEM, arbitrary units) are given for rats that received 5 daily injections of vehicle (V), methylphenidate (5 mg/kg; MP), fluoxetine (5 mg/kg; FLX), or methylphenidate + fluoxetine (MP + FLX) ($n = 6–9$ per group). Sectors: m, medial; d, dorsal; dl, dorsolateral; v, ventral; c, central; vl, ventrolateral. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. V controls or as indicated; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, MP + FLX vs. MP ("potentiation").

after repeated methylphenidate + fluoxetine treatment (Van Waes et al., 2013) was also positively correlated with the present increases in dynorphin expression across the 23 striatal sectors ($r = 0.591$, $P < 0.005$). These molecular changes thus had a similar regional distribution across the striatum. Robust changes preferentially occurred in, but were not limited to, sectors of the sensorimotor striatum (Fig. 3).

3.2. Distribution of 5-HT1B and 5-HT2C receptor expression in the striatum

The distribution of 5-HT1B and 5-HT2C receptor mRNAs in striatum and nucleus accumbens was determined in vehicle-treated controls and is depicted in Fig. 4. The distribution of 5-HT1B expression (Fig. 4A) is fairly uniform throughout the rostral, middle and caudal striatum, with somewhat higher levels in the lateral striatum that peak on the middle level (lateral half of dorsolateral and ventrolateral sectors; Fig. 4A). In the nucleus accumbens (rostral level), the highest 5-HT1B expression is seen in the lateral part of the shell (Fig. 4A).

In marked contrast, the distribution of 5-HT2C mRNA in the striatum is distinctly uneven (Fig. 4B). Overall, there is a rostrocaudal gradient, with high expression rostrally that fades towards background levels in most of the caudal striatum. The expression is considerably higher in ventral and medial (limbic, associative) than in dorsal/lateral (sensorimotor) sectors. On all three rostrocaudal levels, there are distinct small areas of high-density labeling present, probably reflecting the patches of the patch/matrix compartments (Eberle-Wang et al., 1997). In the nucleus accumbens, 5-HT2C expression is by far highest in the rostral pole (data not shown), fairly high in the medial shell and medial core and lowest in the lateral shell (Fig. 4B).

3.3. Fluoxetine potentiates repeated methylphenidate-induced increases in the expression of 5-HT1B, but not 5-HT2C

The present drug treatments (experiment 1) had differential effects on 5-HT1B vs. 5-HT2C expression in the striatum (Figs. 5 and 6). The 5-day repeated treatment with methylphenidate alone produced significantly increased 5-HT1B expression in 6 sectors of the caudate-putamen (Fig. 5A), with similar tendencies in several additional sectors (Fig. 6A). This effect occurred in the middle and caudal striatum and was maximal in the dorsal sector on the caudal level (Fig. 5A). No changes in 5-HT1B expression were seen in the nucleus accumbens after repeated methylphenidate treatment (Fig. 5A).

In contrast to methylphenidate, repeated treatment with fluoxetine alone did not alter 5-HT1B expression in any of these striatal sectors (Figs. 5A and 6A). However, fluoxetine given in conjunction with methylphenidate potentiated methylphenidate-

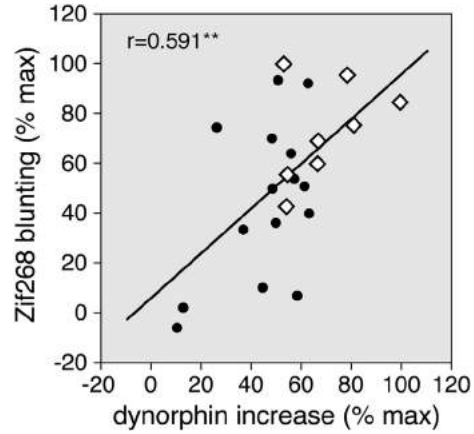

Fig. 3. Striatal distribution of the potentiation of increases in dynorphin expression (present study) vs. that of blunting of *Zif268* induction (Van Waes et al., 2013) after a 5-day repeated methylphenidate plus fluoxetine treatment. The scatterplot shows the correlation between the present dynorphin potentiation (MP + FLX minus MP) and the previously reported potentiation of *Zif268* blunting in the 23 striatal sectors ($r = 0.591$; open diamonds, sensorimotor sectors; full circles, non-sensorimotor sectors). The data are expressed as the percentage of the maximal potentiation for each gene. Values for dynorphin expression were obtained 2 h after the last injection (present study); those for *Zif268* blunting represent values of *Zif268* induction by a cocaine (25 mg/kg) challenge 24 h after a 5-day repeated methylphenidate plus fluoxetine treatment (Van Waes et al., 2013). Potentiation was most robust in sectors of the sensorimotor striatum (open diamonds). $^{**}P < 0.005$.

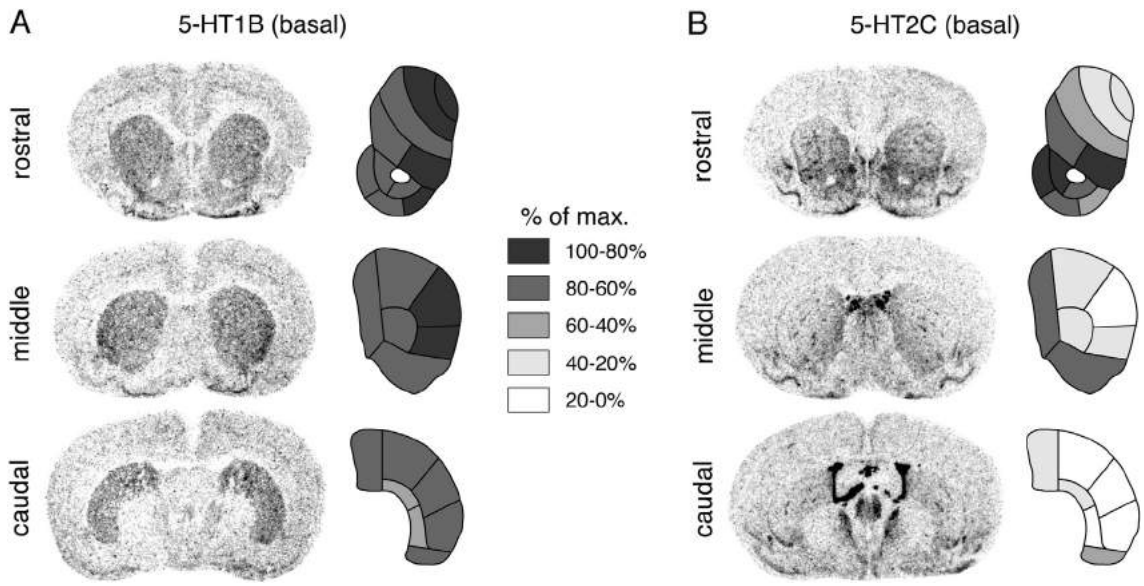

Fig. 4. Expression of 5-HT1B (A) and 5-HT2C (B) receptor mRNAs in the striatum. Illustrations of film autoradiograms (left) depict "basal" 5-HT1B and 5-HT2C expression (i.e., in vehicle-treated rats, $n = 6$) in coronal sections from the rostral, middle and caudal striatum. The maps (right) show the distribution of gene expression across the 23 striatal sectors. Values are given as percentages of maximal expression and are coded as indicated. The maximal hybridization signal is black.

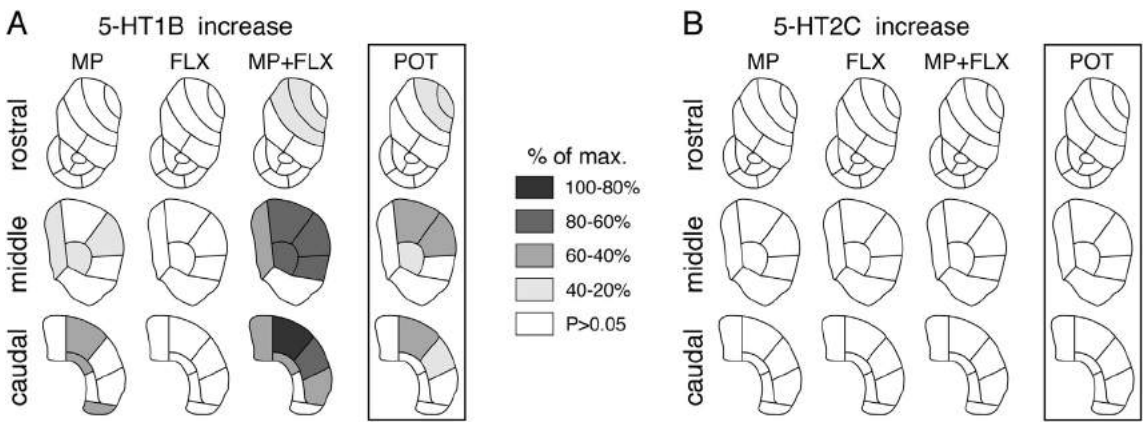

Fig. 5. Topography of changes in 5-HT receptor expression in the striatum after repeated methylphenidate plus fluoxetine treatment. Maps depict the distribution of the increases (vs. vehicle controls, V) in 5-HT1B expression (A) and the lack of changes in 5-HT2C expression (B) in the rostral, middle and caudal striatum after the 5-day treatment with methylphenidate (5 mg/kg, i.p.; MP), fluoxetine (5 mg/kg; FLX) or methylphenidate + fluoxetine (5 mg/kg each; MP + FLX). The potentiation (POT) denotes the difference between methylphenidate + fluoxetine and methylphenidate alone. The differences are expressed relative to the maximal difference for each gene (% of max.). Sectors with significant differences ($P < 0.05$) are coded as indicated. Sectors without significant effects are in white.

induced increases in 5-HT1B expression (Figs. 5A and 6A). Thus, the combination treatment (MP + FLX) produced significantly increased expression (vs. vehicle controls) in 12 of the 18 sectors of the caudate-putamen (compared to 6 sectors for MP alone) and in none of the nucleus accumbens (Fig. 5A). Moreover, MP + FLX animals displayed significantly higher levels of expression than MP animals (potentiation) in 7 sectors. Increased 5-HT1B expression again occurred on all three rostrocaudal levels, but predominantly on middle and caudal levels (Fig. 5A). Increases were present in medial to lateral sectors, but they were most robust dorsally and laterally. Thus, potentiation of 5-HT1B expression was most pronounced in the dorsal/dorsolateral striatum on middle and caudal levels (Fig. 5A).

We also compared the regional distribution of these drug-induced changes in 5-HT1B expression with those in dynorphin and enkephalin expression, by correlation analysis. There was a significant positive correlation between the potentiation of increases in 5-HT1B expression and that of dynorphin expression ($r = 0.634$, $P < 0.002$; Fig. 7), but not that of enkephalin expression ($r = 0.378$, $P > 0.05$). Overall, this analysis confirmed preferential potentiation of changes in 5-HT1B and dynorphin expression in the sensorimotor striatum (Fig. 7).

In contrast to 5-HT1B expression, 5-HT2C expression was not affected by these drug treatments. Neither methylphenidate alone, nor fluoxetine alone or the methylphenidate + fluoxetine combination altered 5-HT2C expression in any of these striatal sectors ($P > 0.05$; Figs. 5B and 6B).

3.4. 5-HT1B receptor stimulation potentiates acute methylphenidate-induced expression of Zif268

Experiment 2 assessed whether the 5-HT1B receptor could modify methylphenidate-induced gene regulation. Our results show that the 5-HT1B receptor agonist CP94253 (3–10 mg/kg)

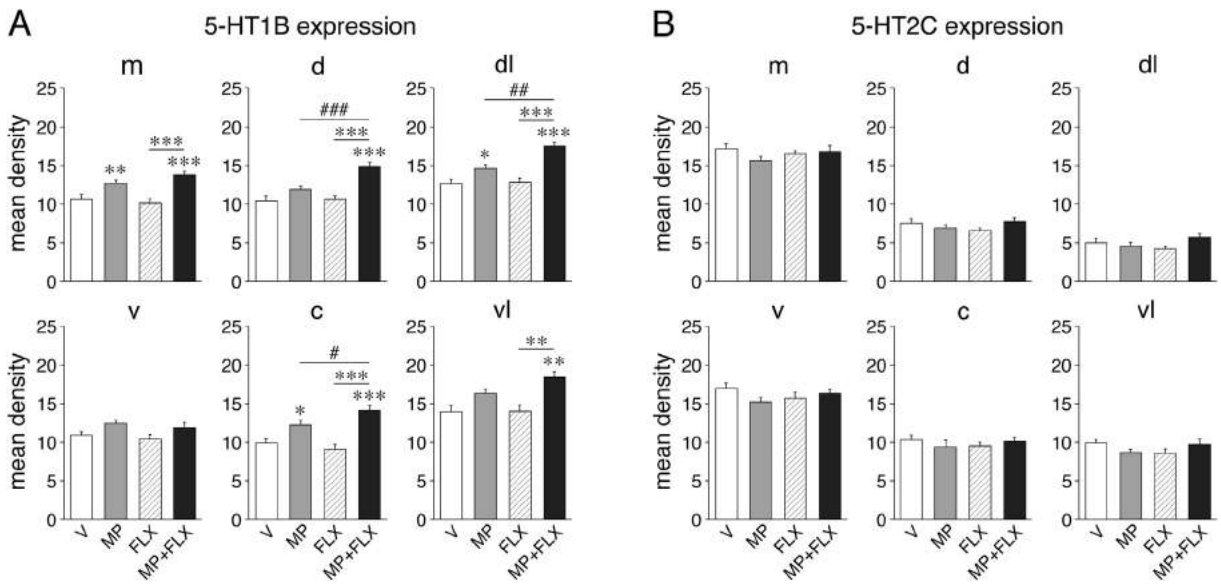

Fig. 6. Changes in striatal 5-HT receptor expression after repeated methylphenidate plus fluoxetine treatment. The changes in 5-HT1B expression (A) and the lack of changes in 5-HT2C expression (B) in the 6 middle striatal sectors are shown. Mean density values (mean ± SEM) are given for rats that received 5 daily injections of vehicle (V), methylphenidate (5 mg/kg; MP), fluoxetine (5 mg/kg; FLX), or methylphenidate + fluoxetine (MP + FLX) ($n = 6–9$ per group). Sectors: m, medial; d, dorsal; dl, dorsolateral; v, ventral; c, central; vl, ventrolateral. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. V controls or as indicated; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, MP + FLX vs. MP (potentiation).

indeed potentiated acute methylphenidate-induced expression of *Zif268* in the striatum in a dose-dependent manner (Fig. 8). This effect was present in many striatal regions, but was maximal in the lateral striatum. Stimulating 5-HT1B receptors thus mimicked fluoxetine effects on gene regulation, consistent with a role for 5-HT1B in the fluoxetine potentiation of methylphenidate-induced gene regulation.

4. Discussion

The goal of the present study was to determine whether repeated combination treatment with methylphenidate plus fluoxetine would produce potentiated changes in gene regulation in both striatal output pathways, by assessing effects on the cell-type markers dynorphin and enkephalin. We here demonstrate that the 5-day repeated treatment with methylphenidate alone or fluoxetine alone with the present subthreshold doses had no effect on either neuropeptide, but that the combined treatment produced increases in the expression for both markers. These results indicate that gene regulation in both pathways is affected by this combination treatment. In addition, we show that these increases in neuropeptide expression were associated with increases in the expression of the 5-HT1B, but not 5-HT2C, receptor subtype in the same striatal regions, and that 5-HT1B receptor stimulation mimicked fluoxetine effects on gene regulation.

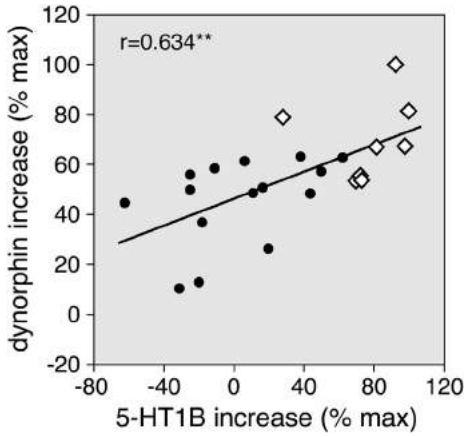

Fig. 7. Striatal distribution of the potentiation of increases in 5-HT1B expression vs. that in dynorphin expression after the 5-day repeated methylphenidate plus fluoxetine treatment. The scatterplot depicts the correlation between the 5-HT1B potentiation and the dynorphin potentiation (MP + FLX minus MP) in the 23 striatal sectors ($r = 0.634$). The data are expressed as the percentage of the maximal potentiation for each gene. Potentiation was most robust in sectors of the sensorimotor striatum (open diamonds). $^{**}P < 0.002$.

4.1. Fluoxetine potentiation of gene regulation by methylphenidate in the striatum: pathways affected and potential significance

Illicit psychostimulants such as cocaine and amphetamine alter gene regulation in the striatum preferentially in the subtype of medium spiny projection neurons that express D1 dopamine receptors and project to the substantia nigra/internal pallidum (direct pathway) (Steiner, 2010; Lobo and Nestler, 2011; Steiner and Van Waes, 2013). Gene regulation in the projection neurons that target the globus pallidus (external pallidum) and contain mostly D2 receptors (first link of the indirect pathway) is also affected, but generally less, and these effects tend to be more context-dependent (Steiner, 2010). This selectivity, confirmed with various double-labeling approaches (Steiner, 2010), was first demonstrated by studies that assessed drug actions on neuropeptide markers that are differentially localized in the two striatal output pathways (Steiner and Gerfen, 1998). Direct pathway neurons predominantly express the neuropeptides substance P and dynorphin, whereas indirect pathway neurons express enkephalin. While not absolute, the principal segregation of these neuropeptides between the two pathways has been demonstrated by a variety of molecular techniques (e.g., Gerfen and Young, 1988; Gerfen et al., 1990, 1991; Surmeier et al., 1996; Heiman et al., 2008).

Numerous studies have shown that drugs such as cocaine and amphetamine produce pronounced increases in the expression of substance P and dynorphin (direct pathway), while expression of

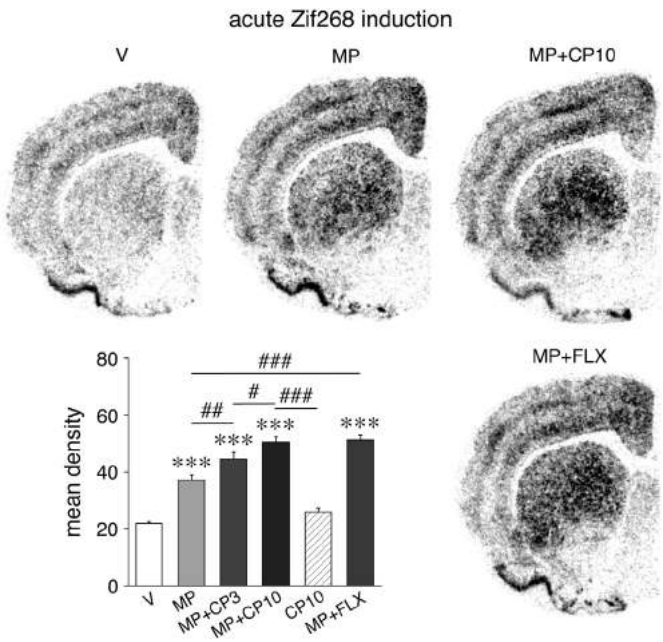

Fig. 8. Stimulation of 5-HT1B receptors potentiates acute induction of *Zif268* by methylphenidate. Illustrations of film autoradiograms depict *Zif268* expression in the middle striatum in rats that were treated with vehicle (V) (upper left), methylphenidate (5 mg/kg, MP) (upper middle), and methylphenidate (5 mg/kg) plus 5-HT1B agonist CP94253 (10 mg/kg, MP + CP10) (upper right) and were killed 40 min later. For comparison, *Zif268* expression after methylphenidate plus fluoxetine (5 mg/kg each, MP + FLX) treatment (lower right) is also shown. The graph lower left presents mean density values (mean ± SEM) in the dorsolateral and ventrolateral sectors pooled for rats that were treated with vehicle, methylphenidate and/or CP94253 (3 or 10 mg/kg), or methylphenidate plus fluoxetine ($n = 5–9$). $^{***}P < 0.001$ vs. V controls; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, as indicated.

enkephalin (indirect pathway) is increased to a considerably lesser extent (see Yano and Steiner, 2007; Steiner, 2010; for reviews). [Note that, in contrast, enkephalin expression is very responsive to antipsychotic/D2 receptor antagonist treatment or dopamine loss (Steiner and Gerfen, 1998)]. Importantly, increased dynorphin mRNA and peptide levels have also been demonstrated in human cocaine abusers (Hurd and Herkenham, 1993; Frankel et al., 2008).

In our previous studies, we found that acute and repeated treatment with methylphenidate induced robust changes in substance P expression (Brandon and Steiner, 2003; Yano and Steiner, 2005a; Van Waes et al., 2012). Dynorphin expression was marginally affected by acute methylphenidate (Yano and Steiner, 2005a; Van Waes et al., 2012), but increased levels of striatal dynorphin mRNA (Brandon and Steiner, 2003) and dynorphin peptide in striatum and substantia nigra (Alburges et al., 2011) were found after repeated treatment with high doses of methylphenidate (10 mg/kg). In contrast, in these studies, enkephalin expression was minimally or not altered (Brandon and Steiner, 2003; Yano and Steiner, 2005a; Van Waes et al., 2012). These differential effects between the two pathways are consistent with findings by others. For example, Kim et al. demonstrated that repeated methylphenidate treatment increased deltaFosB expression in direct, but not in indirect, pathway neurons, as shown by double-labeling techniques (Kim et al., 2009). These findings thus indicate that methylphenidate exposure alone more selectively affects the direct pathway than cocaine or amphetamine do.

Our more recent studies show that adding an SSRI (serotonin action) to methylphenidate (dopamine action) potentiates striatal gene regulation by methylphenidate (see Introduction; Steiner and Van Waes, 2013). This effect is robust in direct pathway neurons, but, as indicated by our neuropeptide marker in the present study, also occurs in indirect pathway neurons with repeated treatment. Thus, repeated methylphenidate plus fluoxetine treatment increases the expression of dynorphin and, to some lesser extent, also enkephalin. This pattern is reminiscent of the effects of cocaine and amphetamine, which have significant effects (if more moderate compared with dynorphin) also on enkephalin expression (e.g., Hurd and Herkenham, 1993; Steiner and Gerfen, 1993; Wang and McGinty, 1996; Spangler et al., 1997). In this sense, the methylphenidate plus fluoxetine combination treatment produces more "cocaine-like" gene regulation than methylphenidate alone.

The present findings also confirm and extend our earlier observations (Van Waes et al., 2010; Van Waes et al., 2012; Van Waes et al., 2013) that this drug-induced gene regulation predominantly occurs in, but is not limited to, sensorimotor sectors mostly in the middle to caudal striatum. Among the affected non-sensorimotor sectors are medial and central striatal sectors that receive inputs from the prefrontal and cingulate cortex (see Willuhn et al., 2003; Steiner and Van Waes, 2013) and are important for goal-directed behavior. The lateral (sensorimotor) striatum, on the other hand, is critical for 'automatic' and habitual behavior (Yin and Knowlton, 2006). Psychostimulant-induced changes in gene regulation (and resulting structural plasticity; Jedynak et al., 2007) in lateral striatal circuits are implicated in habitual and compulsive aspects of drug addiction (Berke and Hyman, 2000; Everitt et al., 2001; Everitt and Robbins, 2013) as well as in relapse to drug taking after abstinence (Vanderschuren et al., 2005; Fuchs et al., 2006; See et al., 2007).

Although overlapping, the regional distribution of methylphenidate plus fluoxetine-induced changes in enkephalin expression was not identical to that of dynorphin expression (not correlated). The basis for this apparent dissociation in distribution (and magnitude) is presently unknown. However, there are various differences in the regulation of gene expression in neurons of these two pathways. These include the dopamine receptors involved (D1 vs. D2) and their respective second messenger signaling pathways, a differential sensitivity to and possibly origin of cortical inputs that drive these changes, differential thalamic inputs and others (see Steiner, 2010; Van Waes et al., 2012; for discussion). For example, stimulation of D1 receptors (direct pathway neurons) facilitates gene regulation driven by cortical (or thalamic) inputs in these neurons, while stimulation of D2 receptors (indirect pathway neurons) dampens such gene regulation in those neurons (Steiner, 2010). This latter effect may account for the more limited changes in enkephalin expression, especially in the lateral striatum, where D2 receptor levels are considerably higher than in the medial striatum (see Steiner and Gerfen, 1999). Future studies will have to elucidate the exact mechanisms involved.

The functional consequences of increased dynorphin and enkephalin expression after psychostimulant treatments remain to be established. However, dynorphin and enkephalin are neurotransmitters released from these neurons. There is evidence that both neuropeptides act, at least in part, as negative feedback systems ('brake') (Steiner and Gerfen, 1998) to limit dopamine and glutamate (e.g., Atwood et al., 2014) input to striatal neurons and help maintain systems homeostasis (Hyman and Nestler, 1996; Steiner and Gerfen, 1998; Steiner, 2010). Increased dynorphin function in these neurons has been implicated in addiction processes. Thus, there is good evidence that dynorphin inhibits dopamine release via kappa opioid receptors on dopamine terminals and dendrites/cell bodies (e.g., Di Chiara and Imperato, 1988; Spanagel et al., 1992; Marinelli et al., 1998; see Shippenberg et al., 2007; for review). Increased dynorphin signaling after psychostimulant treatments may thus excessively inhibit such inputs to the striatum (Hyman and Nestler, 1996; Steiner and Gerfen, 1998; Shippenberg et al., 2007). Based on these and other findings, it

has been proposed that increased dynorphin function (in the ventral striatum) may contribute to somatic signs of withdrawal, such as dysphoria, anxiety, anhedonia and depression (Nestler and Carlezon, 2006; Shippenberg et al., 2007). The behavioral consequences of increased dynorphin (and enkephalin) signaling in the dorsal striatum remain to be investigated.

4.2. Role for the 5-HT1B receptor in the fluoxetine potentiation?

In the present study, we started to investigate the mechanisms underlying the fluoxetine potentiation of methylphenidate-induced gene regulation, by assessing a potential role for specific serotonin receptor subtypes. It is clear that dopamine is critical for gene regulation by psychostimulants such as cocaine (Steiner and Van Waes, 2013) as well as methylphenidate (Yano et al., 2006; Alburges et al., 2011). However, serotonin facilitates these effects of cocaine. For example, it has been shown that attenuation of the serotonin neurotransmission by transmitter depletion (Bhat and Baraban, 1993) or receptor antagonism (e.g., Lucas et al., 1997; Castanon et al., 2000) reduces IEG induction by cocaine in the striatum. A similar effect has been demonstrated for cocaine action on striatal neuropeptide expression (Morris et al., 1988; Walker et al., 1996; Horner et al., 2005).

Serotonin is known to enhance activity in the mesostriatal and mesolimbic dopamine pathways by complex interactions in both the dopamine terminal regions and the somatodendritic areas in the midbrain (for reviews, see Muller and Huston, 2006; Weikop et al., 2007; Bubar and Cunningham, 2008). Therefore, the SSRI potentiation of methylphenidate-induced gene regulation in the striatum could reflect potentiated dopamine action mediated by serotonin receptors in the striatum and/or other brain areas. It is unclear that dopamine neurons express serotonin receptors (Hoyer et al., 1994; Barnes and Sharp, 1999), so these interactions are likely indirect. For example, several serotonin receptor subtypes are expressed by striatal projection neurons themselves (Hoyer et al., 1994; Barnes and Sharp, 1999). Among the most highly expressed are 5-HT1B and 5-HT2C. We first investigated whether the effects of the repeated methylphenidate + fluoxetine treatment on the neuropeptide expression were associated with the distribution of and/or changes in 5-HT1B or 5-HT2C expression.

Expression of 5-HT2C (formerly named 5-HT1C) shows a distinctly uneven distribution throughout the striatum, with a rostrocaudal gradient and preferential expression in medial and ventral ("limbic") regions (e.g., Mengod et al., 1990; Eberle-Wang et al., 1997). This distribution thus does not match the observed distribution of the fluoxetine potentiation of methylphenidate-induced gene regulation in our studies (most robust in dorsal/lateral, sensorimotor regions). Furthermore, our present results show that 5-HT2C expression was not affected by either drug treatment.

In contrast, in agreement with previous studies (e.g., Voigt et al., 1991; Bruinvels et al., 1994), we found that the distribution of 5-HT1B expression is relatively homogeneous throughout the striatum. There is a somewhat higher expression in the lateral striatum on the middle level (Voigt et al., 1991), roughly matching the localization of maximally potentiated IEG expression after acute methylphenidate + fluoxetine treatment (Van Waes et al., 2010).

Moreover, we here demonstrate for the first time that methylphenidate also alters 5-HT1B expression in the striatum. The 5-day repeated treatment with methylphenidate (5 mg/kg) alone was sufficient to increase the expression of 5-HT1B, mostly in the middle to caudal striatum. In contrast, fluoxetine (5 mg/kg, 5 days) alone had no effect [note that a more aggressive treatment (8 mg/kg, 21 days) did increase striatal 5-HT1B expression (Le Poul et al., 2000)]. Adding fluoxetine (5 mg/kg) to methylphenidate (5 mg/kg), however, potentiated the methylphenidate-induced increases. Overall, this potentiation of 5-HT1B expression occurred in the same striatal regions as (i.e., was correlated with) the potentiation of dynorphin expression (maximal in sensorimotor striatum).

These findings of methylphenidate + fluoxetine-induced increases in 5-HT1B expression are consistent with previous studies showing increased 5-HT1B expression in the striatum after repeated cocaine exposure (Hoplight et al., 2007; Neumaier et al., 2009). Increased 5-HT1B expression thus represents a further example of mimicked cocaine effects of the methylphenidate + fluoxetine exposure. In summary, both basal distribution and our drug-induced changes for 5-HT1B expression are consistent with a role for striatal 5-HT1B in the fluoxetine effects on methylphenidate-induced gene regulation.

We therefore further investigated whether 5-HT1B receptor stimulation could modify methylphenidate-induced gene regulation. Indeed, our results show that the 5-HT1B receptor agonist CP94253 potentiated acute *Zif268* induction by methylphenidate, and that this effect was maximal in the lateral striatum. 5-HT1B receptor activation thus mimicked the fluoxetine effects on gene regulation. These findings are the first to demonstrate that 5-HT1B receptors can facilitate methylphenidate-induced gene regulation. They extend previous findings showing that 5-HT1B receptor stimulation enhances methylphenidate-induced locomotor activity (Borycz et al., 2008). Overall, these findings are consistent with previous results demonstrating that 5-HT1B receptors contribute to cocaine-induced gene expression (Lucas et al., 1997; Castanon et al., 2000) and regulate behavioral responses to cocaine (e.g., Neisewander et al., 2014).

At the cellular level, the 5-HT1B receptor subtype is predominantly located on axon terminals to regulate (inhibit) neurotransmitter release (Boschert et al., 1994). There is evidence that 5-HT1B receptors expressed by direct pathway (striatonigral) neurons mediate serotonin-induced inhibition of GABA release from their terminals, and that this effect results in disinhibition of mesostriatal dopamine neurons and increased striatal dopamine release (c.f. Castanon et al., 2000; Hoplight et al., 2007). Alternatively, 5-HT1B-mediated inhibition of GABA release from local striatal axon terminals of striatal projection neurons (Gerfen and Bolam, 2010) may directly disinhibit striatal neurons. Either mechanism could thus be expected to produce potentiated (disinhibited) gene induction in striatal neurons. However, given their fairly widespread distribution in the brain (e.g., cortex; Bruinvels et al., 1994), it is conceivable that 5-HT1B signaling in other brain areas might contribute to the SSRI potentiation of methylphenidate-induced gene regulation in the striatum. Future studies with local experimental manipulations will have to clarify which 5-HT1B receptors are involved.

5. Conclusion

Repeated methylphenidate-induced changes in gene regulation in striatal circuits (Brandon and Steiner, 2003; see Steiner and Van Waes, 2013; for review) are associated with a facilitation of subsequent cocaine seeking and taking in the cocaine self-administration model (Brandon et al., 2001; Schenk and Izenwasser, 2002; Crawford et al., 2011). Potentiated gene regulation by fluoxetine may thus enhance this effect. It has been shown that methylphenidate + fluoxetine co-exposure in juvenile rats enhances their sensitivity to cocaine and natural reward (among other behavioral effects) in adulthood (Warren et al., 2011). Future studies will have to determine whether such co-exposure to fluoxetine, either in the treatment of mental disorders or, more likely, during medication abuse which typically involves higher-level drug exposure (Steiner and Van Waes, 2013), will increase

the abuse/addiction liability of methylphenidate, and whether the 5-HT1B receptor may offer a pharmacological target to attenuate these effects.

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CB1 cannabinoid receptor expression in the striatum: association with corticostriatal circuits and developmental regulation

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Corticostriatal circuits mediate various aspects of goal-directed behavior and are critically important for basal ganglia-related disorders. Activity in these circuits is regulated by the endocannabinoid system via stimulation of CB1 cannabinoid receptors. CB1 receptors are highly expressed in projection neurons and select interneurons of the striatum, but expression levels vary considerably between different striatal regions (functional domains). We investigated CB1 receptor expression within specific corticostriatal circuits by mapping CB1 mRNA levels in striatal sectors defined by their cortical inputs in rats. We also assessed changes in CB1 expression in the striatum during development. Our results show that CB1 expression is highest in juveniles (P25) and then progressively decreases toward adolescent (P40) and adult (P70) levels. At every age, CB1 receptors are predominantly expressed in sensorimotor striatal sectors, with considerably lower expression in associative and limbic sectors. Moreover, for most corticostriatal circuits there is an inverse relationship between cortical and striatal expression levels. Thus, striatal sectors with high CB1 expression (sensorimotor sectors) tend to receive inputs from cortical areas with low expression, while striatal sectors with low expression (associative/limbic sectors) receive inputs from cortical regions with higher expression (medial prefrontal cortex). In so far as CB1 mRNA levels reflect receptor function, our findings suggest differential CB1 signaling between different developmental stages and between sensorimotor and associative/limbic circuits. The regional distribution of CB1 receptor expression in the striatum further suggests that, in sensorimotor sectors, CB1 receptors mostly regulate GABA inputs from local axon collaterals of projection neurons, whereas in associative/limbic sectors, CB1 regulation of GABA inputs from interneurons and glutamate inputs may be more important.

Keywords: CB1 cannabinoid receptor, corticostriatal, development, gene expression, striatum

INTRODUCTION

Anatomical circuits interconnecting the cerebral cortex and the basal ganglia are critical for the organization of goal-directed behavior, and dysfunction in these circuits is associated with numerous brain disorders, ranging from movement disorders to obsessive compulsive disorder, schizophrenia, and drug addiction, depending on the particular circuits affected (e.g., Albin et al., 1989; DeLong, 1990; Hyman and Nestler, 1996; Graybiel and Rauch, 2000; Steiner, 2010). Cortico-basal ganglia circuits arise from all parts of the cortex and project in a topographical manner to the striatum (caudate–putamen, nucleus accumbens), and from there, via basal ganglia output nuclei and thalamus, back to the cortex (Alexander et al., 1986, 1990; Albin et al., 1989; Groenewegen et al., 1990; Joel and Weiner, 1994). Activity within these circuits is modulated by a variety of G-protein-coupled receptors. Among these, the G_i-protein-coupled CB1 cannabinoid receptor is of highest abundance (Herkenham et al., 1990, 1991b). CB1 receptors are the main target for endocannabinoids (fatty acid signaling molecules) and mediate the pharmacological actions of cannabinoid drugs (for reviews, see Kreitzer and Regehr, 2002; Freund et al., 2003; Szabo and Schlicker, 2005; Lovinger et al., 2010). In the basal ganglia, CB1 receptors are predominantly expressed by striatal neurons, and are heavily localized on axon terminals within the striatum, as well as in target nuclei of striatal projection neurons, the substantia nigra and globus pallidus (Herkenham et al., 1991a,b; Mailleux and Vanderhaeghen, 1992; Matsuda et al., 1993). Lower levels of CB1 receptors are also present throughout the cortex (Herkenham et al., 1991b; Mailleux and Vanderhaeghen, 1992; Matsuda et al., 1993; Heng et al., 2011), including in corticostriatal neurons (Uchigashima et al., 2007).

Research over the last decade has shown that endocannabinoid/CB1 receptor signaling exerts powerful inhibitory effects on both glutamatergic and GABAergic synaptic transmission in the basal ganglia and many other brain regions. Typically, endocannabinoids are formed in activated neurons, released from the postsynaptic membrane, and diffuse to and stimulate CB1 receptors on presynaptic terminals to modulate transmitter release (Kreitzer and Regehr, 2002; Freund et al., 2003; Szabo and Schlicker, 2005; Lovinger et al., 2010). Considering the prominence of CB1 receptors in cortico-basal ganglia circuits, it is not surprising that

cannabinoids have been implicated in pathophysiology and/or treatment of many of the disorders associated with these circuits, including Parkinson's and Huntington's disease, levodopa-induced dyskinesia, psychostimulant addiction, and schizophrenia (e.g., Romero et al., 2002; Brotchie, 2003; van der Stelt and Di Marzo, 2003; Wiskerke et al., 2008; Koethe et al., 2009; McCallum and Cheer, 2009; Lovinger et al., 2010; Casadio et al., 2011). The precise roles of CB1 receptors in these disorders remain to be determined; however, they will depend on the particular corticostriatal circuits involved.

The present study assessed the expression of CB1 receptors in the various corticostriatal circuits, by mapping CB1 mRNA throughout the striatum and associating this striatal expression with CB1 expression in cortical input regions (Heng et al., 2011). Our mapping approach used here was previously developed to assign drug-induced molecular changes in the striatum to specific corticostriatal circuits (Willuhn et al., 2003; Yano and Steiner, 2005). Studies indicate that some of the effects of cannabinoid drugs are especially critical during early brain development (see Heng et al., 2011). We thus measured and compared CB1 expression in these circuits at three different postnatal ages, in juveniles (postnatal day 25, P25), adolescents (P40), and adults (P70).

MATERIALS AND METHODS

SUBJECTS

CB1 mRNA expression was studied in male Sprague–Dawley rats (Harlan, Madison, WI, USA) at P25, P40, and P70 ($n = 6$ each). All procedures met the NIH guidelines for the care and use of laboratory animals and were approved by the Rosalind Franklin University Animal Care and Use Committee.

TISSUE PREPARATION AND *IN SITU* HYBRIDIZATION HISTOCHEMISTRY

The rats were killed with CO_2, and their brain was rapidly removed, frozen in isopentane cooled on dry ice and then stored at −30°C until cryostat sectioning. Twelve micrometer thick coronal sections were thaw-mounted onto glass slides (Superfrost/Plus, Daigger, Wheeling, IL, USA) and dried on a slide warmer. In preparation for the *in situ* hybridization histochemistry, the sections were fixed in 4% paraformaldehyde/0.9% saline for 10 min at room temperature, incubated in a fresh solution of 0.25% acetic anhydride in 0.1 M triethanolamine/0.9% saline (pH 8.0) for 10 min, dehydrated, defatted for 2 × 5 min in chloroform, rehydrated, and air-dried. The slides were then stored at −30°C until hybridization.

The oligonucleotide probe (48-mer; Invitrogen, Rockville, MD, USA) was labeled with [35S]-dATP as described earlier (Willuhn et al., 2003). The probe was complementary to bases 1051–1098 of the CB1 mRNA (GenBank accession number X55812). One hundred microliter of hybridization buffer containing labeled probe (~3×10^6 cpm) was added to each slide. The sections were coverslipped and incubated at 37°C overnight. After incubation, the slides were first rinsed in four washes of 1X saline citrate (150 mM sodium chloride, 15 mM sodium citrate), and then washed 3 times 20 min each in 2X saline citrate/50% formamide at 40°C, followed by two washes of 30 min each in 1X saline citrate at room temperature. After a brief water rinse, the sections were air-dried and then apposed to X-ray film (BioMax MR-2, Kodak) for 3 days.

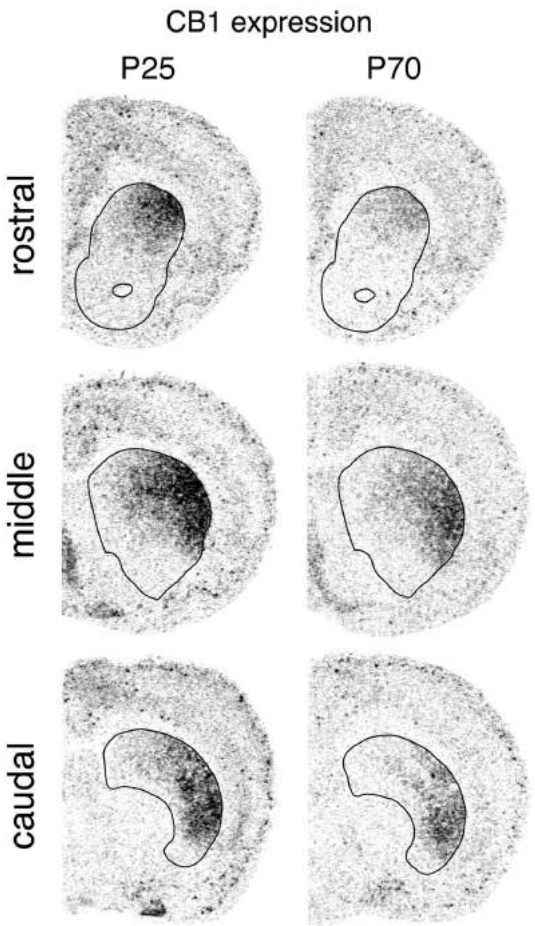

FIGURE 1 | Regional distribution of CB1 receptor expression in the striatum. Illustrations of film autoradiograms show distribution of CB1 mRNA in coronal sections from the rostral, middle and caudal striatum (outlined) in juvenile (P25) and adult (P70) rats.

ANALYSIS OF AUTORADIOGRAMS

Gene expression was assessed in sections from 3 rostrocaudal levels (rostral, approximately at +1.6 mm relative to bregma, Paxinos and Watson, 1998; middle, +0.4; and caudal, −0.8; **Figure 1**), in a total of 23 striatal sectors mostly defined by their predominant cortical inputs (**Figure 2**; Willuhn et al., 2003). Eighteen of these sectors represented the caudate–putamen and five the nucleus accumbens (Yano and Steiner, 2005). Hybridization signals on film autoradiograms were measured by densitometry (NIH Image; Wayne Rasband, NIMH, Bethesda, MD, USA). The film images were captured using a light table (Northern Light, Imaging Research, St. Catharines, ON, Canada) and a Sony CCD camera (Imaging Research). The "mean density" value of a region of interest was measured by placing a template over the captured image. Mean densities were corrected for background by subtracting mean density values measured over white matter (corpus callosum). Values from corresponding regions in the two hemispheres were then averaged.

Treatment effects were determined by one- or two-factor ANOVA, followed by Newman–Keuls *post hoc* tests to describe

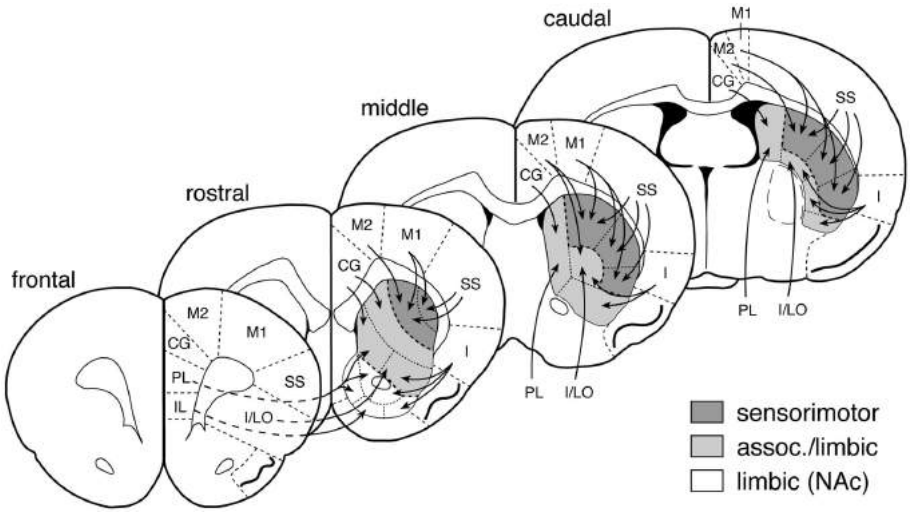

FIGURE 2 | Striatal sectors and their cortical inputs. Schematic illustration depicts the 23 striatal sectors in which CB1 expression was measured. The predominant cortical inputs to these sectors are indicated by arrows (simplified; see Willuhn et al., 2003). Sensorimotor, associative/limbic, and limbic/nucleus accumbens (NAc) circuits are indicated by shading. Abbreviations (medial–lateral): IL, infralimbic; PL, prelimbic; CG, cingulate; M2, medial agranular; M1, motor; SS, somatosensory; I/LO, insular/lateral orbital; I, insular.

differences between individual groups (Statistica, StatSoft, Tulsa, OK, USA). For illustrations of topographies (maps), gene expression in a given region was expressed relative to the maximal value (% of max.) observed in the P25 group. The illustrations of film autoradiograms displayed in **Figure 1** are computer-generated images, and are contrast-enhanced. Maximal hybridization signal is black.

RESULTS

REGIONAL DISTRIBUTION OF CB1 RECEPTOR EXPRESSION IN THE STRIATUM

CB1 receptor expression in the striatum displayed distinctive regional variations that were very similar in the three age groups (**Figures 1**, **3**, and **4**). CB1 mRNA levels were minimal ventrally and medially, and highest in the dorsolateral and ventrolateral sectors. Minimal CB1 mRNA expression was detected in the nucleus accumbens at any age (**Figures 1**, **3**, and **4**). These results show that the CB1 receptor is predominantly expressed in the sensorimotor sectors of the striatum, with minor to minimal expression in associative/limbic striatal regions.

DEVELOPMENTAL TRAJECTORY OF CB1 mRNA EXPRESSION

Despite the dramatic differences in expression levels between different striatal sectors, changes in CB1 expression across development were, overall, similar for all sectors. CB1 mRNA levels were maximal at P25 (**Figure 1**) and then decreased progressively to P40 and to P70 (**Figures 3** and **4**). Levels in adult animals were approximately 50–70% of those in juveniles. Statistical analysis revealed that, at P70, 16 of the 18 sectors of the caudate–putamen and one nucleus accumbens sector showed significantly lower levels, as compared to that at P25 ($P < 0.05$; **Figures 3** and **4**). From P25 to P40, a significant decrease was seen in 12 of the 18 caudate–putamen sectors and 2 of the nucleus accumbens sectors (medial and lateral core; **Figure 4**), and from P40 to P70, in 11 of the caudate–putamen sectors and none of the nucleus accumbens sectors. Exceptions to this general developmental trajectory were found in the ventrolateral sectors on the middle and caudal levels. These sectors displayed either no statistically significant decrease (middle; **Figures 3** and **4**), or a significant but minor decrease (caudal; **Figure 3**) in CB1 expression across development, despite fairly high levels at P25.

CB1 mRNA EXPRESSION IN SPECIFIC CORTICOSTRIATAL CIRCUITS

To assess whether there was a relationship between cortical and striatal CB1 expression, we compared the marked regional variations in striatal CB1 mRNA levels shown here with variations in CB1 expression observed in the cortex (Heng et al., 2011). An overview indicated that striatal sectors with high levels of CB1 expression (e.g., sensorimotor sectors) received inputs mostly from cortical areas with relatively low CB1 levels (motor, somatosensory), whereas striatal sectors with low CB1 mRNA levels (associative/limbic) received afferents from cortical areas with higher CB1 expression (cingulate, insular). We thus assessed whether such an inverse relationship between cortical and striatal CB1 expression existed for specific corticostriatal circuits. We compared, for all 23 corticostriatal circuits, CB1 mRNA levels in cortical areas (Heng et al., 2011) with those in their striatal target sectors, according to the connectivity depicted in **Figure 2**. For striatal sectors with more than one cortical input region (**Figure 2**), the values of these cortical regions were averaged. Data from P25, P40, and P70 animals were initially averaged for this analysis.

Figure 5 shows the relationship between cortical and striatal CB1 mRNA levels for the 8 sensorimotor and 15 associative/limbic circuits (defined by their striatal target sectors, **Figure 2**; see Willuhn et al., 2003). For these 23 circuits pooled, there was only a tendency for a negative correlation between cortical and striatal CB1 expression ($r = -0.35$, $P = 0.10$). However, visual inspection

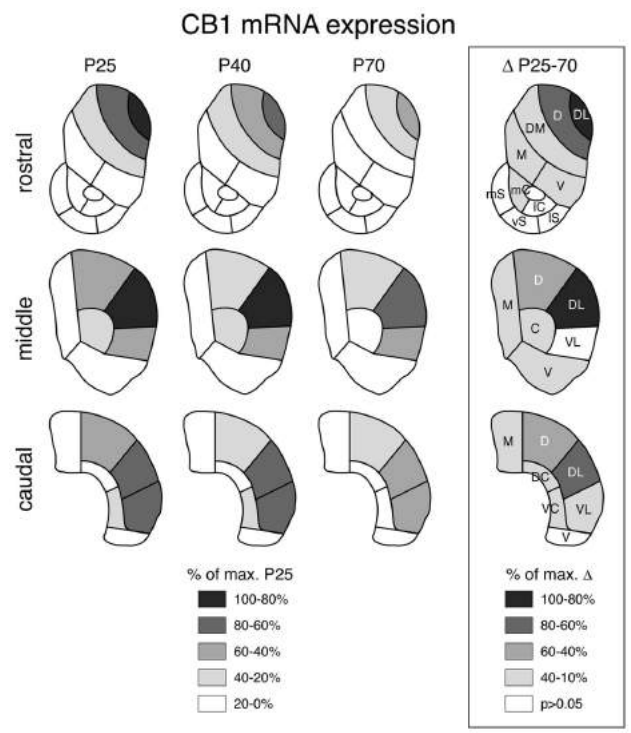

FIGURE 3 | Topography of striatal CB1 receptor expression at different postnatal ages and decreases in expression between juvenile and adult rats. Maps depict the distribution of CB1 mRNA in the rostral, middle, and caudal striatum in juveniles (P25), adolescents (P40) and adults (P70) (left) and the differences in expression between P25 and P70 animals (boxed; right). The data are normalized relative to the maximal value at P25 (left) or the maximal difference (right) (% of max.), and the values and significant decreases ($P < 0.05$) are coded as indicated. Abbreviations: caudate–putamen: C, central; D, dorsal; DC, dorsal central; DL, dorsolateral; DM, dorsomedial; M, medial; V, ventral; VC, ventral central; VL, ventrolateral; nucleus accumbens: mC, medial core; lC, lateral core; mS, medial shell; vS, ventral shell; lS, lateral shell.

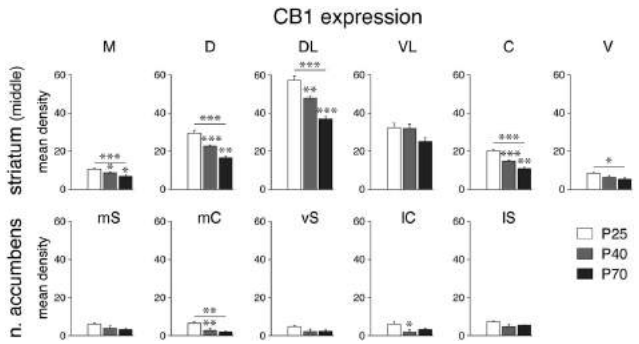

FIGURE 4 | Decrease in CB1 receptor expression during development in select striatal sectors. Mean density values (mean ± SEM) for CB1 expression at P25, P40 and P70 are given for the six sectors of the middle striatum (top) and the five sectors of the nucleus accumbens (bottom) (for abbreviations, see **Figure 3**). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, vs. preceding age group or as indicated.

revealed three different clusters in the value distribution, indicating that cohorts of circuits with different relationships existed (**Figure 5A**). Further *post hoc* analysis showed that the eight sensorimotor circuits by themselves displayed a more robust tendency for a negative correlation ($r = -0.67$, $P = 0.07$). In contrast, no such relationship was seen when the 15 associative/limbic circuits were considered as a group ($r = -0.16$). However, the scatterplot (**Figure 5A**) shows that these latter circuits consisted of two clusters, seven circuits involving projections from medial prefrontal cortical areas (cingulate, prelimbic, infralimbic; **Figure 2**), which have relatively high levels of CB1 expression (Heng et al., 2011), and eight circuits with connections from lateral (insular) cortical areas, which show relatively low CB1 expression (**Figure 5A**). The seven medial prefrontal–striatal circuits indeed displayed the same inverse relationship between cortical and striatal CB1 expression as the sensorimotor circuits. Thus, when pooled ($n = 15$, **Figure 5B**), these circuits showed a very robust negative correlation for cortical and striatal CB1 expression ($r = -0.92$, $P < 0.0001$). This relationship was age-independent; it was also found when the different age groups were analyzed separately (P25, $r = -0.90$, $P < 0.0001$; P40, $r = -0.91$, $P < 0.0001$; P70, $r = -0.81$, $P < 0.0001$). These findings suggest that the regional expression of CB1 receptors is related between cortical areas and their functionally associated striatal sectors, an effect that is already present at P25 and remains unchanged through adolescence to adulthood.

DISCUSSION

The main findings of our present study include (1) detailed expression maps of CB1 receptors in the striatum with reference to functional domains/corticostriatal circuits; (2) a description of the developmental changes in striatal CB1 expression from juveniles to adolescents to adults; and (3) the relationship between CB1 expression in specific striatal sectors and CB1 expression in their cortical input regions.

ASSOCIATION OF CB1 RECEPTOR EXPRESSION WITH FUNCTIONAL DOMAINS IN THE STRIATUM

Our present study associated striatal CB1 expression with specific functional domains/corticostriatal circuits. The functional domains of the striatum are determined by inputs from the cortex (Joel and Weiner, 1994; Parent and Hazrati, 1995). We thus mapped the distribution of CB1 mRNA using 23 striatal sectors on 3 rostrocaudal levels that were previously designed to mostly reflect cortical inputs, based on anatomical tract tracing and functional imaging studies (see Willuhn et al., 2003; Yano and Steiner, 2005). Functional validation and limitations of this mapping approach have been discussed in detail elsewhere (Steiner, 2010). Given the topographical organization of corticostriatal projections, these sectors denote to some degree independent corticostriatal circuits; however, for descriptive purposes, these circuits are also grouped here into the broader categories of "sensorimotor" and "associative/limbic" (Joel and Weiner, 1994, 2000).

Our findings are overall consistent with previous work showing enriched localization of CB1 mRNA in the lateral striatum in rodents (Mailleux and Vanderhaeghen, 1992; Matsuda et al., 1993; Marsicano and Lutz, 1999; Steiner et al., 1999). Our study extends previous findings by providing more fine-grained distribution maps. For example, our results show that CB1 expression is largely

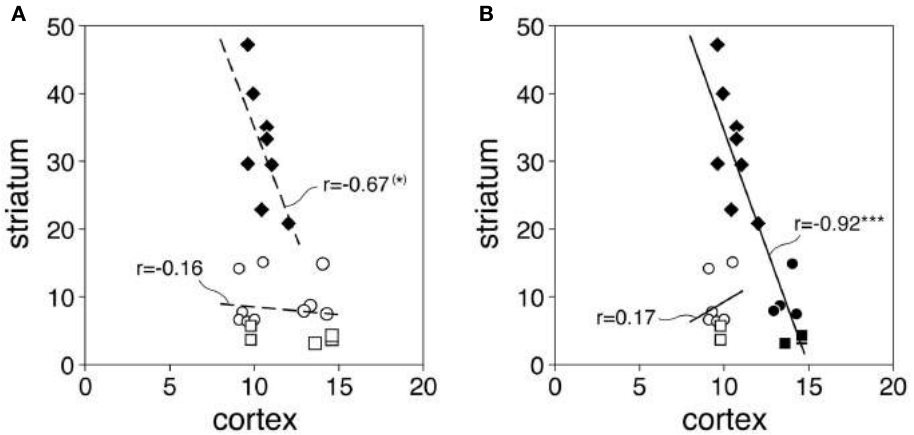

FIGURE 5 | Inverse relationship between cortical and striatal CB1 expression in specific corticostriatal circuits. The scatterplots **(A,B)** depict CB1 expression (mean density; P25, P40, P70 averaged) in cortical areas and their striatal target sectors for the 23 corticostriatal circuits examined. Values for the 8 sensorimotor circuits (diamonds), 10 associative/limbic circuits (circles), and 5 limbic/NAc circuits (squares) are displayed. Results **(B)** show a negative correlation between cortical and striatal CB1 expression for pooled sensorimotor circuits plus associative/limbic/NAc circuits with inputs from medial prefrontal cortex (IL, PL, CG; see **Figure 2**) (filled symbols, $n = 15$; $r = -0.92$, $***P < 0.0001$). $^{(*)}P = 0.07$.

restricted to the sensorimotor striatum, but expression differs between different sectors of the sensorimotor striatum. Thus, within the sensorimotor striatum, expression is highest in the dorsolateral and ventrolateral sectors that receive convergent inputs from the primary motor and somatosensory cortex (e.g., McGeorge and Faull, 1989; Brown et al., 1998; see Willuhn et al., 2003). CB1 expression is considerably lower in the dorsal sensorimotor sectors which differ in that they receive dense inputs also from the medial agranular (premotor) cortex, in addition to sensory and motor inputs (Reep et al., 1987, 2003). The functional significance of this differential expression is presently unclear, but we note that these same dorsal sectors show much more robust molecular adaptations after treatment with psychostimulants such as cocaine and methylphenidate than other parts of the sensorimotor striatum (Willuhn et al., 2003; Yano and Steiner, 2005; Steiner, 2010). Future studies will have to clarify the potential role of these striatal CB1 receptors in psychostimulant-induced neuroplasticity.

In contrast to the sensorimotor striatum, CB1 mRNA levels in associative/limbic sectors, including the nucleus accumbens, were found to be minimal to undetectable, in agreement with previous studies (Mailleux and Vanderhaeghen, 1992; Marsicano and Lutz, 1999; Steiner et al., 1999; Hohmann and Herkenham, 2000). These findings suggest that CB1 effects in these regions are likely mediated by CB1 receptors located on striatal afferents rather than on intrinsic neurons (see below).

In summary, to the extent that the here observed regional variations in CB1 mRNA levels are matched by CB1 receptor function, these findings indicate that CB1 receptors expressed by striatal neurons predominantly regulate sensorimotor processes of the striatum.

DEVELOPMENTAL TRAJECTORY OF CB1 mRNA EXPRESSION IN CORTICOSTRIATAL CIRCUITS

Our developmental analysis indicates that striatal CB1 expression is maximal in juvenile animals and then decreases toward adulthood. This effect was largely independent of the (greatly varying) expression levels observed in the different striatal sectors. This trajectory is similar to developmental changes found for other G-protein-coupled receptors. For example, early overexpression followed by pruning back to adult levels has also been shown for D1 and D2 dopamine receptors (Teicher et al., 1995; Andersen et al., 1997; Tarazi et al., 1999), $A2_A$ adenosine receptors (Johansson et al., 1997), and the putative signaling molecule GPR88 (Van Waes et al., 2011).

A similar developmental decrease in CB1 expression from juveniles to adults was previously observed in cortical input regions of the various corticostriatal circuits (Heng et al., 2011). However, in that study, we noted a differential trajectory for changes in associative/limbic vs. sensorimotor cortical regions. Associative/limbic cortical regions showed a progressive reduction in CB1 expression from P25 to P40 to P70 (Heng et al., 2011), similar to most striatal sectors in the present study. In contrast, decreases in sensorimotor cortical regions mostly occurred only after the adolescent transition period (between P40 and P70; Heng et al., 2011), a developmental pattern of CB1 expression resembling that observed in the ventrolateral sensorimotor sectors of the middle-to-caudal striatum. Collectively, these findings suggest that the maturation of CB1 expression within the associative/limbic corticostriatal circuits has an earlier onset than that in the sensorimotor domain, which occurs during adolescence.

ARE CHANGES IN CB1 mRNA LEVELS REFLECTED BY CHANGES IN CB1 RECEPTOR FUNCTION?

Previous findings showed that, in the striatum, the regional differences in CB1 receptor mRNA are matched by differences in CB1 receptor immunoreactivity and binding (Herkenham et al., 1991a,b; Mailleux and Vanderhaeghen, 1992; Egertová and Elphick, 2000; Julian et al., 2003). Are these molecular variations associated with corresponding differences in CB1 receptor function?

Our previous study addressed the physiological significance of changes in CB1 mRNA levels by whole-cell patch clamp recordings (Heng et al., 2011). These experiments measured postsynaptic depolarization-induced suppression of excitation (DSE) in deep-layer pyramidal neurons of the medial prefrontal cortex during development (Heng et al., 2011). DSE is a well-established electrophysiological response used to determine endocannabinoid (CB1) regulation of synaptic activity (Kreitzer and Regehr, 2002; Lovinger et al., 2010). Our results demonstrated that DSE is indeed markedly reduced in the adult prefrontal cortex as compared with that in adolescent rats (Heng et al., 2011). These changes in DSE thus paralleled the changes in CB1 mRNA levels. DSE was associated with a facilitation of the paired-pulse ratio (Heng et al., 2011), indicating involvement of changes in presynaptic neurotransmitter release (Thomson, 2000). Moreover, DSE was inhibited by the CB1 receptor antagonist AM-251, confirming that this effect was mediated by activation of CB1 receptors (Heng et al., 2011). Collectively, these electrophysiological findings indicate that this CB1 receptor function in the cortex is down-regulated during development, in parallel with the decrease in local CB1 receptor expression.

Nevertheless, the relationship between CB1 mRNA and function may be different in the striatum. It will be important to establish whether the differential CB1 mRNA levels, between different striatal regions and between developmental stages, are similarly accompanied by differential CB1 receptor function in striatal neurons.

CELLULAR LOCALIZATION OF CB1 RECEPTORS IN THE STRIATUM: DIFFERENTIAL REGULATION OF GABA VS. GLUTAMATE SIGNALING IN DIFFERENT CORTICOSTRIATAL CIRCUITS?

The distinctive differences in CB1 mRNA levels between striatal sectors in association with those in their cortical input regions allow for speculations regarding differential CB1 receptor signaling in the different corticostriatal circuits/functional domains.

The functional significance of these CB1 receptors is dependent on their cellular localization. Striatal CB1 binding reflects a combination of CB1 receptors located (1) on terminals of striatal afferents; (2) on striatal interneurons; and (3) on striatal projection neurons. We hypothesize that CB1 receptor binding in striatal sectors with high CB1 mRNA levels (and binding) mostly reflects receptors on striatal projection neurons, more precisely, on local axon collateral terminals of these projection neurons. This is supported by the following findings.

CB1 receptors on afferent terminals can not account for the distinct striatal binding patterns. For example, afferents from the cortex, although subject to significant presynaptic cannabinoid regulation (e.g., Lovinger et al., 2010, for review), express relatively low levels of CB1 receptors (e.g., Uchigashima et al., 2007; see also discussion in Freund et al., 2003). In fact, our results indicate an inverse relationship between cortical and striatal CB1 expression (i.e., striatal sectors with high CB1 mRNA levels and binding tend to receive inputs from cortical areas with low CB1 expression, and vice versa; **Figure 5**). Similarly, CB1 receptors on the other major afferents to the striatum, those from midbrain dopamine neurons and thalamic neurons, can hardly explain the observed patterns of striatal CB1 receptor binding, as these neurons show minimal or no CB1 mRNA/protein expression (Herkenham et al., 1991a; Mailleux and Vanderhaeghen, 1992; Julian et al., 2003; Uchigashima et al., 2007). CB1 receptors are expressed at greater levels in hippocampus and amygdala, although it remains unclear to what extent this expression is restricted to interneurons (Freund et al., 2003). However, inputs from these nuclei predominantly target the associative/limbic striatum (Joel and Weiner, 1994). They may thus contribute to CB1 binding in those sectors, but not in sensorimotor sectors.

Regarding the contribution of CB1 receptors on projection neurons vs. interneurons, colocalization studies demonstrate that CB1 receptors are very highly expressed in striatal projection neurons (Marsicano and Lutz, 1999; Hohmann and Herkenham, 2000; Martín et al., 2008). CB1 receptor expression is also established in (some) fast-spiking, parvalbumin-containing interneurons (Marsicano and Lutz, 1999; Hohmann and Herkenham, 2000; Martín et al., 2008) and may be present in a minority of NOS-containing and cholinergic interneurons (Fusco et al., 2004; Uchigashima et al., 2007). However, given that striatal interneurons in total only comprise a small fraction (∼3%, Oorschot, 2010) of striatal neurons, the regional patterns of CB1 mRNA as depicted by film autoradiography (our studies) predominantly reflect expression in striatal projection neurons.

Where on projection neurons are these CB1 receptors localized? A prominent feature of striatal projection neurons is their extensive local axon collaterals that distribute roughly in a tissue volume similar to, and partly overlapping with, that of their dendrites (Gerfen and Bolam, 2010). These axon collaterals connect to interneurons and other projection neurons (**Figure 6**) and form numerous inhibitory (GABA) synapses onto spines and dendritic

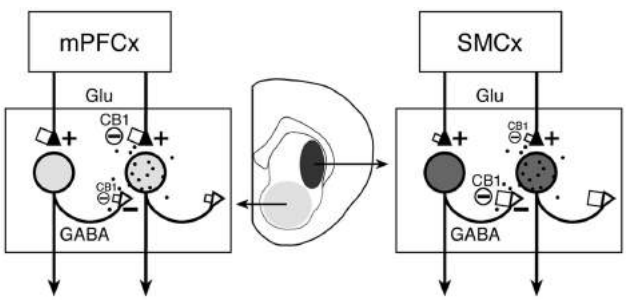

FIGURE 6 | Proposed differential CB1 regulation of collateral (GABA) inputs and cortical (glutamate, Glu) inputs to striatal projection neurons in associative/limbic vs. sensorimotor corticostriatal circuits. The proposed number of CB1 receptors on afferent terminals (see text) is indicated by the size of squares. Our present findings indicate an inverse relationship between cortical and striatal CB1 expression in associative/limbic circuits with inputs from medial prefrontal cortex (mPFCx) (left) and sensorimotor circuits (right), with relatively high cortical and low striatal CB1 expression in the former and low cortical and high striatal expression in the latter. It is argued that, in sensorimotor corticostriatal circuits, striatal CB1 receptors are mainly located presynaptically on local axon collateral terminals of projection neurons (open triangles) and regulate GABA release from these terminals (see text). On the other hand, striatal CB1 receptors in associative/limbic circuits may mostly regulate excitatory inputs from the cortex (filled triangles) and other areas (and GABA inputs from interneurons; not shown). The degree of shading in striatal areas and respective neurons reflects the amount of CB1 receptor expression in these neurons. The black dots symbolize endocannabinoid release from striatal neurons. SMCx, sensorimotor cortex.

shafts of these neurons (Kubota and Kawaguchi, 2000; Wilson, 2007). Studies show that CB1 receptors are preferentially localized on axon terminals as opposed to postsynaptic elements ("polarized" distribution; Irving et al., 2000; McDonald et al., 2007). While some CB1 receptors have been found in dendritic profiles of striatal neurons (e.g., Rodriguez et al., 2001), this polarized distribution (Irving et al., 2000; McDonald et al., 2007) thus predicts much higher CB1 receptor levels on axon terminals of these neurons (Herkenham et al., 1991a). The importance of CB1 receptors on striatal axon collaterals has been demonstrated by electrophysiological findings. For example, stimulation of cannabinoid receptors inhibits GABAergic synaptic transmission between paired striatal projection neurons (**Figure 6**), and this effect is mediated by presynaptic CB1 receptors on axon terminals (Freiman et al., 2006).

Taken together, the above findings suggest that CB1 receptor binding in striatal sectors with high CB1 mRNA levels (sensorimotor sectors) largely reflects presynaptic CB1 receptors on local collateral terminals of projection neurons that regulate interactions between such neurons (Freiman et al., 2006; **Figure 6**). On the other hand, CB1 receptors in striatal areas with low CB1 mRNA levels (associative/limbic sectors, nucleus accumbens; e.g., Hoffman and Lupica, 2001; Manzoni and Bockaert, 2001) may mostly regulate GABA inputs from interneurons (e.g., Freiman et al., 2006), in addition to glutamate inputs from associative/limbic cortex and other brain regions.

LIMITATIONS

The above hypothesis will need to be tested experimentally. This hypothesis is based on measurement of mRNA, which is better quantifiable than receptor immunohistochemistry and thus better suited for mapping studies. Moreover, mRNA "imaging" allows identification of the neurons (cell bodies) that express the receptor. In contrast, receptor binding signals can not differentiate between receptors on afferent terminals and those on postsynaptic neurons. Such a differentiation is necessary for the above considerations. However, the main caveat of the mRNA approach is that mRNA levels not always correlate with receptor levels and function. As stated above, while we have demonstrated parallel changes in CB1 mRNA levels and function for cortical neurons, a similar relationship in striatal neurons remains to be demonstrated.

CONCLUSION: DIFFERENTIAL CB1 SIGNALING IN DIFFERENT CORTICOSTRIATAL CIRCUITS

Our anatomical comparison of CB1 expression in cortex and striatum indicates an inverse relationship between cortical and striatal nodes of sensorimotor corticostriatal circuits and associative/limbic circuits receiving inputs from medial prefrontal cortex, with low cortical and high striatal expression and vice versa, respectively. These findings indicate that CB1 receptors differentially regulate corticostriatal transmission for these groups of circuits. Moreover, based on the above considerations, it can be hypothesized that, in sensorimotor corticostriatal circuits, exposure to cannabinoid drugs will predominantly affect striatal GABA release from local axon collaterals, whereas in associative/limbic circuits, cortical glutamate inputs are expected to be more affected.

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GPR88: A putative signaling molecule predominantly expressed in the striatum: Cellular localization and developmental regulation

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ABSTRACT

GPR88 is a putative G-protein-coupled receptor that is highly and almost exclusively expressed in the striatum. Its function remains unknown. We investigated GPR88 cellular localization and expression levels across development in different functional domains of the striatum in juvenile (P25), adolescent (P40), and adult (P70) rats, by in situ hybridization histochemistry. At all ages, GPR88 mRNA expression was most robust in the sensorimotor (lateral) striatum and was detected in virtually every neuron. Expression was highest in juveniles and decreased thereafter with regionally distinct trajectories. Thus, in the dorsal striatum, there was a progressive decrease from juveniles to adolescents to adults. In contrast, in the nucleus accumbens, the only (modest) decrease occurred between juveniles and adolescents. These findings indicate that GPR88 is expressed in all striatal neurons, but is differentially regulated across development in different striatal regions.

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Introduction

The basal ganglia comprise a group of forebrain nuclei that are interconnected with the cerebral cortex, thalamus and brainstem [1,2]. Basal ganglia circuits mediate diverse brain functions including motor control, sensorimotor integration, attention, reward and cognition. Thus, dysfunction in basal ganglia circuits is implicated in a wide spectrum of disorders, including Parkinson's disease, Huntington's disease, obsessive–compulsive disorder, attention–deficit hyperactivity disorder, and psychostimulant addiction (e.g., [2–7]). The striatum (caudate–putamen, nucleus accumbens) constitutes the main input station of the basal ganglia and is thus critically important for the function of basal ganglia circuits. The striatum is composed of medium-sized GABAergic projection neurons and a small proportion of interneurons (<3% of striatal neurons) [8]. The projection neurons are divided into two subtypes, which give rise to the "direct" (striatonigral) and "indirect" (striatopallidal) output pathways [9]. The principal inputs to striatal neurons include excitatory (glutamate) afferents from various cortical and thalamic areas that drive striatal activity, and dopamine inputs from the midbrain that modulate activity flow in striatal output pathways [10].

The pharmacological strategies used in the treatment of disorders involving the striatum often focus on the dopamine (and glutamate) systems. For example, 3,4-dihydroxyphenyl-L-alanine (L-DOPA), a dopamine precursor, and antipsychotic medications targeting the D2 dopamine receptor have been mainstays in the treatment of Parkinson's disease and schizophrenia, respectively, for decades. However, these treatments have several limitations. For one, dopamine and glutamate systems are present in many brain areas and mediate many brain functions. Systemically administered pharmacological agents targeting dopamine or glutamate signaling will thus also affect brain functions unrelated to basal ganglia circuits and produce unwanted side effects. Moreover, these drugs are often not fully effective. For example, after long-term treatment with L-DOPA or dopamine receptor antagonists, disruptive motor side effects typically emerge, which restricts the usefulness of these drugs [11]. Therefore, considerable emphasis has been placed on identifying alternative mechanisms that regulate basal ganglia systems, with the hope that these will lead to the development of better medications for such disorders.

One approach used to develop more selective agents is to find and evaluate new candidate molecules whose anatomical localization is regionally more restricted. GPR88 is such a molecule; it is almost exclusively localized in the striatum and, within this structure, is preferentially expressed in lateral parts [12,13]. Based on sequence homology, GPR88 is proposed to encode a novel G-protein-coupled receptor [12]. GPR88 expression has been shown in humans and other primates, rats and mice [12–15].

Presently, almost nothing is known on the function of GPR88. A recent study reported that, in GPR88 knockout mice, basal extracellular dopamine levels in the striatum were lower, while amphetamine-induced dopamine release was normal [15]. These

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mice also displayed increased apomorphine-induced stereotypy and amphetamine-stimulated locomotor activity [15]. These results suggest that GPR88 plays a role in the regulation of dopamine signaling in the striatum. Consistent with an interaction with the dopamine transmission, another study found that dopamine depletion or receptor stimulation produced (moderate) changes in GPR88 expression in striatal neurons [13]. However, manipulation of glutamate input to the striatum or various other drug treatments also altered the expression of GPR88 [13].

Beyond its gross distribution in the striatum, little is known on subregional differences in expression, cellular localization [13] and developmental regulation of GPR88. In this study, we investigated the cellular localization of GPR88 mRNA and its association with specific functional domains (as defined by their predominant cortical inputs [16]) of the rat striatum, in order to determine the striatal targets of potential GPR88-selective pharmacological agents. We further assessed the developmental regulation of GPR88 by comparing expression in juveniles (P25), adolescents (P40), and adults (P70). The cellular localization was established by fluorescence in situ hybridization histochemistry in combination with immunohistochemistry. Expression levels were measured by radioactive in situ hybridization histochemistry.

Materials and methods

Subjects

Cellular localization and expression levels of GPR88 mRNA in the striatum were investigated in juvenile (postnatal day 25, P25), adolescent (P40), and adult (P70) male Sprague–Dawley rats (Harlan, Madison, WI, USA) (n = 6 per group). The animals were allowed to habituate for 3 days after arrival before they were killed for tissue processing. All procedures met the NIH guidelines for the care and use of laboratory animals and were approved by the Rosalind Franklin University Animal Care and Use Committee.

Tissue preparation

The rats were killed with CO_2. The brain was rapidly removed, frozen in isopentane cooled on dry ice, and stored at −30 °C until cryostat sectioning. Twelve micron thick coronal sections were thaw-mounted onto glass slides (Superfrost/Plus, Daigger, Wheeling, IL, USA) and dried on a slide warmer. In preparation for the in situ hybridization histochemistry, the sections were fixed in 4% paraformaldehyde/0.9% saline for 10 min at room temperature, incubated in a fresh solution of 0.25% acetic anhydride in 0.1 M triethanolamine/0.9% saline (pH 8.0) for 10 min, dehydrated, defatted for 2 times 5 min in chloroform, rehydrated, and air-dried. The slides were then stored at −30 °C until hybridization.

Double labeling by fluorescence in situ hybridization histochemistry combined with immunohistochemistry

Oligonucleotide probes ($GPR88_1$, $GPR88_2$, enkephalin; 48-mers; Invitrogen, Rockville, MD, USA) were labeled using the DIG-ddUTP oligonucleotide 3′-end labeling kit (Roche, Mannheim, Germany). The probes had the following sequence: $GPR88_1$, complementary to bases 451–498; $GPR88_2$, bases 613–660, GenBank Accession No. AB042407; enkephalin, bases 436–483, M28263. The two GPR88 probes produced the same signal distribution; they were combined to increase the signal strength. No signal was observed with corresponding sense probes.

One hundred microliters of hybridization buffer containing a mix of the two digoxigenin-labeled GPR88 probes (10 pmol each), or the digoxigenin-labeled enkephalin probe (10 pmol), was added to each slide. The sections were coverslipped and incubated at 37 °C

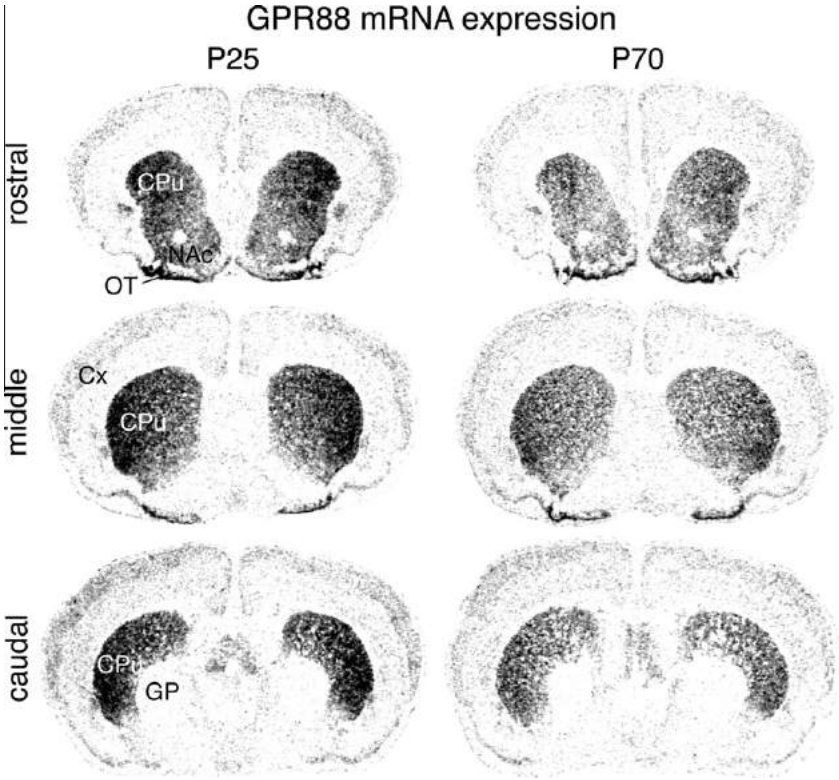

Fig. 1. Distribution of GPR88 expression in the striatum. Illustrations of film autoradiograms depict the distribution of GPR88 mRNA in coronal sections from the rostral, middle and caudal striatum in juvenile (P25) and adult (P70) rats. The maximal hybridization signal is black. CPu, caudate–putamen; Cx, cortex; GP, globus pallidus; NAc, nucleus accumbens; OT, olfactory tubercle.

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overnight. After incubation, the slides were first rinsed in four washes of 1X saline citrate, and then washed 3 times 20 min each in 2X saline citrate/50% formamide at 40 °C, followed by 2 washes of 30 min each in 1X saline citrate at room temperature. The sections were then incubated 15 min in 2% H_2O_2 to remove endogenous peroxidase activity, rinsed 5 min in TNT washing buffer (0.1 M TRIS–HCl, 0.15 M sodium chloride, and 0.05% Tween 20), incubated 30 min in TNB blocking buffer (0.1 M TRIS–HCl, 0.15 M sodium chloride, and 0.5% blocking reagent; PerkinElmer, Waltham, MA, USA), and 30 min in anti-digoxigenin antibody conjugated with horseradish peroxidase (1:40, in TNB blocking buffer, Fab fragments; Roche). After 3 times 5 min rinses in TNT washing buffer, 200 μl of fluorophore tyramidine solution (TSA Plus fluorescence system; PerkinElmer) was added to each slide for 10 min, and the slides were then rinsed 3 times 5 min in TNT washing buffer. Slides were incubated overnight in mouse anti-neuronal nuclei (NeuN) antibody (1:500, clone A60; Millipore, Temecula, CA, USA), rinsed 3 times 5 min in TNT buffer, and incubated 2 h in Alexa Fluor 594 goat anti-mouse antibody (1:200; Invitrogen, Carlsbad, CA, USA). The sections were finally washed 3 times 5 min in TNT buffer, briefly rinsed in water, air-dried and coverslipped with PVA-DABCO (Sigma–Aldrich; Allentown, PA, USA).

Sections were examined with a fluorescence microscope (Nikon ECLIPSE E400) linked to a high resolution Hamamatsu Orca-ER digital camera (C4742-80). Images were captured using Stereo Investigator software (MBF Science, Williston, VT, USA). Gene expression in the striatum was evaluated in sections from three rostrocaudal levels (rostral, approximately at +1.6 mm relative to bregma, [17]; middle, +0.4; caudal, −0.8; Fig. 1), in a total of 23 sectors mostly defined by their predominant cortical inputs [16]. Eighteen of these sectors represented the caudate–putamen and 5 the nucleus accumbens.

Radioactive in situ hybridization histochemistry

The $GPR88_1$ oligonucleotide probe was labeled with [^{35}S]-dATP as described earlier [16]. One hundred microliter of hybridization buffer containing labeled probe (~3×10^6 cpm) was added to each slide. The sections were coverslipped and incubated at 37 °C overnight. After incubation, the slides were washed as described above. The sections were air-dried and then apposed to X-ray film (Bio-Max MR-2, Kodak) for 3 days.

Hybridization signals on film autoradiograms were measured by densitometry (NIH Image; Wayne Rasband, NIMH, Bethesda,

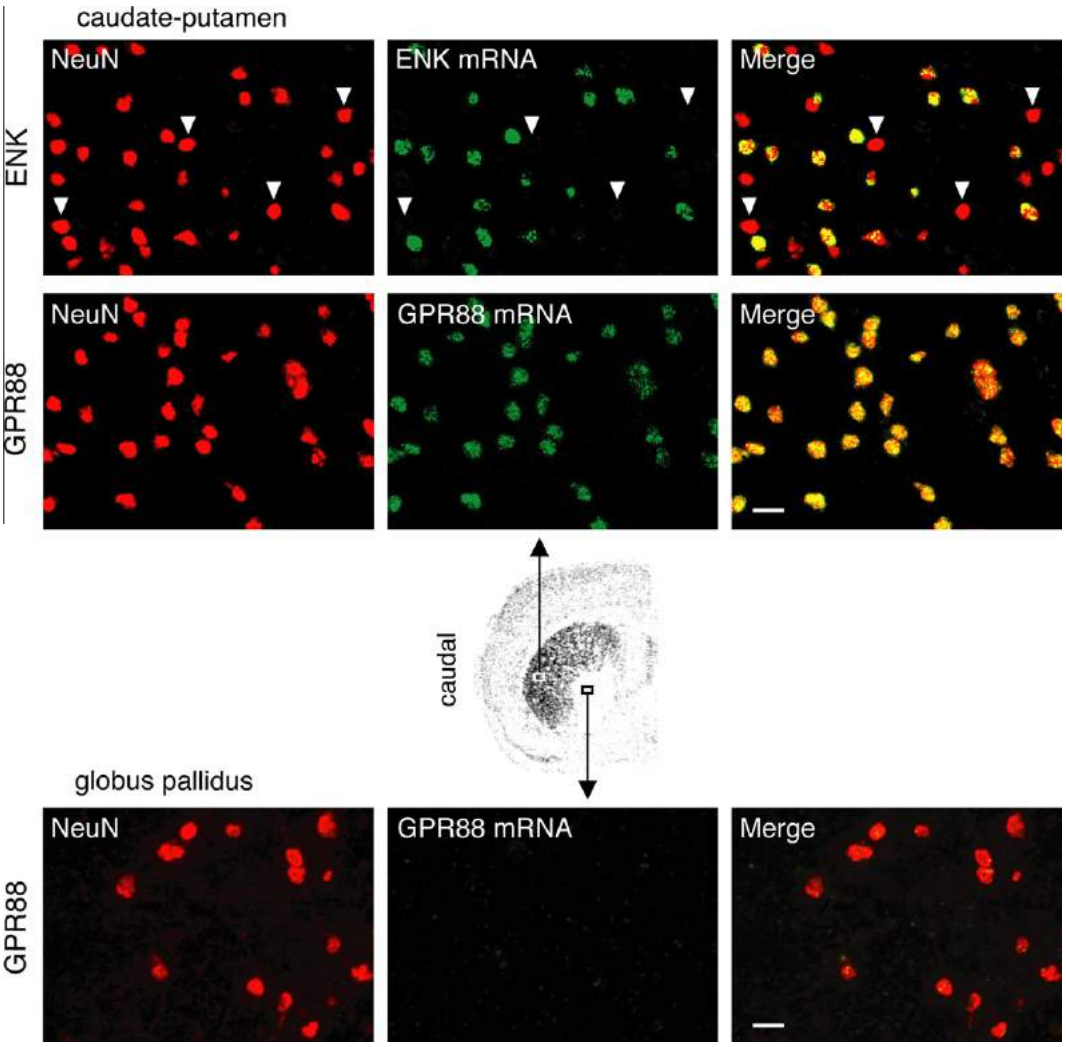

Fig. 2. Cellular localization of GPR88 mRNA in the striatum. Examples of neurons labeled for NeuN (neuronal marker; fluorescence immunohistochemistry, red) (left column) and for enkephalin (ENK) mRNA or GPR88 mRNA (fluorescence in situ hybridization histochemistry, green) (center column) in the caudal caudate–putamen and in the globus pallidus are shown for adult rats (P70). Double-labeled neurons (merge, yellow) are depicted in the right column. GPR88 mRNA is expressed in all striatal neurons (middle row), but is not expressed in the globus pallidus (bottom row). White arrowheads indicate examples of NeuN-positive/ENK-negative cells (top row). Scale bars = 25 μm.

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MD, USA). The films were captured using a light table (Northern Light, Imaging Research, St. Catharines, Ontario, Canada) and a Sony CCD camera (Imaging Research). The "mean density" value of a region of interest was measured by placing a template over the captured image. Mean densities were corrected for background by subtracting mean density values measured over white matter (corpus callosum). Values from corresponding regions in the two hemispheres were then averaged. The images of film autoradiograms displayed in Fig. 1 are computer-generated and contrast-enhanced (linear). Maximal hybridization signal is black.

Statistics

The effect of age on GPR88 mRNA levels in the different striatal sectors was determined by one-factor ANOVA. The developmental trajectories in the caudate–putamen vs. nucleus accumbens were compared by two-factor ANOVA with age as between-subject variable and striatal region as within-subject variable. Newman–Keuls post hoc tests were used to describe differences between individual groups (Statistica, StatSoft, Tulsa, OK, USA). For illustrations of topographies (maps), gene expression in a given region was expressed relative to the maximal expression observed in the P25 group (% of max. P25). The difference in GPR88 expression between P25 and P70 was expressed as the percentage of the maximal change (% of max. Δ).

Results

GPR88 expression in the forebrain

Expression of GPR88 mRNA was very pronounced in the striatum (caudate–putamen, nucleus accumbens) on all rostrocaudal levels (Fig. 1). Robust expression was also present in the olfactory tubercle. In the cortex, modest GPR88 mRNA levels were found in superficial layers (layers 2 and 3), from the cingulate (dorsomedial) to the piriform areas (ventrolateral), while deep layers showed minimal or no expression (Fig. 1). In marked contrast to the striatum, no GPR88 expression was detected in the globus pallidus (Figs. 1 and 2).

GPR88 expression in striatal neurons

At the cellular level, the fluorescent GPR88 mRNA signal had a distinctive granular appearance (Figs. 2 and 3). Both in the caudate–putamen (Fig. 2, middle row) and in the nucleus accumbens (not shown), GPR88 expression was detected in every cell labeled with the neuronal marker NeuN, independent of age. For comparison, approximately 50% of the neurons expressed enkephalin mRNA (i.e., indirect pathway neurons) (Fig. 2, top row). In contrast, in the globus pallidus, NeuN-positive cells did not express detectable levels of GPR88 mRNA (Fig. 2, bottom row). Therefore, our data indicate that, in the striatum, GPR88 is localized in both direct and indirect pathway neurons, as well as in interneurons. Notably, GPR88 mRNA was detected in large neurons (Fig. 3), presumably cholinergic interneurons. The intensity of the signal in these large neurons was typically in the lower range of signals detected in medium-sized neurons (Fig. 3). Overall, we did not observe a difference in the cellular localization of GPR88 mRNA between the 23 sectors of the striatum.

Regional and developmental variations in GPR88 expression in the striatum

Despite GPR88 expression in all striatal neurons, there were distinct regional variations and developmental changes present on all three rostrocaudal levels examined (Figs. 1 and 4). Independent of

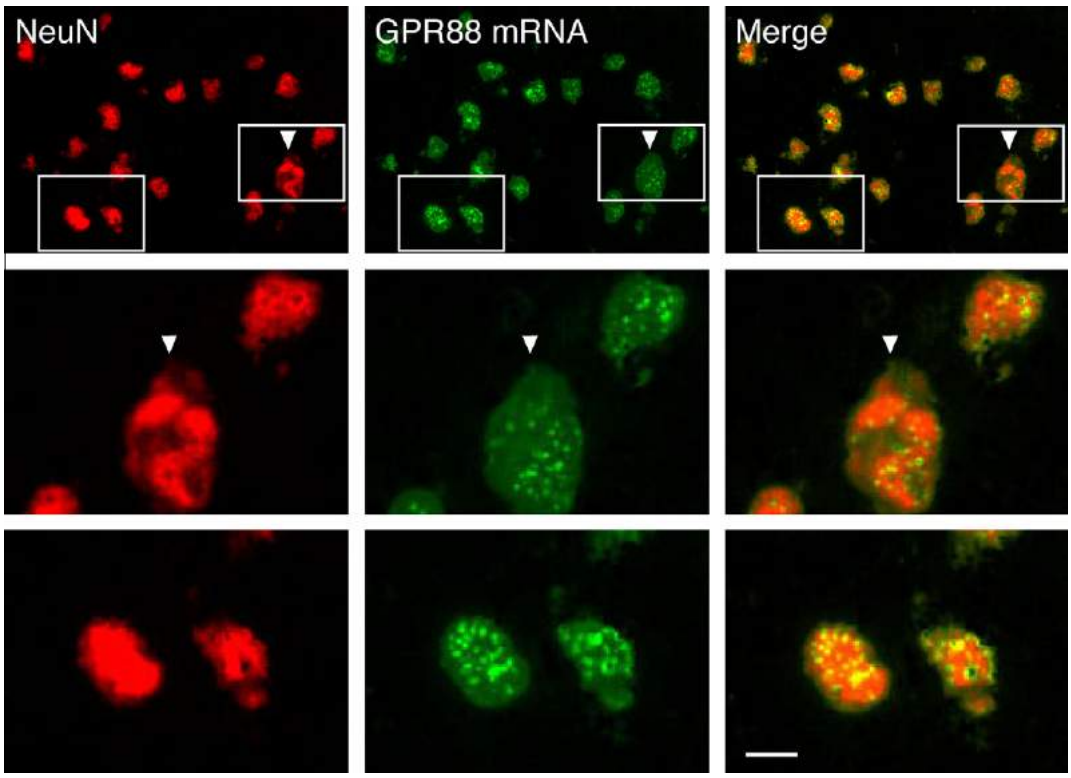

Fig. 3. Localization of GPR88 mRNA in medium-sized and large neurons of the striatum. Lower power photomicrographs (top row) depict neurons labeled for NeuN (red; left column), or GPR88 mRNA (green; center column), and double-labeled neurons (merge, yellow; right column) in the caudate–putamen in adults (P70). Higher power photomicrographs (middle and bottom rows) display the neurons in the boxed areas in the top images. Examples of neurons with a relatively low (middle row) or high (bottom row) GPR88 mRNA signal are shown. The white arrowhead indicates a large neuron, presumably a cholinergic interneuron. Scale bar = 10 μm.

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age, GPR88 mRNA levels displayed a robust medial–lateral gradient, with highest levels laterally, in the sensorimotor striatum (dorsolateral, ventrolateral sectors) and more moderate levels in medial, associative sectors (Figs. 1 and 4). GPR88 expression was also more moderate in the nucleus accumbens (Figs. 1, 4 and 5).

Contrasting the overall similar regional patterns between the age groups (e.g., medial–lateral gradient), there were marked differences in GPR88 expression levels across development. GPR88 expression was highest at P25 and then decreased with age (Figs. 4 and 5). Regional analysis revealed a significant decrease in expression ($P < 0.05$) from P25 to P40 in 21, and from P40 to P70 in 17 of the 23 striatal sectors (Figs. 4 and 5). However, unlike in the sectors of the caudate–putamen, the decrease in GPR88 expression in the nucleus accumbens predominantly occurred between P25 and P40 and was limited to the core and the medial shell (Fig. 5). No statistically significant decrease between P25 and P70 was observed in the ventral and lateral shell. Overall these findings demonstrate a robust and progressive decrease from P25 to P70 for the caudate–putamen, intermediate changes in the core of the nucleus accumbens and minor or no changes in the shell (Figs. 4 and 5).

Differential developmental trajectories for GPR88 expression in the caudate–putamen vs. nucleus accumbens

We further determined the developmental regulation of GPR88 expression in caudate–putamen and nucleus accumbens by comparing their developmental trajectories statistically, using pooled values from the respective sectors in these two regions (Fig. 6). This analysis confirmed a differential regulation across age in caudate–putamen vs. nucleus accumbens (main effect of age, $F(2, 15) = 84.3$, $P < 0.001$; main effect of region, $F(1, 15) = 0.78$, $P > 0.05$; age × region interaction, $F(2, 15) = 6.7$, $P < 0.01$). Post hoc comparisons showed a similar relative reduction in GPR88 levels between P25 and P40 for caudate–putamen and nucleus accumbens (P25 vs. P40, $P < 0.001$; at P40, caudate–putamen vs. nucleus accumbens, $P > 0.05$). However, GPR88 expression further decreased between P40 and P70 in the caudate–putamen ($P < 0.001$), whereas no change occurred in the nucleus accumbens during this period ($P > 0.05$). This differential decrease resulted in a lower relative GPR88 level in the caudate–putamen than in the nucleus accumbens at P70 ($P < 0.01$).

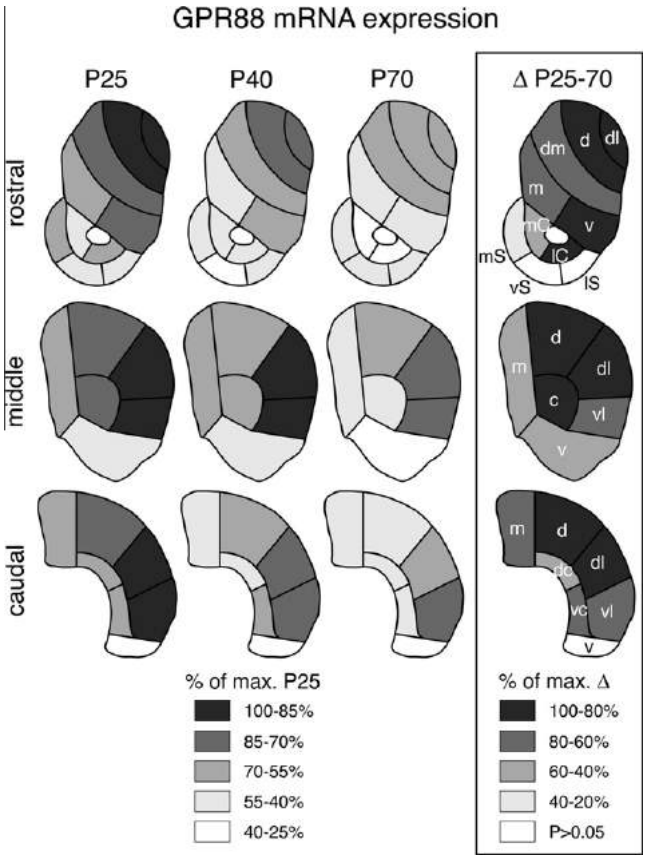

Fig. 4. Topography of GPR88 expression in the striatum at different postnatal ages (left) and differences in expression between P25 and P70 animals (boxed; right). Maps depict the distribution of GPR88 expression in the rostral, middle and caudal striatum in juvenile (P25), adolescent (P40) and adult (P70) rats. For P25, P40 and P70 groups, the data are expressed relative to the maximal value observed in the P25 group (% of max. P25). The differences in GPR88 expression between P25 and P70 rats (Δ P25-70) are expressed as the percentage of the maximal change (% of max. Δ). Sectors with significant differences ($P < 0.05$) are coded as indicated. Sectors without a significant decrease are in white. Caudate–putamen: c, central; d, dorsal; dc, dorsal central; dl, dorsolateral; dm, dorsomedial; m, medial; v, ventral; vc, ventral central; vl, ventrolateral. Nucleus accumbens: mC, medial core; lC, lateral core; mS, medial shell; vS, ventral shell; lS, lateral shell.

Discussion

In the present study, we investigated the cellular localization and developmental changes in expression of GPR88, a putative novel G-protein-coupled receptor, in the striatum of juvenile, adolescent, and adult rats. We found that GPR88 expression is most robust in juveniles and decreases thereafter towards adult levels. GPR88 is expressed in all striatal neurons, but with distinct regional variations. Our results also indicate a differential developmental regulation for GPR88 expression between regions of the caudate–putamen and the nucleus accumbens. A progressive decrease from P25 to P40 to P70 was observed in the caudate–putamen, whereas a more moderate decrease occurred in the nucleus accumbens, mostly between P25 and P40.

Regional and cellular localization of GPR88 expression

The expression of GPR88 was evaluated both with qualitative (fluorescence) and quantitative (radioactive) in situ hybridization histochemistry. Consistent with previous studies [12–15], GPR88 expression was very pronounced in the striatum (caudate–putamen, nucleus accumbens) at all ages. Robust expression was also present in the olfactory tubercle. A modest signal was found in superficial layers of the cortical mantle, also consistent with the earlier reports (see above).

Our study provides the first detailed description of the regional distribution of GPR88 mRNA expression throughout the striatum. The expression was mapped in striatal sectors mostly defined by their predominant cortical inputs (see [16]), in order to determine expression levels across the different functional domains. GPR88 is expressed with a distinctive medial–lateral gradient. Expression was most robust in the sensorimotor (lateral) striatum and was more moderate in the associative (medial) and limbic (ventral) striatum. This regional distribution thus matches that of certain G-protein-coupled receptors, for example, the CB1 cannabinoid receptor [18,19] and the D2 dopamine receptor [20–22], but also the distribution of some neuropeptides (e.g., substance P [23]). Given that the cell density is similar between these striatal regions, neurons of the sensorimotor striatum appear to express higher levels of GPR88. Pharmacological agents targeting GPR88 would thus be expected to preferentially affect the sensorimotor striatum.

At the cellular level, GPR88 mRNA expression was restricted to NeuN-positive cells in all age groups, thus demonstrating exclusively neuronal expression, consistent with a recent study by Massart and collaborators that used a new GPR88 polyclonal antibody to localize GPR88 protein [13]. GPR88 mRNA expression in all

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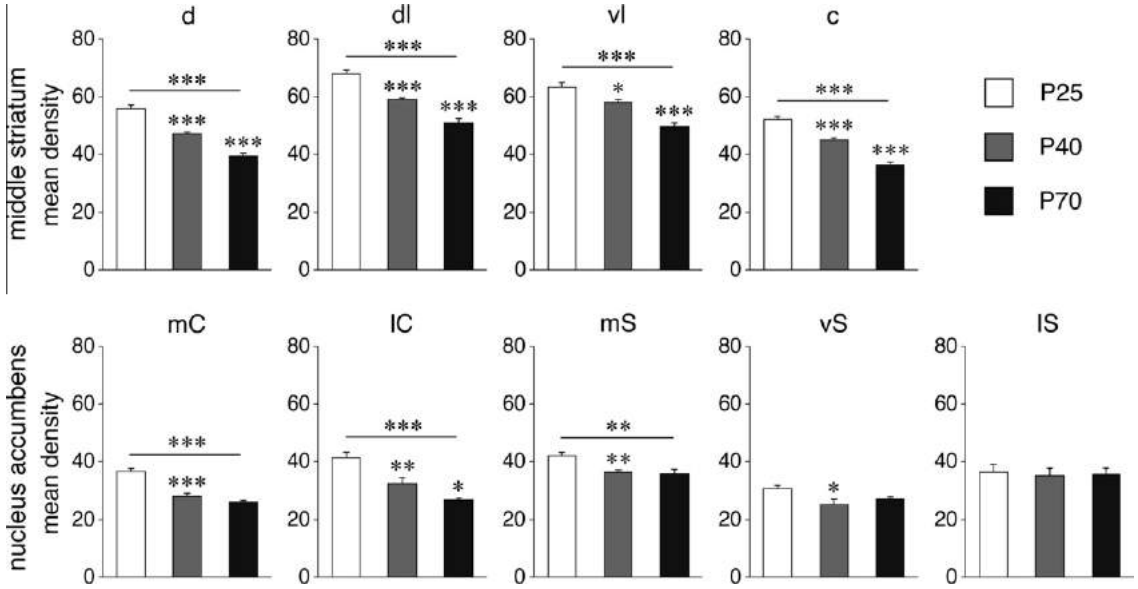

Fig. 5. Expression of GPR88 across development in select areas of the striatum and nucleus accumbens. Mean density values (mean ± SEM) for GPR88 mRNA levels in juvenile (P25), adolescent (P40) and adult (P70) rats are depicted for 4 sectors from the middle striatum (top) and the 5 sectors of the nucleus accumbens (bottom). Caudate–putamen: d, dorsal; dl, dorsolateral; vl, ventrolateral; c, central. Nucleus accumbens: mC, medial core; lC, lateral core; mS, medial shell; vS, ventral shell; lS, lateral shell. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, vs. preceding age group or as indicated.

striatal neurons indicates that GPR88 is localized in both projection neuron types as well as interneurons. This is in agreement with the main observation in the above immunohistochemical study that showed robust GPR88 expression in striatopallidal and striatonigral projection neurons [13]. However, our findings differ in part from this study regarding labeling of interneurons. Although Massart et al. [13] also found a (weak) immuno-signal in parvalbumin-positive interneurons (the most numerous type), they reported lack of a signal in interneurons double-labeled for somatostatin, calretinin and choline acetyltransferase (cholinergic interneurons). The reasons for this discrepancy are presently unclear. The lack of an immuno-signal in some interneurons may indicate a lack of GPR88 mRNA translation to GPR88 protein in these interneurons. Alternatively, this difference may reflect a greater sensitivity of the present in situ hybridization histochemistry. For example, we did consistently find a GPR88 signal in large neurons, which had the size of cholinergic interneurons [8], and the GPR88 mRNA levels in these neurons tended to be in the lowest range of labeling in medium-sized neurons. Thus, our findings, together with the lack of GPR88 labeling in the globus pallidus (negative control), argue for GPR88 expression in striatal projection neurons and interneurons.

Developmental regulation of GPR88 expression

Our study is the first to describe the developmental regulation of GPR88 expression in the striatum. In almost all striatal regions, the expression decreases from juveniles to adolescents to adults. This pattern is reminiscent of developmental changes for other G-protein-coupled receptors. For example, D1 and D2 receptors are over-expressed before puberty and are then pruned back to adult levels [24–26]. A similar pattern was observed for adenosine A2A receptors [22]. While not all of these receptors show identical early changes, the regulation of GPR88 resembles that of D1 and D2 receptor expression between adolescence and adulthood. Similar to D1 and D2 receptors [24,26], GPR88 expression is also differentially regulated between caudate–putamen and nucleus accumbens during this developmental period. In fact, such differential developmental trajectories in anatomically related, but functionally different, brain regions are not uncommon for metabotropic receptors. For example, we have shown that CB1 cannabinoid receptors in the cortex, which are also downregulated from prepuberty to adulthood, are differentially regulated in limbic/associative versus sensorimotor cortical areas [27].

In summary, these findings indicate that, similar to a number of G-protein-coupled receptors, GPR88 undergoes pruning across postnatal development, but that the exact trajectory is brain region-specific. Therefore, these findings suggest that the effects of GPR88-selective drugs would be, at least to some degree, age- and region-dependent.

Is GPR88 a G-protein-coupled receptor?

GPR88 was initially proposed to be an orphan G-protein-coupled receptor based on its predicted primary protein sequence, which suggests seven transmembrane domains, as indicated by hydrophobicity analysis [12]. According to this study, the amino acid sequence of GPR88 shows significant homology with ß-3 adrenergic and 5HT1D receptors [12]. Based on an analysis of the chemical structure of the putative transmembrane domains, a more recent study clustered GPR88 with metabotropic glutamate and GABA-B receptors [28]. The genomic organization of the human and mouse GPR88 gene was also found to be very similar to that of a number of G-protein-coupled receptors, including several muscarinic, histamine (H1), and dopamine (D1, D5) receptors [12]. Our finding of a developmental regulation similar to that of other G-protein-coupled receptors (early over-expression, followed by pruning; see above) thus extends the list of similarities between GPR88 and such receptors.

A recent ultrastructural study supports an association of GPR88 protein with postsynaptic sites [13]. This study showed that GPR88 immunolabeling is concentrated along the somatodendritic surface of striatal projection neurons, with a pronounced preference for dendrites and dendritic spines. Within dendrites, GPR88 protein was localized in the postsynaptic densities of mostly asymmetrical synapses contacted by terminals immunoreactive for the vesicular glutamate transporter (VGluT) 1, but not VGluT2 or tyrosine hydroxylase [13]. These findings indicate that GPR88 is preferen-

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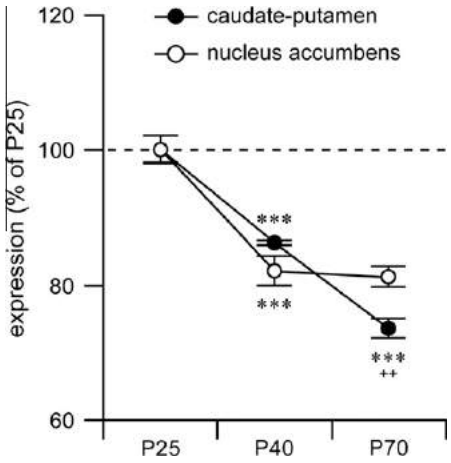

Fig. 6. Differential regulation of GPR88 expression in the caudate–putamen and nucleus accumbens across development. Normalized mean density values (relative to P25; mean ± SEM) for GPR88 mRNA levels in the whole caudate–putamen (all sectors pooled, filled circles) and the whole nucleus accumbens (open circles) at P25, P40 and P70 are shown. $^{***}P < 0.001$, vs. preceding age group; $^{++}P < 0.01$, caudate–putamen vs. nucleus accumbens.

tially associated with synapses receiving glutamate input from the cortex, but not glutamate input from the thalamus (VGluT2), GABA or dopamine inputs.

However, other characteristics of GPR88 are unusual for a functional G-protein-coupled receptor. In particular, the predicted GPR88 product lacks the tripeptide motif DRY at the intracellular boundary of the transmembrane domain 3 [12], which seems to be critical for receptor activation [29,30]. Also lacking are cysteine residues that are necessary to form disulfide bonds between extracellular loops, as is often seen in biogenic amine receptors [30]. Therefore, GPR88 may be a novel kind of G-protein-coupled receptor. Alternatively, given the apparent futility of a more than 10-year search for an endogenous ligand despite the profound interest in putative orphan G-protein-coupled receptors as potential drug targets, GPR88 may be another type [31] of membrane-bound molecule associated with synaptic signaling.

Conclusions

GPR88 may be an attractive target for pharmacological interventions in pathologies involving the striatum due to its restricted localization to this brain region. Here, we provide a description of the cellular localization of GPR88, indicating that GPR88 mRNA is expressed in neurons of both direct and indirect striatal output pathways, as well as in interneurons. Furthermore, we show that the developmental regulation of GPR88 expression is similar to that of G-protein-coupled receptors. The functional consequences of this GPR88 regulation over the postnatal development remain to be determined.

Acknowledgements

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Repeated Transcranial Direct Current Stimulation Prevents Abnormal Behaviors Associated with Abstinence from Chronic Nicotine Consumption

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Successful available treatments to quit smoking remain scarce. Recently, the potential of transcranial direct current stimulation (tDCS) as a tool to reduce craving for nicotine has gained interest. However, there is no documented animal model to assess the neurobiological mechanisms of tDCS on addiction-related behaviors. To address this topic, we have developed a model of repeated tDCS in mice and used it to validate its effectiveness in relieving nicotine addiction. Anodal repeated tDCS was applied over the frontal cortex of Swiss female mice. The stimulation electrode (anode) was fixed directly onto the cranium, and the reference electrode was placed onto the ventral thorax. A 2 × 20 min/day stimulation paradigm for five consecutive days was used (0.2 mA). In the first study, we screened for behaviors altered by the stimulation. Second, we tested whether tDCS could alleviate abnormal behaviors associated with abstinence from nicotine consumption. In naive animals, repeated tDCS had antidepressant-like properties 3 weeks after the last stimulation, improved working memory, and decreased conditioned place preference for nicotine without affecting locomotor activity and anxiety-related behavior. Importantly, abnormal behaviors associated with chronic nicotine exposure (ie, depression-like behavior, increase in nicotine-induced place preference) were normalized by repeated tDCS. Our data show for the first time in an animal model that repeated tDCS is a promising, non-expensive clinical tool that could be used to reduce smoking craving and facilitate smoking cessation. Our animal model will be useful to investigate the mechanisms underlying the effects of tDCS on addiction and other psychiatric disorders.
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INTRODUCTION

Transcranial direct current stimulation (tDCS) is a neuromodulatory technique that consists in stimulating the cerebral cortex with a weak constant electric current in a non-invasive and painless manner. Since a decade ago, tDCS has been used experimentally to treat several psychiatric disorders, including depressive symptoms (Brunoni *et al*, 2012; Nitsche *et al*, 2009). The mechanisms underlying its effects are not well understood, but early studies in animals using current directly applied to the cortex suggest that anodal stimulation causes a depolarization of the resting membrane potential and increases the firing rates of cortical neurons in the tissue under the electrode (Bindman *et al*, 1964; Purpura and McMurtry, 1965; Stagg and Nitsche, 2011).

Recently, the use of tDCS-induced modulation of cortical excitability has gained interest in the scientific community as a means to decrease maladaptive behaviors in drug-dependent patients (Feil and Zangen, 2010). Two preliminary clinical studies carried out in chronic smokers support/exemplify tDCS efficacy in relieving addiction-related behaviors (Boggio *et al*, 2009; Fregni *et al*, 2008). In these studies, the dorsolateral prefrontal cortex (DLPFC) was targeted because smoking craving processing has been associated with enhanced activity of this area (Brody *et al*, 2002; Due *et al*, 2002; McBride *et al*, 2006; Wilson *et al*, 2004). In the first clinical trial, Fregni *et al* (2008) showed that a single-tDCS session over the DLPFC reduced cue-induced smoking craving in tobacco users. Participants received three different types of tDCS: sham tDCS, anodal tDCS of the left DLPFC, and anodal tDCS of the right DLPFC (a single session of 2 mA for 20 min). Before and right after the electrical stimulation, they completed a visual analog scale (VAS) to evaluate mood and a nicotine-based VAS to measure craving levels. The authors found that stimulation of both left and right DLPFC with active, but not sham, tDCS reduced general and smoking cue-induced nicotine craving with no other significant mood changes associated with the tDCS treatment. In the second study (Boggio *et al*, 2009), chronic smokers were randomized to receive either sham tDCS or active anodal tDCS of the left DLPFC for five

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consecutive days (2 mA, 1 session of 20 min per day). The results indicated a cumulative effect of tDCS such that the magnitude of tDCS on reducing cue-induced craving increased after each session. Nonetheless, the duration of this phenomenon was not evaluated in this work.

These preliminary findings are very encouraging because current available treatment options for smoking cessation remain limited and/or are associated with poor long-term success rates (O'Brien, 2008). Several approaches, such as nicotine replacement therapy (patch, chewing gum, electronic cigarette), drugs (eg, brupopion, varenicline), and psychotherapy (behavioral and motivational interventions) are used for the purpose of decreasing smoking craving (see Stead and Lancaster, 2012); however, their relative poor efficacy and potential drug side effects limit the use of these methods. In this context, electrical stimulation of specific brain regions appears as innovative and attractive technique worth of further investigation to reduce smoking craving (Fraser and Rosen, 2012).

In spite of these promising results, a suitable experimental animal model with translational value is presently lacking in order to investigate the mechanisms underlying tDCS effects on addiction-related behavior. The aim of the present work is to characterize an animal model that can be used to study the neurobiological mechanisms associated with the efficacy of the tDCS treatment. The first step toward this goal is to demonstrate that protocols of tDCS in laboratory animal can result in positive/negative outcomes on a variety of behavioral measures that are clinically relevant. On the basis of the work of Liebetanz *et al* (2006; 2009), we have developed a model of repeated tDCS in mice with an experimental paradigm similar to that used in clinical trials. In a first experiment, we screened naive animals (never exposed to nicotine) for behaviors altered by repeated tDCS (depression, anxiety, memory, and reinforcing effect of nicotine). In a second set of experiments, we tested whether tDCS could alleviate behaviors associated with abstinence from chronic nicotine consumption during adolescence (postnatal day, PND 30–43), a period of high vulnerability to nicotine exposure (Iniguez *et al*, 2009). Our data show for the first time in an animal model that repeated tDCS has antidepressant properties and decreases the reinforcing effect of nicotine consistent with the outcomes observed after repeated tDCS in humans.

MATERIALS AND METHODS

Subjects

Swiss female mice (Janvier, Laval, France) were housed 4–5 per cage under standard laboratory conditions (12:12-hour light/dark cycle; lights on at 0700 hours) with food and water available *ad libitum*. Before the surgery, mice were allowed 1 week of acclimation, during which they were repeatedly handled. All procedures met the NIH guidelines for the care and use of laboratory animals and were approved by the University of Franche-Comté Animal Care and Use Committee.

Surgery

A tubular plastic jacket (internal diameter: 2.1 mm) was surgically fixed onto the skull 1 week before the stimulation protocol (Figure 1). Animals were anesthetized with ketamine hydrochloride/xylazine (80 mg/12 mg/kg, respectively; i.p.) and were placed in a stereotaxic apparatus. The center of the plastic jacket was positioned over the left frontal cortex 1 mm anterior to the coronal fissure and 1 mm left of the sagittal fissure (Figure 1b) and fixed with a coating of glass ionomer cement (GC Fuji I, Leuven, Belgium). After surgery, all animals were allowed to recover for 1 week before undergoing tDCS. During this period as well as during the electrical stimulations, mice were placed in individual cages. Experimental design of experiments 1 and 2 is summarized in Figures 2a and 3a.

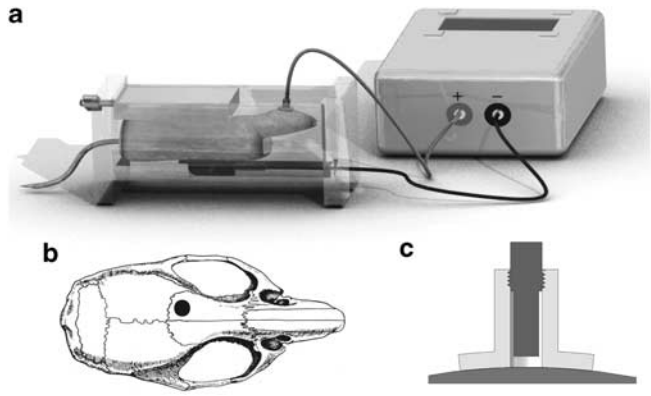

Figure 1 Illustration of the tDCS device used to deliver the current stimulation. (a) The mouse is placed in a custom-made restraint box. The anode (contact area 3.5 mm^2) is positioned over the left frontal cortex and the cathode (rubber-plate electrode, 9.5 cm^2) onto the ventral thorax. A 2×20 min/day constant current of 0.2 mA is applied transcranially using a direct current stimulator (DC-Stimulator Plus) for five consecutive days, with a linear fade in/fade out of 10s. (b) The center of the electrode is positioned over the left frontal cortex 1 mm anterior to the coronal fissure and 1 mm left of the sagittal fissure (adapted from Paxinos and Franklin, 2001). (c) Anode: a tubular plastic jacket (internal diameter: 2.1 mm) is surgically fixed onto the skull and filled with saline solution before the stimulation. The stimulation electrode is screwed into the tubular plastic jacket and dip in the saline solution. Only the saline solution is in contact with the skull.

Stimulation Protocol

The jacket was filled with saline solution (NaCl 0.9%) before the stimulation to establish a contact area of 3.5 mm^2 toward the skull. The stimulation electrode (anode, Figure 1c, DIXI Medical, Besançon, France) was screwed into the tubular plastic jacket. A larger conventional rubber-plate electrode (cathode, 9.5 cm^2; Physiomed Elektromedizin AG, Schnaittach, Germany) served as the counter-electrode and was placed onto the ventral thorax (adapted from Liebetanz *et al*, 2009, Figure 1a). This setting prevented the bypassing of currents (shunting effect) that would occur in the case of two juxtaposed encephalic electrodes in mice. An anodal 2×20 min/day constant current of 0.2 mA was applied transcranially over the frontal cortex using a DC-Stimulator Plus (NeuroConn, Ilnemau, Germany) for five consecutive days, with a linear fade in/fade out of 10s (Ferrucci *et al*, 2009; Rigonatti *et al*, 2008). Animals were awake and restrained during the tDCS (Figure 1a) to prevent possible interactions between tDCS effects and anesthetic drugs. Control animals were subjected

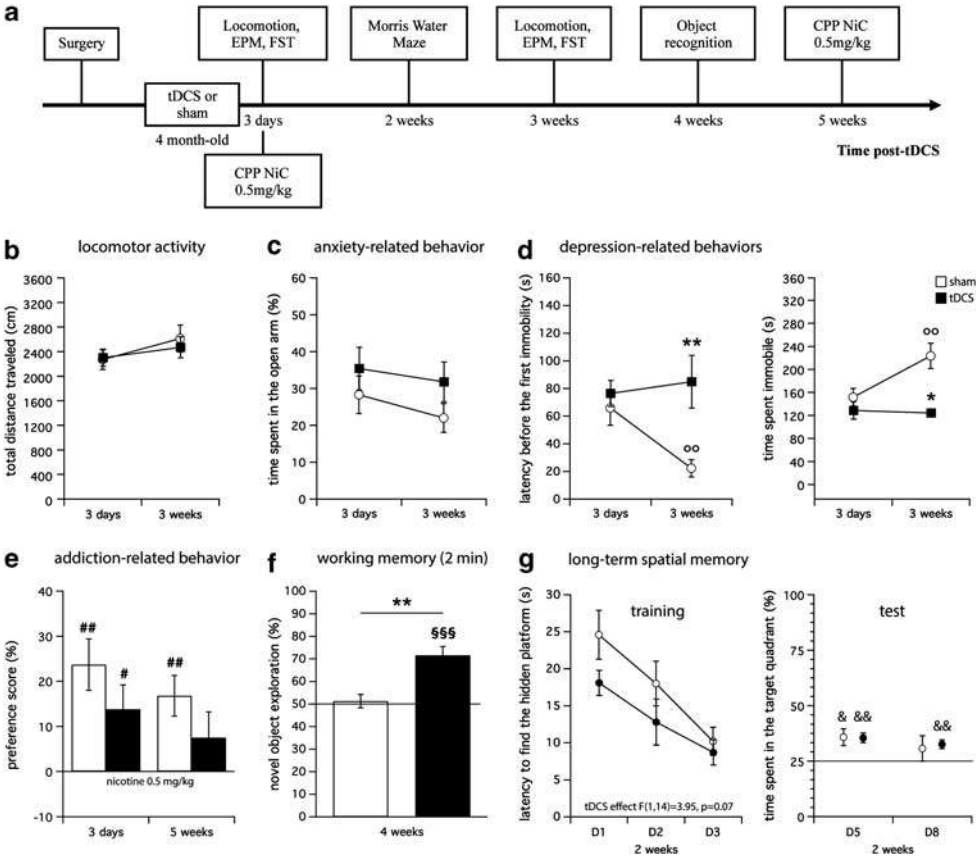

Figure 2 Experiment 1. (a) Experimental design. Four-month-old Swiss female mice never exposed to nicotine were subjected to repeated anodal tDCS for five consecutive days (2 × 20 min/day constant current, 0.2 mA). Behavioral effects of tDCS were screened from 3 days to 5 weeks following the last electrical stimulation. The same animals were used in all behavioral tests (sham $N = 8$, tDCS $N = 8$) except in the CPP test at 3 days for which another batch of mice was used (sham $N = 10$, tDCS $N = 10$). EPM: elevated plus maze, FST: forced swim test, CPP: conditioned place preference, NiC: nicotine. Effect of repeated anodal tDCS on (b) locomotor activity, (c) anxiety-related behavior, (d) depression-related behaviors, and (e) addiction-related behavior 3 days and 3–5 weeks following the last electrical stimulation. (f) Effect of repeated anodal tDCS on working memory 4 weeks after the last stimulation and (g) effect of repeated anodal tDCS on long-term spatial memory (training, left; test, right) 2 weeks after the last stimulation. $^{*}p < 0.05$ and $^{**}p < 0.01$ *vs* sham, $^{\circ\circ}p < 0.01$ 3 days *vs* 3 weeks, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ *vs* 0%, $^{\S\S\S}p < 0.001$ *vs* 50%, $^{\&}p < 0.05$ and $^{\&\&}p < 0.01$ *vs* 25%.

to the same procedure (surgeries, restraint box, electrode montage), but current was not delivered.

Nicotine Treatment during Adolescence

Adolescent mice (PND 30–43) were treated twice a day for 14 consecutive days with nicotine (1 mg/kg i.p. in 0.2 ml, time between injections: 6 h; (−)-nicotine hydrogen tartrate salt, SIGMA, France; Figure 3a). Control mice were injected with NaCl 0.9% following the same schedule.

Behavioral Tests

Body weight, food, and water consumption. The weight of mice and food/fluid intake during the 24-h period were monitored throughout the experiments.

Locomotor activity. A system that automatically analyzes locomotor activity of mice in a cage similar to their home cage (Activmeter, Bioseb, France) was used to record the total distance traveled (in cm) by the mice during 16 min. This system consists of a transparent plastic cage (17 × 21 × 14 cm) and uses cage vibrations to measure locomotion.

Elevated plus maze. Anxiety-related behavior was examined in the elevated plus maze (EPM) test (Walf and Frye, 2007). The arms (two open without walls and two enclosed by 45 cm high walls) were 30 cm long, 6 cm wide, and raised off the floor by 50 cm. Each animal was placed in the center square of the apparatus facing a closed arm and was allowed to move freely for 5 min. The test was recorded with a video camera and analyzed using the Ethovision system (video-tracking, Noldus, Paris, France). The parameter used to assess anxiety-related behavior was the percentage of time spent in the open arms during the 5 min.

Forced swim test. We adapted the forced swim test (FST) method from Porsolt *et al* (1977). Each mouse was placed into a beaker (height 26 cm, diameter 18 cm) containing water at a temperature of 32 ± 2 °C and a depth of 17 cm so that the mouse could neither escape nor touch the bottom. Each test lasted 6 min and was video recorded for subsequent scoring by a blind observer of the latency before the first episode of immobility and the total time spent immobile. Mice were considered immobile when they ceased struggling and remained floating motionless in the water for at least 2 s.

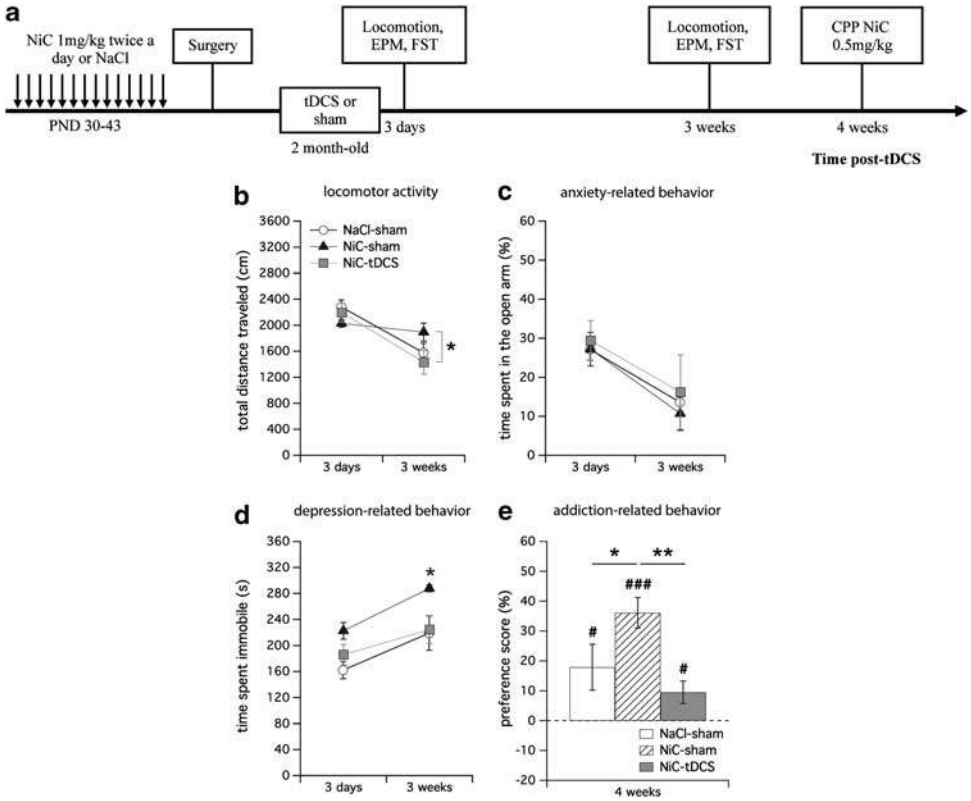

Figure 3 Experiment 2. (a) Experimental design. Adolescent mice were treated twice a day for 14 consecutive days with nicotine (1 mg/kg i.p.) postnatal day (PND) 30–43. Control mice were injected with NaCl 0.9% following the same schedule. Nicotine and NaCl-treated animals were submitted to repeated anodal tDCS or sham stimulations for five days (PND 60–64). Animals were then tested for locomotor activity, anxiety- and depression-related behaviors, and for their sensitivity to the rewarding effects of nicotine. CPP, conditioned place preference; EPM, elevated plus maze; FST, forced swim test; NiC, nicotine. NaCl-sham ($N=10$), NiC-sham ($N=9$), NiC-tDCS ($N=10$). (b) Effect of nicotine exposure during adolescence and subsequent repeated anodal tDCS on locomotor activity, (c) anxiety-related behavior, (d) depression-related behaviors, and (e) addiction-related behavior 3 days and 3-4 weeks following the last electrical stimulation. $^{*}p<0.05$ and $^{**}p<0.01$ *vs* NaCl-sham or as indicated, $^{\#}p<0.05$ and $^{\#\#\#}p<0.001$ *vs* 0%.

Novel object recognition task. Two objects (figurines) with different forms and colors were used for the experiments. There were two copies of each object. A preliminary test was carried out to verify there was no preexisting preference for any of the figurines. Mice were habituated to the empty test arena (diameter: 47 cm) 10 min per day for four consecutive days before the object recognition test. During the exposure phase, two identical copies of the sample object were placed in the arena, and mice were allowed to explore the objects for 10 min. After an intertrial interval of 2 min in the home cage, mice were placed again in the test arena for 5 min (test phase). During this phase, the arena contained one object used in the exposure phase and one novel object. The arena and objects were wiped down with 70% ethanol between trials to minimize olfactory cues. Novel object exploration during the test phase was determined as the percentage of time spent with the nose not more than 1 cm away from the novel object divided by the total time spent to explore the two objects. The test was recorded with a video camera and analyzed using the Ethovision system.

Morris water maze. A circular pool (diameter: 130 cm, height: 30 cm) was filled to a depth of 10 cm with water ($32\pm2\,^{\circ}$C) and placed in a room with visual cues. Over three consecutive days, mice were given 12 training trials per day. A clear platform (diameter: 9 cm) was placed at the midpoint of one quadrant, submerged 0.5 cm below the water surface, and fixed in the same place throughout the training trials. The point of entry of the mouse into the pool was randomized. When a mouse located the platform, it was allowed to remain on it for 20 s. If the mouse could not locate the platform within 60 s, it was gently navigated to the platform and remained on it for 20 s. The parameter evaluated was the latency to find the hidden platform in seconds. A test trial was then carried out 2 and 5 days after the last training trial. During the test trial, the platform was removed, and mice were allowed to swim freely for 60 s. The percentage of time spent in the target quadrant (containing the hidden platform during the training trials) was recorded and compared with 25%. The test was monitored using the Ethovision system.

Nicotine-induced conditioned place preference. The conditioned place preference apparatus consists of two main compartments linked by a corridor displaying each different features: visual (wall patterns) and tactile (floor texture). On day 1 (pre-conditioning), mice were placed in the corridor and allowed free access to the compartments for 10 min. The time spent in each compartment was recorded using the

Ethovision system. On days 2–4 (conditioning phase) mice received injections of nicotine (0.5 mg/kg, i.p.) or vehicle and were immediately confined into one of the two conditioning compartments for 15 min (drug pairing was biased to the least preferred chamber). On day 5 (post-conditioning), mice were once again allowed free access to the compartments for 10 min. Percentage of time spent in the drug-paired compartment was calculated during the pre-conditioning and the post-conditioning phases as follows: drug-paired compartment (s)/(drug-paired compartment (s) + NaCl-paired compartment (s)) × 100. The preference score was determined as the difference between the percentages of time spent in the drug-paired compartment during the post-conditioning and pre-conditioning sessions.

Statistical Analysis

The results were expressed as mean ± SEM. Significance was set at $p \leqslant 0.05$. Repeated analysis of variance (ANOVA) was used to analyze body weight, food/fluid consumption, locomotor activity, anxiety-, and depression-related behaviors with group as between-subject variable (experiment 1: sham, tDCS; experiment 2: NaCl-sham, NiC-sham, Nic-tDCS) and time as within-subject variable (3 days, 3 weeks). Repeated ANOVA was also used to analyze long-term spatial memory in experiment 1 with tDCS as between-subject variable (sham, tDCS) and time as within-subject variable (D1, D2, D3; D5, D8). Student's *t*-test (experiment 1) or one-way ANOVA (experiment 2) was used to analyze addiction-related behavior at 3 days, 4, or 5 weeks. Newman–Keuls *post hoc* tests were used to describe differences between individual groups. Student's *t*-tests were also used to compare the means of each group with a standard value (ie, 0% for the conditioned place preference, 50% for the novel object recognition task, and 25% for the test trial in the Morris water maze).

RESULTS

Experiment 1

Body weight, locomotor activity, and anxiety-related behavior. tDCS had no impact on body weight or on food and fluid consumption 3 days and 3 weeks after the last electrical stimulation (data not shown). Neither locomotor activity (total distance traveled during 16 min, Figure 2b), nor anxiety-related behavior (percentage of time spent in the open arm of the EPM during 5 min, Figure 2c) was affected by tDCS. Although tDCS caused a slight drop in anxiety-related behavior (Figure 2c), this effect was not significant (ANOVA tDCS effect: $F(1,14) = 1.93$, $p = 0.19$; time effect: $F(1,14) = 1.57$, $p = 0.23$; tDCS x time interaction: $F(1,14) = 0.11$, $p = 0.74$).

Depression-related behaviors. In the FST, tDCS differently affected the latency before the first immobility (Figure 2d, left) and the immobility time (Figure 2d, right) depending on the time post stimulation (ANOVA tDCS x time interaction: $F(1,14) = 7.67$, $p < 0.05$ and $F(1,14) = 7.14$, $p < 0.05$, respectively). *Post hoc* Newman–Keuls analysis revealed that, relative to sham controls, tDCS significantly increased the latency before the first immobility (Figure 2d, left, $^{**}p < 0.01$) and decreased immobility time (Figure 2d, right, $^{*}p < 0.05$) 3 weeks after the last stimulation, but not after 3 days (all $p > 0.05$). The sham group was affected by the repetition of the test as evidenced by a significant decrease in the latency before the first immobility and increase in the time spent immobile between 3 days and 3 weeks ($^{\circ\circ}p < 0.01$, Figure 2d). A complementary experiment with a different batch of mice tested only 3 weeks after the last stimulation confirmed the antidepressive-like property of tDCS in the FST (data not shown).

Addiction-related behavior. Nicotine (0.5 mg/kg) induced a significant place preference in the sham group 3 days ($p < 0.01$ *vs* 0%, Figure 2e) and 5 weeks ($p < 0.01$ *vs* 0%) following sham stimulations. Animals submitted to repeated anodal tDCS also displayed a place preference for nicotine 3 days after the last stimulation ($p < 0.05$ *vs* 0%). However, 3 weeks after the last stimulation, the preference score for nicotine was not significantly different from 0% ($p = 0.23$). Student's *t*-test did not reveal a significant direct difference between sham and tDCS groups.

Working memory. The percentage of exploration of the novel object during the test phase was not significantly different from 50% in the sham group, indicating that animals did not differentiate the novel object from the object already presented with an intertrial interval of 2 min (sham group: $p = 0.68$ *vs* 50%, Figure 2f). In contrast, mice exposed to active tDCS explored significantly more the novel object than the object already presented (tDCS group: $p < 0.001$ *vs* 50%).

Long-term spatial memory. There was only a trend for a beneficial global effect of tDCS on learning performances (ANOVA tDCS effect: $F(1,14) = 3.95$, $p = 0.07$, Figure 2g, left). This effect was reflected in the test trials in which both groups spent significantly more time (compared to 25%) in the quadrant associated with the platform (target quadrant) than in the other quadrants at day 5 (all $p < 0.05$) but not at day 8 when only the tDCS group performed well (sham $p = 0.35$, tDCS $p < 0.01$ *vs* 25%; Figure 2g, right). Repeated ANOVA, however, did not reveal any significant effect of tDCS on spatial memory in the test trials (ANOVA tDCS effect: $F(1,14) = 0.03$, $p = 0.86$, time effect: $F(1,14) = 2.23$, $p = 0.16$, tDCS x time interaction: $F(1,14) = 0.17$, $p = 0.68$).

Experiment 2

Locomotor activity and anxiety-related behavior. Locomotor activity in adults tended to be affected differently by the group depending on the time post stimulation (ANOVA group x time interaction: $F(2,26) = 3.09$, $p = 0.06$, Figure 3b). *Post hoc* Newman–Keuls analysis indicated that tDCS significantly reduced locomotor activity in nicotine-treated animals 3 weeks after the last stimulation (NiC-sham *vs* NiC-tDCS, $p < 0.05$). The percentage of time spent in the open arm of the EPM after nicotine withdrawal was not affected by the group (ANOVA group effect: $F(2,26) = 0.27$, $p = 0.77$, group × time interaction: $F(2,26) = 0.05$, $p = 0.95$, Figure 3c).

Depression-related behaviors. The time spent immobile in the FST during adulthood was significantly affected by the experimental conditions (ANOVA group effect: $F(2,26) = 4.66$, $p < 0.05$, Figure 3d). *Post hoc* Newman–Keuls analysis revealed that nicotine exposure during adolescence increased the time spent immobile in the FST during adulthood (NaCl-sham *vs* NiC-sham, $p < 0.05$). Exposure to repeated tDCS normalized this parameter (NiC-sham *vs* NiC-tDCS, $p < 0.05$, NaCl-sham *vs* NiC-tDCS, $p = 0.51$). When time was taken into consideration, tDCS significantly reduced the immobility in nicotine-pretreated animal only 3 weeks after the stimulation (NaCl-sham *vs* NiC-sham, $p < 0.05$ and NiC-sham *vs* NiC-tDCS at 3 weeks, $p < 0.05$). Three weeks after the stimulation, animals exposed to nicotine during adolescence and stimulated during the withdrawal period did not differ from animals never exposed to nicotine and not stimulated (NiC-tDCS *vs* NaCl-sham, $p = 0.98$).

Addiction-related behavior. As in experiment 1, injections of nicotine (0.5 mg/kg, i.p) induced a place preference in the sham group never exposed to nicotine (NaCl-sham, $p < 0.05$ *vs* 0%, Figure 3e). Exposure to nicotine during adolescence robustly increased the place preference induced by nicotine in adults (ANOVA group effect: $F(2,26) = 5.21$, $p < 0.05$; Nic-sham *vs* NaCl-sham, $p < 0.05$; *vs* 0%, $p < 0.001$). tDCS significantly reduced nicotine-induced place preference in nicotine-pretreated animals (NiC-sham *vs* Nic-tDCS, $p < 0.01$). Animals exposed to nicotine during adolescence and stimulated during the withdrawal period did not differ from animals never exposed to nicotine and not stimulated (Nic-tDCS *vs* 0%, $p < 0.05$; NiC-tDCS *vs* NaCl-sham, $p = 0.32$).

DISCUSSION

The present study is the first to show that repeated anodal tDCS over the frontal lobe induces long-lasting modulation of the behavior in mice. Notably, tDCS decreases abnormal behaviors associated with abstinence in an animal model of chronic nicotine consumption. These results provide important preclinical evidence for the use of electrical brain stimulation in promoting smoking cessation and potentially ameliorating other addiction-related behaviors in dependent patients (Boggio *et al*, 2009; Fregni *et al*, 2008).

There is a growing body of evidence to suggest that tDCS has an antidepressant effect in humans (for review see Brunoni *et al*, 2012; Nitsche *et al*, 2009), but, to the best of our knowledge, this has never been shown in an animal model. Our study demonstrates that repeated anodal tDCS *per se* produces significant antidepressant-like effects in mice in the most frequently used test of learned helplessness (3 weeks after the stimulations). This effect seems relatively specific as body weight, food/drink consumption, locomotor activity, and anxiety-related behavior were unaffected both 3 days and 3 weeks following the treatment. It is known that the 'acute' physiological effects of a single tDCS session are not limited to modulation of cortical excitability during stimulation and may outlast the stimulation period by several minutes or even hours (Bindman and Richardson, 1969; Nitsche *et al*, 2003; Nitsche and Paulus, 2000; Ohn *et al*, 2008); however, the long-lasting effects of repeated stimulation on behavior have never been reported before. The effects of such repeated sessions of anodal tDCS on the immobility in the FST were present at 3 weeks but absent 3 days after the last stimulation. Interestingly, this delayed response is reminiscent of the delayed therapeutic onset of antidepressants typically reported in humans (Lam, 2012). However, in rodents, the behavioral effects of antidepressant drugs (eg, selective serotonine reuptake inhibitors, SSRIs) are usually observed acutely after a single injection, suggesting different mechanisms of action between SSRI antidepressants and tDCS.

Repeated anodal tDCS had minimal impact on spatial learning and memory in the Morris Water Maze. Conversely, our stimulation protocol significantly improved mice performance in a working memory task. This result is in accordance with a growing number of studies, indicating an improvement of working memory by anodal tDCS (Boggio *et al*, 2006; Fregni *et al*, 2005; Ohn *et al*, 2008). In a recent work, Zaehle *et al* (2011) studied working memory function in humans several minutes after a single tDCS over the DLPFC (15 min, 1mA) in combination with neurophysiological methods. Working memory was improved, and oscillatory brain activity was affected, as evidenced by amplified oscillatory power in the theta and alpha bands after the stimulation. Our study assessed for the first time the impact of repeated anodal tDCS (2 sessions per days, 5 consecutive days) and demonstrates that this protocol has long-lasting beneficial consequences in working memory of mice for at least 4 weeks. Longer time points remain to be tested to determine more precisely the duration of this effect.

Antidepressants can be used to facilitate smoking cessation (Hughes *et al*, 2007). What about tDCS? Chronic exposure to nicotine during adolescence induces depression-related behavior in adult rats (1 month after nicotine withdrawal (Iniguez *et al*, 2009)). Interestingly, Iniguez *et al* (2009) demonstrated that the altered behaviors observed after nicotine withdrawal can be prevented in rats by either subsequent re-exposure to nicotine or antidepressant treatment (fluoxetine or bupropion; 10 mg/kg). Similarly, our results show that mice exposed to nicotine during adolescence display increased immobility time in the FST after nicotine withdrawal. In agreement with the previous work, this alteration was normalized by our repeated anodal tDCS protocol. Our study also shows that exposure to nicotine during adolescence dramatically increases nicotine-induced place preference (0.5 mg/kg) in adult mice, an effect that was completely abolished by the electrical stimulations.

In the brain, nicotine binds to nicotinic cholinergic receptors, which are ligand-gated ion channels that normally bind acetylcholine. Stimulation of nicotinic cholinergic receptors is known to evoke dopamine release in the cortical and subcortical dopaminergic systems, which are critical for the reinforcing effects of nicotine. This effect can be direct *via* the activation of dopaminergic neurons or indirect *via* the stimulation of other neurotransmitters' release including glutamate. Animal experiments have shown that descending pathways from the frontal cortex modulate the release of dopamine in subcortical areas such as the striatum (Karreman and Moghaddam, 1996; Taber

and Fibiger, 1993, 1995). There is evidence that this occurs both directly via glutamatergic corticostriatal projections (Taber and Fibiger, 1995) and indirectly through an effect on mesostriatal dopamine neurons in the midbrain (Karreman and Moghaddam, 1996). As a possible mechanism of action, we hypothesize that anodal tDCS could promote a direct or indirect modulation of subcortical dopamine release involved in nicotine addiction. However, to definitively conclude that tDCS impacts nicotine consumption, it will be necessary to substantiate our findings in an intravenous self-administration paradigm.

Important questions remain to be answered, such as whether tDCS effects are specific for the reinforcing effect of nicotine, whether it would also work for other drugs of abuse (eg, alcohol, psychostimulants), and whether it affects the response to natural rewards (eg, sexual activity, appetite). Our preliminary data suggest that the reinforcing effect of food is not altered by transcranial stimulations (no change in food/fluid consumption), but more detailed work is necessary to address this question. A limit in our work is that all of our experiments were carried out in female mice; therefore, additional studies are warranted to determine if our findings can be extrapolated to male mice. A final important consideration is the equivalence of our stimulation paradigm in animal *vs* humans. Indeed, our stimulation protocol (time, length, repetition) is the same as the one used in clinical trials, but the intensity is lower: 0.2 mA *vs* 2 mA. However, in our animal model, the current density is much more elevated due to the small size of the electrode (57.1 *vs* 0.57 A/m^2 for clinical trials). This is of importance because the area stimulated by the current might be significantly different in mice and in humans, in particular if the size of the brain is taken into account. Future studies will need to determine the minimal amount of current necessary in our model to maintain the efficiency in alleviating abnormal behaviors associated with chronic nicotine consumption, to explore the importance of the polarity, and to assess whether the effects are specific to the area stimulated or simply due to a generalized stimulation of the mouse brain.

Taken together, our findings indicate that (1) our tDCS protocol results in behavioral outcomes similar to the ones observed in clinical trials, (2) exposure to nicotine during adolescence promotes abnormal behaviors during adulthood (depressive-like behavior, increase in the rewarding effect of nicotine), and (3) that this detrimental effect might be prevented by repeated anodal tDCS treatment. Our results also highlight the time dependence of the tDCS effects, which seems to be more prominent 3 weeks after the last tDCS session. On the basis of the research presented here, our experimental animal model provides a framework to investigate the effects of tDCS on smoking craving and, more importantly, to explore the neurobiological changes that underlie the beneficial effects of tDCS on this and other addiction-related behaviors.

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Publication 10

Addiction Biology

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ORIGINAL ARTICLE

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Transcranial direct current stimulation produces long-lasting attenuation of cocaine-induced behavioral responses and gene regulation in corticostriatal circuits

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ABSTRACT

Transcranial direct current stimulation (tDCS) is a non-invasive method to modulate cortical excitability. This technique is a promising emerging tool to treat several neuropathologies, including addiction. We have previously shown in mice that repeated tDCS normalizes pathological behaviors associated with chronic nicotine exposure. Here, we evaluated, in adult female mice, the impact of tDCS on cocaine-induced behavior and gene regulation in corticostriatal circuits implicated in psychostimulant addiction. Anodal tDCS was applied transcranially over the frontal cortex. Three weeks after repeated tDCS, we investigated the induction of a gene expression marker (*Zif268*) by cocaine (25 mg/kg) in 26 cortical and 23 striatal regions using *in situ* hybridization histochemistry. We also assessed place preference conditioning by cocaine (5, 10 and 25 mg/kg). tDCS pretreatment increased basal expression and attenuated cocaine (25 mg/kg)-induced expression of *Zif268* in specific corticostriatal circuits. Cocaine-induced locomotor activation (25 mg/kg) and place preference conditioning (5 and 25 mg/kg) were also reduced. These results demonstrate that tDCS can attenuate molecular and behavioral responses to cocaine for several weeks. Together, our findings provide pre-clinical evidence that such electrical brain stimulation may be useful to modify the psychostimulant addiction risk.

Keywords Cocaine, conditioned place preference, corticostriatal circuits, gene expression, neuromodulation, tDCS.

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INTRODUCTION

Transcranial direct current stimulation (tDCS) is a non-invasive and painless neuromodulatory technique that uses weak constant electrical current to stimulate specific areas of the cerebral cortex. During the last decade, tDCS has emerged as a successful approach to alleviate symptoms of various psychiatric and neurological conditions, including depression (e.g. Brunoni *et al.* 2012; Berlim, Van den Eynde, & Daskalakis 2013; Kuo, Paulus, & Nitsche 2014), memory disorders (e.g. Bennabi *et al.* 2014) and addiction (Feil & Zangen 2010). For example, studies found that repeated tDCS over the prefrontal cortex decreased craving for, and the consumption of, cigarettes (Fregni *et al.* 2008; Boggio *et al.* 2009; Fecteau *et al.* 2014) and alcohol (Boggio *et al.* 2008; Klauss *et al.* 2014) in chronic users. The mechanisms underlying these behavioral modifications are unknown. We recently developed an animal model for tDCS (Pedron *et al.* 2014) to investigate the neuronal processes affected by this technique. Consistent with the above clinical findings, our early work shows that repeated tDCS for five days in mice has antidepressant-like properties, improves working memory and decreases nicotine-induced place preference conditioning, three weeks after tDCS (Pedron *et al.* 2014).

The potential effects of tDCS in cocaine addiction remain poorly explored (Conti & Nakamura-Palacios 2014). However, reduced cocaine craving has been reported after another kind of non-invasive cortical stimulation, repeated transcranial magnetic stimulation (rTMS) (Camprodon *et al.* 2007; Politi *et al.* 2008), suggesting that modifying cortical activity may also alter psychostimulant-induced processes. The mechanisms

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underlying these effects of tDCS or rTMS, and whether or not other subcortical addiction-related brain structures are also impacted, remain unclear.

It has been shown that corticostriatal circuits play a critical role in several aspects of addiction, including abnormal reward processing, habit formation and compulsive behavior (Berke & Hyman 2000; Everitt & Robbins 2005; Wise 2009). A large literature implicates changes in gene regulation in specific corticostriatal circuits in addiction (Renthal & Nestler 2008). Among the many genes affected by psychostimulants such as cocaine in the cortex and striatum is *Zif268* (Steiner & Van Waes 2013), which encodes a transcription factor (Knapska & Kaczmarek 2004) that is critical for cocaine-induced behavioral changes (Lee *et al.* 2005; Valjent *et al.* 2006; Theberge *et al.* 2010)

In the present study, we determined, in mice, whether tDCS can modify cocaine-induced behavior and/or normal or cocaine-induced gene regulation in the cortex and striatum, using *Zif268* as a gene regulation marker. Our mapping study also assessed the spread of such molecular changes across different corticostriatal circuits.

MATERIALS AND METHODS

Subjects

Swiss female mice (8 weeks at the beginning of tDCS; Janvier, France) were housed 8–10 per cage under standard laboratory conditions (12:12-hour light/dark cycle; lights on at 7:00 am) with food and water available *ad libitum*. Prior to the surgery, mice were allowed 1 week of acclimation, during which they were repeatedly handled. In experiment 1 (locomotor activity, gene expression), animals were divided into eight experimental groups: Sham-Veh (Vehicle) ($N = 8$), tDCS-Veh ($N = 6$), Sham-5 (Cocaine 5 mg/kg, i.p.) ($N = 9$), tDCS-5 ($N = 10$), Sham-10 (Cocaine 10 mg/kg) ($N = 9$), tDCS-10 ($N = 10$), Sham-25 (Cocaine 25 mg/kg) ($N = 8$) and tDCS-25 ($N = 8$). In experiment 2 (place preference conditioning by cocaine), animals were divided into eight groups: Sham-Veh ($N = 8$), tDCS-Veh ($N = 10$), Sham-5 ($N = 14$), tDCS-5 ($N = 12$), Sham-10 ($N = 14$), tDCS-10 ($N = 14$), Sham-25 ($N = 13$) and tDCS-25 ($N = 12$). All procedures met the NIH guidelines for the care and use of laboratory animals and were approved by the University of Franche-Comté Animal Care and Use Committee (CEBEA-58).

Surgery

A tubular plastic jack (internal diameter: 2.1 mm) was surgically fixed onto the skull one week before the stimulation protocol began (Fig. 1a). Animals were anesthetized with ketamine hydrochloride/xylazine (80/12 mg/kg; i.p.) and were placed in a stereotaxic apparatus. The center of the plastic jack was positioned over the left frontal cortex 1 mm rostral and 1 mm left of bregma (Fig. 2) and fixed with a coating of glass ionomer cement (GC Fuji I, Leuven, Belgium)

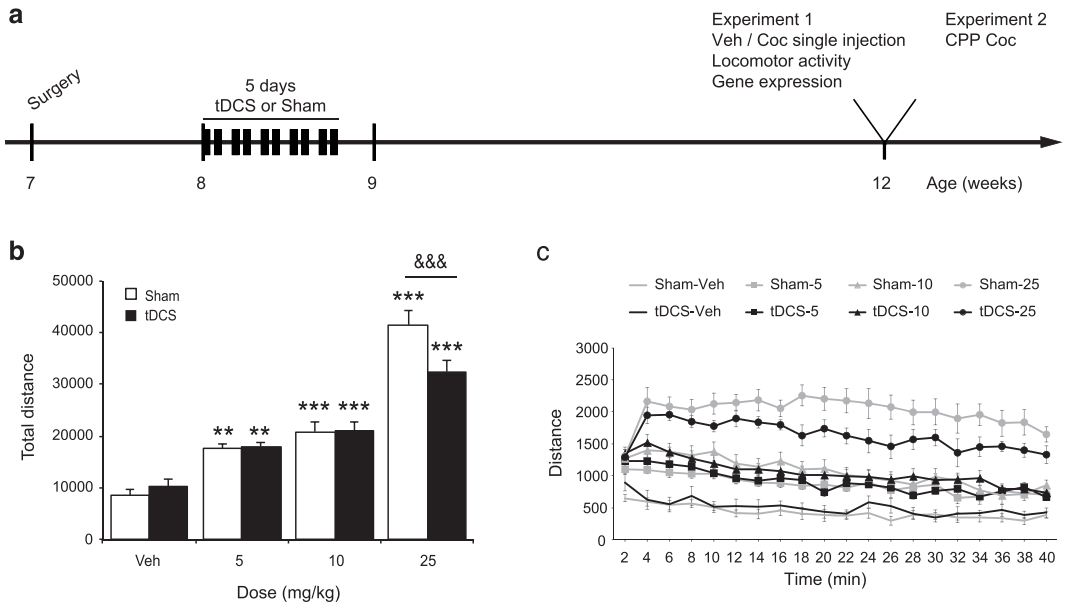

Figure 1 (a) Experimental design (experiments 1 and 2). CPP: conditioned place preference test (b) Total distance traveled (mean ± SEM, in cm) in a novel open field is shown for animals that were subjected to tDCS (twice daily, 5 days) or Sham stimulation and, 3 weeks after stimulation, received an injection of cocaine (Coc) (5, 10 or 25 mg/kg) or vehicle (Veh), followed by the 40-min behavioral test. (c) Time course of the distance traveled is given for each group. ** $p < 0.01$ and *** $p < 0.001$, *versus* respective control group (Sham-Veh or tDCS-Veh); &&& $p < 0.001$, Sham-25 *versus* tDCS-25. $N = 6$–10 per group

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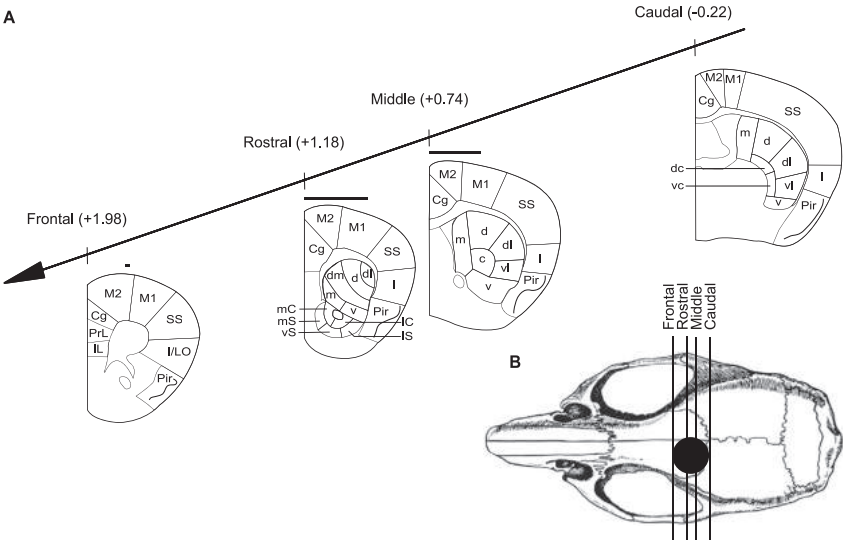

Figure 2 (a) Schematic illustration of the 26 cortical areas (Paxinos & Franklin 2001) and the 23 striatal sectors (mostly defined by their predominant cortical inputs, Willuhn *et al.* 2003) used to measure *Zif268* expression. Gene expression was assessed in coronal sections from four rostrocaudal levels: frontal, rostral, middle and caudal (ranging from approximately +1.98 to −0.22 mm relative to bregma; Paxinos & Franklin 2001). Horizontal black lines indicate the position of the anode. Cortical areas (from medial to lateral): IL, infralimbic; PrL, prelimbic; Cg, cingulate; M2, secondary motor; M1, primary motor; SS, somatosensory; I/LO, insular/lateral orbital; I, insular; Pir, piriform. Striatal sectors: m, medial; dm, dorsomedial; d, dorsal; dl, dorsolateral; dc, dorsal central; c, central; vc, ventral central; vl, ventrolateral; v, ventral. Nucleus accumbens: mC, medial core; lC, lateral core; mS, medial shell; vS, ventral shell; lS, lateral shell. (b) The center of the stimulation electrode (anode, filled circle) was positioned over the left frontal cortex 1 mm rostral and 1 mm left of bregma. The anode (diameter: 2.1 mm) had a contact area of 3.5 mm^2, and the cathode (rubber-plate electrode, 9.5 cm^2) was positioned onto the ventral thorax (not shown). A 2 × 20 minutes/day constant current of 0.2 mA, with a linear fade in/fade out of 10 seconds, was applied transcranially using a direct current stimulator (DC-Stimulator Plus), on 5 consecutive days. The position of the assessed brain sections (frontal to caudal) relative to the stimulation electrode is also shown

(Pedron *et al.* 2014). After surgery, all animals were allowed to recover for 1 week before undergoing tDCS. During this period and tDCS, mice were placed in individual cages.

Stimulation protocol

The plastic jack was filled with saline (NaCl 0.9 percent) to establish a contact area of 3.5 mm^2 with the skull. The stimulation electrode (anode, diameter: 2.1 mm; DIXI Medical, Besançon, France) was then screwed into the jack. A larger conventional rubber-plate electrode (cathode, 9.5 cm^2; Physiomed Elektromedizin AG, Schnaittach, Germany) served as the counterelectrode and was placed onto the ventral thorax (Pedron *et al.* 2014). On 5 consecutive days, an anodal constant current (0.2 mA; 2 × 20 minutes/day, 5 hour interstimulation interval) was applied transcranially over the frontal cortex, using a DC-Stimulator Plus (NeuroConn, Ilmenau, Germany) with a linear fade in/fade out (10-second ramp). Animals were awake and restrained during tDCS to prevent possible interactions between tDCS effects and anesthetic drugs. The design of the custom-made restraining box is shown in Pedron *et al.* 2014. Control (Sham) animals were subjected to the same procedure (surgery, restraining box, electrode connected to the jack), except the current was not delivered.

Our protocol of stimulation was chosen based on earlier clinical studies (Rigonatti *et al.* 2008; Boggio *et al.* 2009; Ferrucci *et al.* 2009), as well as our previous work in mice (Pedron *et al.* 2014). We evaluated effects on behavior and gene expression three weeks after tDCS ended, because this time window showed robust effects of tDCS on behavior (Pedron *et al.* 2014).

Experiment 1: Locomotor activity and gene expression

Drug treatment and behavioral testing

Three weeks after the last tDCS session, animals received a single injection of either cocaine (5, 10 or 25 mg/kg in 0.02 percent ascorbic acid, i.p.,1 ml/kg, Sigma-Aldrich, France) or vehicle. Immediately after the injection, the animal was placed in a circular open-field (diameter 47 cm) for 40 minutes in low-light conditions (40 lux). Locomotor activity was analyzed using a video-tracking system (Ethovision, Noldus, France). The parameter assessed was the distance traveled in the open-field. The mice were then killed with CO_2. The brain was rapidly removed, frozen in isopentane cooled on dry ice and stored at −30°C until cryostat sectioning.

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Tissue preparation and in situ hybridization histochemistry

Only the mice treated with cocaine 25 mg/kg or vehicle were used in the gene expression study. Coronal sections (12 μm) were thaw-mounted onto glass slides (Superfrost/Plus, Daigger, Wheeling, IL, USA), dried on a slide warmer and stored at −30°C. In preparation for the *in situ* hybridization histochemistry, the sections were fixed in 4 percent paraformaldehyde/0.9 percent saline for 10 minutes at room temperature, incubated in a fresh solution of 0.25 percent acetic anhydride in 0.1 M triethanolamine/0.9 percent saline (pH 8.0) for 10 minutes, dehydrated, defatted for 2 × 5 minutes in chloroform, rehydrated and air-dried. The slides were then stored at −30°C until hybridization. An oligonucleotide probe (48-mer, Invitrogen, Rockville, MD, USA) was labeled with [α-^{33}P]-dATP, as described earlier (Van Waes *et al.* 2014). The probe had the following sequence: *Zif268* (Egr1), complementary to bases 352–399, GenBank accession number M18416. One hundred microliters of hybridization buffer containing labeled probe (~3 × 10^6 cpm) was added to each slide. The sections were coverslipped and incubated at 37°C overnight. After incubation, the slides were first rinsed in four washes of 1X saline citrate (150 mM sodium chloride, 15 mM sodium citrate), and then washed three times for 20 minutes each in 2X saline citrate/50 percent formamide at 40°C, followed by two washes of 30 minutes each in 1X saline citrate at room temperature. After a brief water rinse, the sections were air-dried and then apposed to X-ray film (BioMax MR-2, Kodak, Rochester, NY, USA) for 6 days.

Analysis of autoradiograms

Gene expression in the cortex was measured in a total of 26 regions (infralimbic, prelimbic, cingulate, secondary motor, primary motor, somatosensory, lateral orbital, insular and piriform, based on Paxinos & Franklin 2001, in coronal sections from four rostrocaudal levels: frontal, approximately at +1.98 mm relative to bregma; rostral, +1.18 mm; middle, +0.74 mm; and caudal, −0.22 mm, Fig. 2a). Striatal gene expression was determined at the rostral, middle and caudal levels in a total of 23 sectors mostly defined by their predominant cortical inputs (Willuhn, Sun, & Steiner 2003). Eighteen of these sectors represented the caudate-putamen (medial, dorsomedial, dorsal, dorsolateral, dorsal central, central, ventral central, ventrolateral, ventral) and five the nucleus accumbens (medial core, lateral core, medial shell, ventral shell and lateral shell, Fig. 2a) (Van Waes *et al.* 2010).

Hybridization signals on film autoradiograms were measured by densitometry (ImageJ, Wayne Rasband, Bethesda, MD, USA). The films were captured using a light table (Northern Light, Imaging Research, St. Catharines, Ontario, Canada) and a Sony CCD camera (Imaging Research). The 'mean density' value of a region of interest was measured by placing a template over the captured image. Mean densities were corrected for background by subtracting mean density values measured over white matter (corpus callosum) of the same hemisphere. Values from corresponding regions in the two hemispheres were averaged when no significant differences in the patterns of *Zif268* induction by tDCS or cocaine were detected between the left (stimulated) and right (contralateral) sides. The illustrations of film autoradiograms displayed in Fig. 3 are computer-generated images, and are contrast-enhanced. The maximal hybridization signal is black.

Experiment 2: Place preference conditioning by cocaine

Animals performed the conditioned place preference (CPP) test three weeks after tDCS, as previously described (Pedron *et al.* 2014). Three doses of cocaine were tested (5, 10 or 25 mg/kg in 0.02 percent ascorbic acid, i.p.). Two groups that received vehicle injections in both compartments were used as controls (Sham-Veh and tDCS-Veh).

Briefly, the CPP apparatus consists of two main compartments linked by a corridor displaying each different features: visual (wall patterns) and tactile (floor texture). On day 1 (pre-conditioning, D1), mice were placed in the corridor and allowed free access to the compartments for 10 minutes. The time spent in each compartment was recorded using the Ethovision system. On days 2–4 (conditioning phase) mice received an injection of cocaine or vehicle (one of each per day, interval between the injections: 6 hours) and were immediately confined into one of the two conditioning compartments for 15 minutes (drug pairing occurred in the least preferred compartment). On day 5 (post-conditioning, D5), mice were again allowed free access to both compartments for 10 minutes, without drug treatment. The percentage of time spent in the drug-paired compartment was calculated for the pre-conditioning (D1) and the post-conditioning (D5) phases as follows: drug-paired compartment (second) / (drug-paired compartment + vehicle-paired compartment (second)) × 100. A significant increase in the percentage of time spent in the drug-paired compartment between the pre-conditioning session (D1) and the post-conditioning session (D5) indicates that the substance induces a place preference.

Statistical analysis

The results were expressed as mean ± standard error of the mean. Significance was set at $p \leq 0.05$. For locomotor

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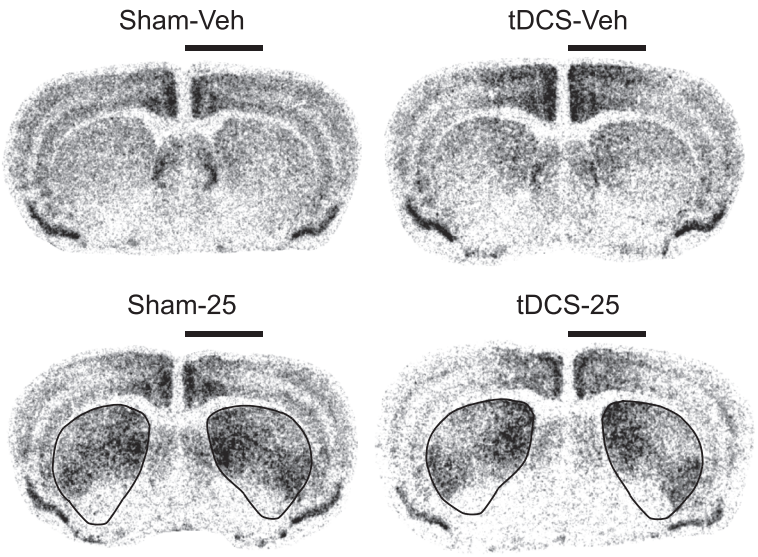

Figure 3 Illustrations of film autoradiograms depict *Zif268* expression in coronal sections from the middle striatum in Sham or tDCS mice treated with vehicle (Veh) or cocaine (25 mg/kg, i.p.). Horizontal black lines illustrate the position of the anode. The maximal hybridization signal is in black

activity two-factor ANOVAs with factors drug (Veh, 5, 10, 25) and stimulation (Sham, tDCS) were performed. To compare *Zif268* densities, we first used three-factor ANOVAs (one ANOVA per cortical area or striatal sector) with factors drug (Veh, 25), stimulation (Sham, tDCS) and side of the brain (right, left; within factor). Then, for the averaged values (right/left hemisphere), two-factor ANOVAs were performed with factors drug and stimulation. Finally, for the CPP experiment, we performed for each dose (0, 5, 10 and 25 mg/kg) two-factor ANOVAs with factor stimulation (Sham, tDCS) and time (pre-(D1), post-(D5) conditioning; within factor). Newman–Keuls post-hoc tests were used to describe differences between individual groups (Statistica, StatSoft, USA). For illustrations of topographies (maps, Figs 4a and 5a), the increase in gene induction (*versus* respective control group) in a given region was expressed as the percentage of the maximal increase observed (% max). The regional distribution of *Zif268* induction in the cortex and striatum was compared by Pearson's correlations, for different experimental conditions.

RESULTS

Experiment 1: Locomotor activity and gene expression

tDCS attenuated cocaine-induced locomotor activity for the highest dose of cocaine

Cocaine increased locomotor activity in both Sham and tDCS mice (Drug effect: $F(3,59) = 80.97$, $p < 0.001$; Fig. 1b,c, Newman–Keuls post-hoc analyses ** $p < 0.01$ and *** $p < 0.001$ *versus* respective control group (Sham-Veh or tDCS-Veh)). tDCS had a differential effect on locomotor activity depending on the dose of cocaine tested (tDCS × drug interaction: $F(3,59) = 3.87$, $p < 0.05$). Newman–Keuls post-hoc analyses revealed that tDCS alone had no effect on locomotor activity (tDCS-Veh *versus* Sham-Veh, $p = 0.50$). For the lower doses, tDCS had also no impact on cocaine-induced locomotor activity (tDCS *versus* Sham: 5 mg/kg, $p = 0.84$; 10 mg/kg, $p = 0.93$). However, tDCS reduced cocaine-induced locomotor activity for the highest cocaine dose tested (25 mg/kg, $p < 0.001$).

No asymmetrical effects of tDCS or cocaine on Zif268 expression in the left versus right hemisphere

Although the stimulation electrode was positioned asymmetrically on the skull (1 mm left of bregma, Fig. 2b), there was no difference between the left (stimulated) and right (contralateral) hemisphere in the expression of *Zif268*, with or without cocaine (Fig. 3). That is, for each area of cortex and striatum, there was no significant interaction with the hemisphere (right or left). Values from corresponding regions in the two hemispheres were therefore averaged for the rest of the study.

tDCS increased basal Zif268 expression

Three weeks after tDCS, vehicle-treated animals subjected to tDCS displayed enhanced expression of *Zif268* compared to sham controls in the striatum (Figs 3 and 4, supplementary Table 2) and to a lesser degree in the cortex (supplementary Table 1). Among the cortical areas, a statistically significant increase was seen in the

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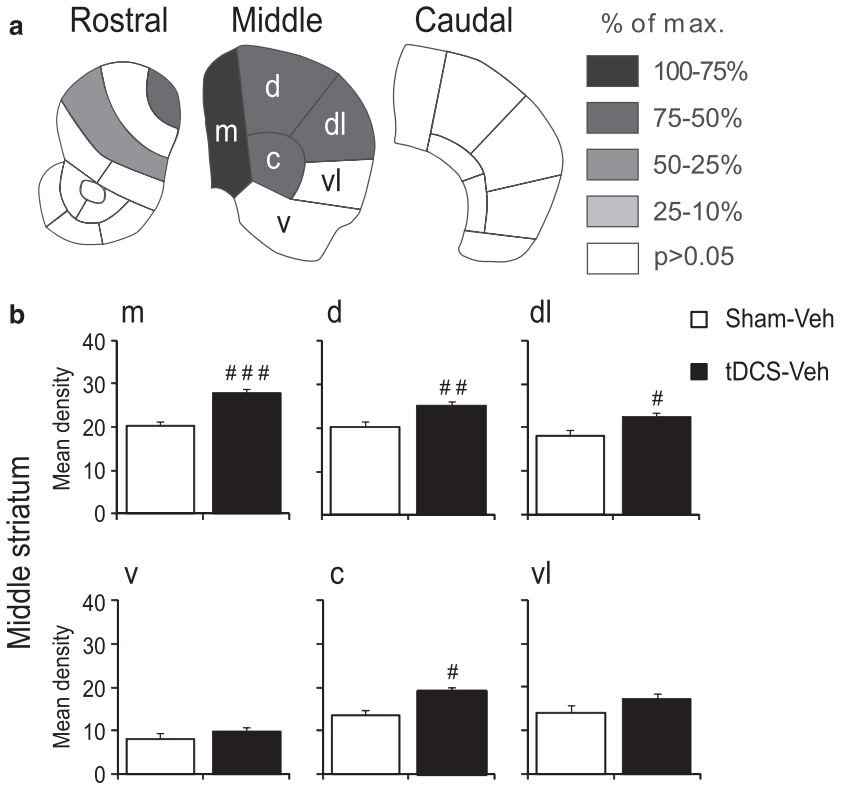

Figure 4 Topography of tDCS-induced *Zif268* expression in the striatum (in vehicle-treated mice). (a) Maps depict the distribution of increases in *Zif268* expression at the rostral, middle and caudal levels of the striatum, 3 weeks after tDCS and following the behavioral test (ipsi- and contralateral values averaged). The values (difference tDCS-Veh minus Sham-Veh) are expressed relative to the maximal increase observed (% of maximum). Sectors with significant differences ($p < 0.05$) are shaded as indicated. Sectors without significant effect are in white. (b) Mean density values (mean ± SEM) for *Zif268* expression in Sham (white) and tDCS (black) mice 3 weeks after tDCS and following the behavioral test are depicted for the six middle striatal sectors. $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$, and $^{\#\#\#}$ $p < 0.001$, *versus* Sham-Veh. $N = 6$–8 per group

piriform cortex on rostral, middle and caudal levels ($p < 0.05$) and in the cingulate cortex (frontal level, $p < 0.05$; supplementary Table 1). A similar tendency was also present in the motor cortex (M1 and M2 on the middle level, $p = 0.06$ and 0.07, respectively), in the infralimbic and prelimbic cortices on the frontal level (each $p = 0.07$) and in the cingulate cortex on the rostral level ($p = 0.07$; supplementary Table 1).

The striatum was more affected by tDCS than the cortex. In tDCS-treated animals, significantly increased *Zif268* expression was observed on the levels situated under the electrode (i.e. in rostral and middle sections, Fig. 4a and b, supplementary Table 2), in dorsal sectors of the striatum (rostral: dorsolateral and dorsomedial; middle: medial, dorsal, dorsolateral and central). Notably, tDCS had no significant effect in the nucleus accumbens (supplementary Table 2).

tDCS attenuated cocaine-induced Zif268 expression

Because significant differences were observed between Sham and tDCS groups in vehicle-treated animals (Fig. 4), the effects of cocaine were expressed relative to the values in the respective Veh control groups (the supplementary Tables 1 and 2 present the absolute values for comparison). In Sham animals, the single cocaine injection induced a minor but statistically significant increase in *Zif268* mRNA expression in a few areas of the cortex (frontal: cingulate; middle: M1, M2; $p < 0.05$, supplementary Table 1), with similar tendencies in other areas. In the striatum, cocaine produced a more robust augmentation in *Zif268* expression on all three rostrocaudal levels (Figs 3 and 5, supplementary Table 2). A significant increase in *Zif268* mRNA levels was observed in 15 of the 23 sectors (Fig. 5a). Gene induction varied considerably between different striatal regions. The most robust increase was observed on the middle (Fig. 5b) and caudal levels, in striatal sectors that receive cingulate, motor and sensorimotor cortical inputs (i.e. middle: medial, dorsal, central and lateral sectors; caudal: dorsal sector, Willuhn *et al.* 2003). In contrast, in accordance with previous findings in the rat (Unal *et al.* 2009), the nucleus accumbens displayed modest or no drug effects. Cocaine significantly increased *Zif268* expression only in the lateral shell ($p < 0.05$).

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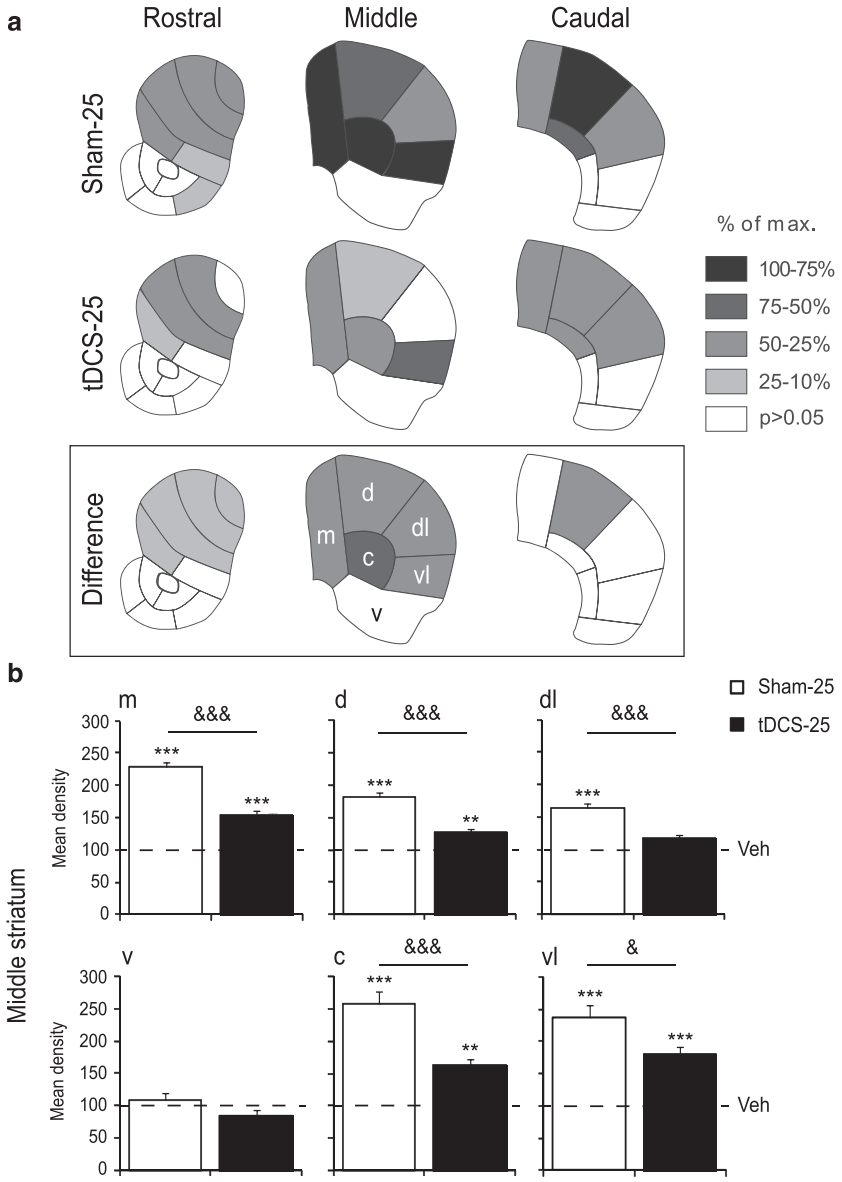

Figure 5 tDCS attenuates cocaine-induced *Zif268* expression in specific areas of the striatum. (a) Maps depict the distribution of *Zif268* expression induced by cocaine (25 mg/kg, i.p.; ipsi- and contralateral values averaged) at the rostral, middle and caudal levels of the striatum, for cocaine-treated Sham (Sham-25) and tDCS (tDCS-25) mice. The data are expressed relative to the maximal increase observed (% of maximum). Sectors with significant differences *versus* respective Veh controls (i.e. Sham-Veh or tDCS-Veh) are shaded as indicated. Sectors without significant effects are in white. The 'difference' (box) indicates significant differences in *Zif268* induction between cocaine alone (Sham-25) and tDCS + cocaine (tDCS-25) groups. (b) Mean density values (mean ± SEM) (expressed as percentage of respective control groups, Veh = 100 percent) for *Zif268* expression are depicted for the six middle striatal sectors. ** $p < 0.01$ and *** $p < 0.001$, *versus* respective Veh control group; & $p < 0.05$ and &&& $p < 0.001$, Sham-25 *versus* tDCS-25. $N = 6$–8 per group

In the tDCS group, cocaine had no significant effect on *Zif268* expression in the cortex. Thus, *Zif268* induction in the cingulate and motor cortex was prevented in animals that received tDCS three weeks before the cocaine treatment (supplementary Table 1). Consistent with this result, in tDCS-treated animals (tDCS-25), cocaine-induced *Zif268* expression in the striatum was markedly attenuated compared to the Sham control (Sham-25). This was reflected, for one, by a lower proportion of the 23 striatal sectors displaying significantly increased *Zif268* expression in the tDCS group than in the Sham group (relative data, 11 sectors *versus* 15 sectors; Fig. 5a, tDCS-25). Direct statistical comparisons showed that *Zif268* induction was significantly weaker in tDCS-25 animals in 10 striatal sectors (Fig. 5a, Difference and Fig. 5b). tDCS had no significant effect on cocaine-induced *Zif268* expression in the nucleus accumbens (Fig. 5a, Difference).

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Correlation analysis was used to compare tDCS- and cocaine-induced increases in *Zif268* expression between striatal sectors and their respective cortical input regions (see Cotterly *et al.* 2007; Van Waes *et al.* 2010; Willuhn *et al.* 2003; Yano & Steiner 2005 for more details). Our results for vehicle-treated mice show that, despite the modest effects in the cortex, tDCS-induced *Zif268* expression in the 23 striatal sectors was positively correlated with that in their anatomically connected cortical areas (Veh animals, $r = 0.415$, $p < 0.05$). Similarly, in these striatal sectors, the magnitude of the reduction in cocaine-induced gene expression produced by tDCS was highly correlated with that in their connected cortical input regions ($r = 0.627$, $p < 0.001$). Therefore, tDCS produced coordinated molecular changes in cortical and striatal nodes of specific corticostriatal circuits.

Experiment 2: Place preference conditioning by cocaine

tDCS abolished cocaine-induced place preference conditioning for 5 and 25 mg/kg

Vehicle injections did not produce any place preference (ANOVA all effects: $p > 0.05$, Fig. 6, Veh). In sham groups, cocaine induced a place preference with all doses tested (5 mg/kg: $p < 0.05$, 10: $p < 0.001$, 25: $p < 0{,}01$), in accordance with previous studies in mice (e.g. Zhang *et al.* 2002; Iniguez *et al.* 2015). In contrast, animals subjected to repeated anodal tDCS failed to show cocaine-induced place preference for the doses of 5 ($p = 0.98$) and 25 mg/kg ($p = 0.53$). Only the 10 mg/kg dose produced statistically significant place preference conditioning ($p < 0.001$, Fig. 6). For the dose of 25 mg/kg, the percentage of time spent in the drug-paired compartment on the post-conditioning day (D5) was significantly lower in tDCS than in sham mice ($p < 0.05$).

DISCUSSION

Our findings show that tDCS produces long-lasting modifications in behavioral responses and gene regulation in corticostriatal circuits induced by cocaine. Thus, repeated anodal tDCS over the frontal cortex increased 'basal' expression of the marker gene *Zif268* and attenuated cocaine-induced gene regulation, locomotion and place preference conditioning, three weeks after tDCS pretreatment.

Cortical and Subcortical Effects of tDCS in Vehicle Controls

One aim of this study was to determine possible tDCS effects on normal gene regulation (i.e. in vehicle controls) and to map the distribution of such effects in the cortex and striatum. Our findings in vehicle-treated mice show that repeated tDCS produced increased expression of *Zif268*, mostly in the striatum, three weeks after tDCS pretreatment. Future studies will have to determine whether these increased *Zif268* mRNA levels represented upregulated gene expression that endured for three weeks, or whether they reflected an altered responsiveness to experimental conditions such as handling or behavioral testing, or other neuronal changes (e.g. increased arousal; see Steiner & Van Waes 2013), in the affected corticostriatal circuits. Regardless of the underlying cause, these findings demonstrate long-lasting effects of tDCS on gene regulation in these circuits.

Although tDCS preferentially impacted dorsal striatal regions under the stimulation electrode, these effects were not strictly related to the position of the electrode. For one, gene regulation changes in striatum (and cortex) were symmetrically distributed in the two hemispheres, despite the asymmetrical electrode placement

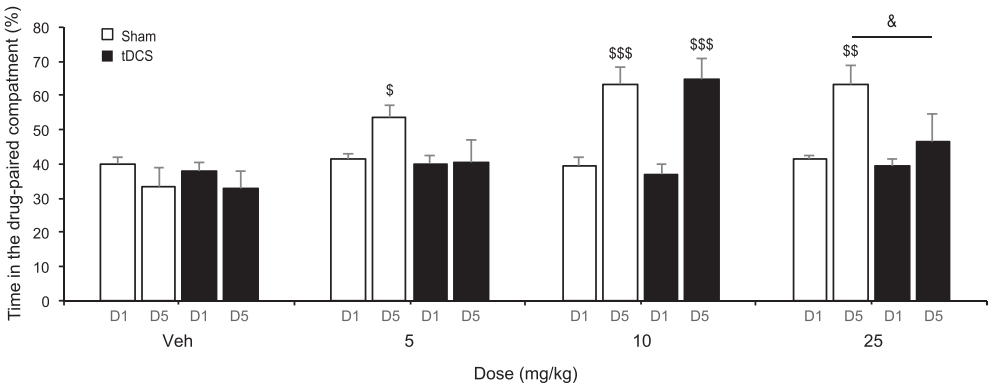

Figure 6 Conditioned place preference induced by cocaine (5, 10 or 25 mg/kg) or vehicle (Veh, control groups) 3 weeks after Sham (white) or tDCS (black) stimulation. A significant increase in the time spent in the drug-paired compartment between day 1 (D1, pre-conditioning session) and day 5 (D5, post-conditioning session) indicates that cocaine induced a place preference. For the Sham groups, cocaine induced a place preference with all doses tested. In contrast, in the tDCS groups, cocaine induced a place preference only with the 10 mg/kg dose.
$^{\$}$ $p < 0.05$, $^{\$\$}$ $p < 0.01$, and $^{\$\$\$}$ $p < 0.001$, D1 *versus* D5; $^{\&}$ $p < 0.05$, Sham-25 D5 *versus* tDCS-25 D5. $N = 8$–14 per group

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(over left cortex). Moreover, in the cortex, the most robust increase in *Zif268* expression was present in the piriform (olfactory) cortex, a ventral brain region. In the striatum, regions on the middle level were considerably more affected than those on the rostral level, despite the similar position of these levels relative to the electrode placement. These findings indicate that specific neuronal circuits, rather than just proximal cortical and striatal tissues, are modified by tDCS. This conclusion is supported by our correlation analysis that showed that changes in gene expression were correlated between cortical areas and their connectionally (functionally) related striatal target sectors (Veh animals, $r = 0.415$, $p < 0.05$) (Willuhn *et al.* 2003) and that these effects preferentially occurred in sensorimotor circuits (Steiner & Van Waes 2013).

The finding that the asymmetrically positioned electrode produced bilaterally symmetrical gene regulation patterns (with and without cocaine) was somewhat unexpected. The contralateral cortical and striatal effects may have been mediated by the pronounced interhemispheric cortico-cortical and cortico-striatal projection systems. Alternatively, they may have resulted in part from bilateral current spread. tDCS is known to be less focal than rTMS (Nitsche *et al.* 2007; Miniussi *et al.* 2008), and the current may thus have spread over both hemispheres to some degree. However, the finding that distinct corticostriatal circuits were affected, without clear relationship to their distance from the electrode (see above), seems to argue against such a nonspecific effect as the sole factor. This is an important question that will have to be addressed in future studies.

Gene Regulation Effects of Cocaine are Attenuated by tDCS

The main goal of this study was to determine whether tDCS might modulate gene regulation by cocaine. Our results show diminished induction of *Zif268* by cocaine in cortex and striatum when examined three weeks after the repeated tDCS pretreatment. Again the magnitude of this effect was correlated between cortical regions and their striatal targets ($r = 0.627$, $p < 0.001$), indicating that specific circuits were affected. The tDCS-mediated decrease in gene induction was maximal in (but not limited to) sensorimotor and associative corticostriatal circuits, which are known to be involved in habit formation and compulsive aspects of drug taking (Everitt & Robbins 2005).

The basis for this altered gene regulation is presently unclear. Early studies in animals using direct current stimulation (current applied directly to the cortex) (Bindman, Lippold, & Redfearn 1964; Purpura & McMurtry 1965), as well as more recent data on tDCS in humans (Nitsche & Paulus 2000), suggest that anodal stimulation increases neuronal excitability, which may increase transmitter release in the striatum. Repeated tDCS may thus induce synaptic plasticity (Stagg & Nitsche 2011) that is usually associated with such changes in neuronal activity and their molecular sequelae, including altered gene regulation. Altered neuronal responsiveness in these corticostriatal circuits is consistent with the here observed reduced behavioral responses, which are modulated by neuronal activity in these circuits, including place preference conditioning (Ilango *et al.* 2014).

The acute induction of immediate-early genes such as *Zif268* by psychostimulants serves as a marker that predicts long-term neuroadaptations after repeated drug exposure, as this acute response is correlated with various neuronal changes after repeated drug treatments (Steiner & Van Waes 2013). However, *Zif268* is also directly implicated in various plasticity processes, including several long-term neurobehavioral changes induced by psychostimulants. For example, this transcription factor likely mediates some drug-induced neuroplastic changes (Knapska & Kaczmarek 2004). Indeed, previous work demonstrated that *Zif268* is critical for place preference conditioning by cocaine (Valjent *et al.* 2006) and for reconsolidation of cocaine memories (Lee *et al.* 2005; Theberge *et al.* 2010). *Zif268* also contributes to processes underlying cocaine-induced behavioral sensitization (Valjent *et al.* 2006).

Our present findings of an association between diminished *Zif268* induction and attenuated locomotor activity and place preference conditioning by cocaine are consistent with these earlier findings. Whether directly affecting the underlying neuronal mechanisms or serving as a marker, the attenuated *Zif268* response after repeated tDCS may indicate a 'protective' effect of tDCS against drug-induced neuronal changes subsequent to tDCS treatment.

Behavioral Effects of Cocaine are Attenuated by tDCS

The impact of tDCS on cocaine-induced behavioral effects was dependent on the dose of cocaine. For locomotor activity, the tDCS effect was selectively observed with the highest dose (25 mg/kg). It could be argued that an increase in focused stereotypies, which are associated with certain psychostimulants, might have contributed to the reduced locomotion in these animals. We did not measure stereotypies and, therefore, cannot rule out (or confirm) a contribution of tDCS-induced stereotypies. It is noteworthy that a previous study in female (but not male) rats found comparable amounts of stereotypies for cocaine doses of 10, 20 and 40 mg/kg (Walker *et al.* 2001), while our effect was observed for the dose of

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25 mg/kg only. Future studies will have to clarify how tDCS modulates cocaine-induced locomotor activity and/or stereotypies.

In the CPP paradigm, which aims to evaluate the motivational properties of cocaine, tDCS suppressed place preference conditioning for the lowest and highest dose of cocaine (5 and 25 mg/kg), but not 10 mg/kg. There is evidence that place preference conditioning by cocaine displays an inverted U-shaped dose–response function, such that very low or high doses of cocaine do not induce place preference (Hnasko, Sotak, & Palmiter 2007). The optimal dose of cocaine for inducing place preference in mice seems to be situated between 7.5 and 15 mg/kg (Hnasko *et al*. 2007). Therefore, the present tDCS treatment seems to have attenuated place preference conditioning for suboptimal doses of cocaine (5 and 25 mg/kg), but was not sufficient to modify place preference conditioning induced by an optimal dose (10 mg/kg).

Regarding the underlying mechanisms, cocaine-induced behavior in the CPP paradigm is determined by opponent processes ('rewarding' *versus* 'aversive' properties of the drug), which appear to have different neuronal substrates (Lammel *et al*. 2012). Thus, lower-dose conditioning (left limb of inverted U) is taken to reflect a rewarding effect of the drug, while upper-dose conditioning (right limb) is governed by increasing aversion. It is therefore tempting to speculate that tDCS may attenuate 'reward' and/or increase 'aversion' by (differentially) modifying their underlying neuronal systems. A potential beneficial effect of tDCS on addiction processes will have to be verified in other drug addiction-related paradigms such as the cocaine self-administration model.

CONCLUSIONS

Overall, our results indicate that repeated tDCS pretreatment produces long-lasting modifications in the molecular and behavioral sensitivity to cocaine, especially for a high dose (25 mg/kg). These findings suggest the intriguing possibility that tDCS pretreatment might attenuate the addiction liability of psychostimulants such as cocaine by attenuating the drugs' molecular impact. This technique of neuromodulation, which is non-invasive, easy to use and affordable, might therefore be useful as an intervention to protect vulnerable individuals from getting addicted.

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Conflicts of Interest

None

Author Contribution

VVW, HS and EH were responsible for the study concept and design. SP, JB and PA contributed to the acquisition of animal data. SP, VVW and HS assisted with data analysis and interpretation of findings. SP, VVW and HS drafted the manuscript. All authors critically reviewed content and approved final version for publication.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table 1 Effects of tDCS on basal and cocaine-induced *Zif268* expression in the cortex

Table 2 Effects of tDCS on basal and cocaine-induced *Zif268* expression in the striatum

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Résumé

L'étude des phénomènes liés à l'addiction constitue le fil conducteur de ce travail. Pour ce faire, j'ai utilisé des modèles animaux (rats, souris) et mon approche était intégrative (du comportement à la biologie cellulaire et moléculaire) et, lorsque cela pouvait s'appliquer, translationnelle (études cliniques et précliniques menées en parallèle). Après une description de mes principales réalisations portant sur les deux versants de mon travail : enseignement et recherche, j'expose une rétrospective de mes recherches. Dans une troisième partie se trouve une sélection représentative de mes principales publications.

Mes premiers travaux portaient sur la différence de vulnérabilité aux drogues entre les individus et aux facteurs qui la contrôlent. Je me suis intéressé à un facteur environnemental, le stress, en particulier lorsqu'il est appliqué de manière précoce au cours du développement de l'individu. Ainsi, j'ai évalué les conséquences d'un stress *in utero* chez le rat sur la sensibilité aux effets de l'alcool et la propension à consommer à l'âge adulte. Ces études sont décrites dans le ***Chapitre 1 (thématique de la thèse, Université de Lille1/France, Université de Rome/Italie)***. Lors de mon post-doctorat, mon travail a porté sur l'implication des circuits cortico-striataux dans l'addiction. J'ai évalué chez le rat les conséquences de l'usage combiné de deux molécules : le méthylphénidate (Ritaline) - un psychostimulant utilisé pour traiter l'hyperactivité - et la fluoxétine (Prozac) - une molécule prescrite en première intention pour traiter la dépression -. Il ressort de cette étude que l'usage concomitant de ces deux traitements provoque des effets moléculaires et comportementaux comparables à ceux de la cocaïne. Mes données révèlent un potentiel effet addictogène de la combinaison de ces deux substances (pourtant largement co-prescrites), et soulèvent un éventuel problème de santé publique. Ces résultats sont exposés dans le ***Chapitre 2 (thématique principale du post-doctorat, Chicago Medical School/USA)***. J'ai par ailleurs lors de mon post-doctorat quantifié l'évolution en fonction de l'âge (pré-pubère, adolescent, adulte) de l'expression de récepteurs impliqués dans les processus addictifs (récepteur CB1) ou dans la modulation de la fonction dopaminergique (récepteur orphelin GPR88) ***(Chapitre 3, thématique secondaire du post-doctorat, Chicago Medical School/USA)***. Pour finir, je développe actuellement, dans le cadre d'études translationnelles, un modèle de stimulation transcrânienne par courant continu (tDCS) chez la souris. Cet outil clinique de neuromodulation innovant est à l'origine de résultats préliminaires enthousiasmants chez l'Homme pour le traitement de divers troubles psychiatriques (ex : dépression, troubles cognitifs, addiction). Cependant, ses mécanismes d'action restent peu connus, nécessitant la mise en place d'études comportementales et neurobiologiques chez l'animal. Ces travaux sont développés dans le ***Chapitre 4 (thématique actuelle, Maître de Conférences, Université de Franche Comté/France)***.

Mots clés:
Addiction, dépression, stress, neurostimulation non invasive, modèles animaux, comportement, expression génique.