Université de Lille 1
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Habilitation à Diriger des Recherches

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Maître de Conférences
en Neurosciences

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Muriel DARNAUDERY, HDR, 2007

STRESS, GESTATION ET REPONSE ALLOSTASIQUE CHEZ LE RAT

L'exposition à un environnement hostile produit chez les organismes vivants une série de réponses organisées ayant pour but d'augmenter les chances de survies. Ces réponses constituent la réponse au stress, qui résulte d'adaptations multiples sur le plan comportemental, neuronal et hormonal, aboutissant à un nouvel état d'équilibre nommé « allostasie ». Chez les mammifères, un environnement de stress précoce lors du développement peut exercer une influence à long terme sur l'adaptation future de l'individu, voire sur celles des générations suivantes. Mes activités de recherche, portent sur l'analyse des conséquences chez le rat, de l'exposition à des stress lors de périodes « critiques», sur les capacités d'adaptations. J'encadre des travaux sur 2 thématiques : 1) Thème « Stress in utero et surcharge allostasique à l'âge adulte : évaluation du stress prénatal en tant que modèle animal pour l'étude de la dépression, du stress post-traumatique, du vieillissement cognitif et la vulnérabilité aux drogues. » ; 2) Thème « Gestation, stress et charge allostasique chez la mère : recherche des origines des désadaptations de la descendance». Dans le cadre de la thèse de Sara Morley-Fletcher, nous avons évalué, en collaboration avec les laboratoires Servier, la sensibilité de ce modèle aux antidépresseurs (tianeptine, imipramine) et démontré qu'il possédait une bonne validité prédictive. Par ailleurs, grâce à une collaboration avec des cliniciens spécialistes du psychotrauma (Pr P Thomas, Pr G Vaiva, Dr Ducros, CHU Hôpital Fontan, Université de Lille 2), nous avons développé un modèle animal d'Etat de Stress Post-Traumatique chez la femelle rat. Dans le cadre de la thèse d'Hélène Louvart, nous avons mis en évidence qu'un stress intense à l'âge adulte (choc électrique inévitable) induisait de profondes altérations comportementales et neuroendocrines à long terme. En particulier, un stress intense provoque des perturbations de l'axe corticotrope proches de celles observées chez les sujets qui développent un état de stress post-traumatique après exposition à un trauma, à savoir une augmentation du rétrocontrôle de l'axe corticotrope. Dans ce modèle, le stress prénatal exacerbe certains désordres comportementaux (sensibilisation du comportement de peur) et endocrines. Plus récemment, dans le cadre du travail de thèse de Vincent Van Waes et du séjour post-doctoral de Mihaela Enache, en collaboration avec des cliniciens spécialisés dans les dépendances (équipe du Pr M Lhermitte, CHU Calmette, Université de Lille 2), nous avons initié des travaux sur la vulnérabilité à l'alcool chez le rat adolescent ayant subi un stress prénatal. Nous avons mis en évidence une sensibilité différentielle aux effets de l'éthanol chez les rats exposés à un stress prénatal. Ainsi, un stress précoce est associé à une hypo-réponse de l'axe corticotrope après une administration d'alcool lors de l'adolescence. De plus, nous avons observé que la prise spontanée d'alcool après un stress intense à l'âge adulte était modulée par le stress précoce. Mes activités de recherche ont également permis de démontrer que la gestation constituait une période critique à haute charge allostasique pour la mère et était associée à de profonds changements de la fonction hippocampique en période *peripartum*. Nous avons observé qu'un stress chronique lors de la gestation avait des conséquences neurobiologiques durables sur le comportement émotionnel de la mère lors de la lactation et plusieurs semaines après. En conclusion, mes recherches contribuent à mettre en évidence qu'un stress à des périodes critiques de la vie, produit une empreinte permanente sur l'individu. L'ensemble des études menées m'a permis d'être co-auteur de 24 publications dans des revues internationales depuis 2000. J'ai participé à l'encadrement de 3 étudiants en formation doctorale sur des projets de recherches financés (S. Morley-Fletcher, H. Louvart et V. Van Waes) et au recrutement de 2 chercheurs post-doctoraux (M. Enache et C. Laloux).

MOTS CLEFS : comportement maternel, lactation, stress in utero, mémoire, dépression, motivation, émotion, stress post-traumatique, alcool, adolescence, vieillissement, sexe, axe corticotrope, estrogènes, facteurs neurotrophiques, neurogenèse, hippocampe, système méso-limbique.

REMERCIEMENTS

Je tiens à exprimer mes profonds remerciements au Professeur Régis Bordet et au Professeur Luis Miguel Garcia-Segura, ainsi qu'au Docteur Anna-Moles pour avoir accepté d'évaluer ce mémoire. Merci Luis Miguel, pour ton aide bienveillante et le temps précieux, que tu m'as accordés au cours de ces années. C'est un grand honneur, que les Professeurs Michel Le Moal et Bernard Bioulac participent à ce jury. Merci au Professeur Michel Le Moal pour ses enseignements et son soutien remarquable à notre petite équipe de lilloises ; merci au Professeur Bernard Bioulac de m'avoir soutenu dans mon projet de rejoindre en délégation le grand laboratoire bordelais qu'il a fondé. Je tiens à exprimer mon profond respect et mon immense reconnaissance envers le Professeur Stéfania Maccari. Stéfania, merci pour ton soutien sans faille et de la confiance que tu m'as accordée. Je tiens à remercier toutes les personnes, enseignant et/ou chercheur, cliniciens, personnels techniques, administratifs et étudiants des universités de Sciences (Lille 1), Médecine (Lille 2) et Psychologie (Lille 3), qui m'ont aidé à réaliser mon travail parfois schizoïde d'enseignant et de chercheur. Un merci chaleureux à Messieurs Jean-Pierre Decottignies, Daniel Deschamps et Serge Lefèvre pour le soin de notre « pouponnière », le centre vital de toute notre recherche : l'animalerie. Je tiens également à remercier la région Nord-Pas de Calais, l'Université de Lille 1 et les différents organismes (l'institut national de la santé, la mission interministérielle de lutte contre les drogues et les toxicomanies, l'institut de recherche sur les boissons, le centre national de la recherche scientifique) et entreprises (Servier, Nestlé) qui ont fait confiance à une jeune maître de conférences et m'ont permis par leurs aides financières de réaliser mes travaux de recherche. Enfin, un très très grand merci, à mes étudiants : Guylaine Bélizaire, Fabien del Faverro, Hélène Louvart, Sara Morley-Fletcher et Vincent Van Waes pour leur investissement remarquable et le travail réalisé.

Merci et pardon à ma famille et aux amis, qui depuis toujours soutiennent par leur amour inconditionnel ma réponse allostasique au stress, au détriment parfois de leur propre plasticité hippocampique !

SOMMAIRE

CURRICULUM VITAE.. 5

LISTE DES PUBLICATIONS 14

CONGRES SCIENTIFIQUES 18

ACTIVITES DE RECHERCHE.. 25

I) ORIENTATIONS GENERALES DE LA RECHERCHE 30
A) Stress et allostasie : « Can't live with it, can't live without it » 30
B) La machinerie de l'axe hippocampo-hypothalamo-hypophyso-corticosurrénalien.................. 33
C) Environnement maternel et adaptation... 37
D) Le stress « périnatal » chez le rat, comme modèle de surcharge allostasique......................... 40
E) Situation des thèmes et questions posées 44

II) RESULTATS.. 46
A) Stress précoces et surcharge allostasique chez la descendance 46
B) Stress, Gestation et allostasie maternelle.. 58

III) CONCLUSIONS ET PERSPECTIVES 63
A) Stress prénatal et modèle animal de PTSD.. 64
B) Stress prénatal et vulnérabilité à l'alcool : étude de l'environnement précoce sur les perturbations des comportements de consommation 66
C) Maternité et plasticité neuro-comportementale : modulation par le stress gestationnel 68

BIBLIOGRAPHIE 70

SELECTION DE PUBLICATIONS 79

CURRICULUM VITAE

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Situation administrative
Maître de Conférences, Enseignant Chercheur en Neurosciences (section 69 du Conseil national des Universités)

FORMATION UNIVERSITAIRE

- **1992 : Licence de Psychologie (options : Psychologie Expérimentale, Psychologie Clinique), Université de Bordeaux 2**
- **1993 : Maîtrise de Psychologie Expérimentale, Université de Bordeaux 2**
- **1994 : DEA de Biologie Santé (option Neurosciences et Pharmacologie), Université de Bordeaux 2**
- **1998 : Doctorat de Biologie Santé (option Neurosciences et Pharmacologie), Université de Bordeaux 2**

ACTIVITES DE RECHERCHE

- **1992-1993 : Maîtrise de Psychologie Expérimentale, Université de Bordeaux 2**
  «Effets de la lésion du cortex perirhinal sur la mémoire de reconnaissance spatiale chez la souris», tuteurs : Dr M Meunier, Pr C Destrade. Laboratoire de Neurosciences Comportementales et Cognitives, Centre National de la Recherche Scientifique (CNRS), Unité Mixte de Recherche 239, Pr. R. Jaffard, Bordeaux.
- **1993-1994 : DEA de Biologie Santé (option Neurosciences et Pharmacologie), Université de Bordeaux 2**
  «Etudes des différences individuelles dans la sensibilité aux propriétés renforçantes de la nourriture chez le rat», tuteurs : Pr H Simon, Dr W Mayo. Laboratoire de Psychobiologie des Comportements Adaptatifs, Institut National de la Santé Et de la Recherche Médicale (INSERM), Unité 259, Pr. M. Le Moal, Bordeaux.

- **1994-1998 : Doctorat de Biologie Santé (option Neurosciences et Pharmacologie), Université de Bordeaux 2**
  «Neurostéroïdes et mémoire : Etude des effets comportementaux, neurochimiques et électroencéphalographiques du sulfate de prégnènolone chez le rat», tuteurs : Dr W Mayo et Pr M Le Moal. Laboratoire de Psychobiologie des Comportements Adaptatifs (INSERM, U259, Pr. M. Le Moal, Bordeaux).

- **1999 : Stage post-doctoral, Instituto Ramon y Cajal, CSIC (Madrid, Spain)**
  «Neurostéroïdes et plasticité cérébrale», Instituto Santiago Ramon y Cajal, Consejo Superior de Investigaciones Cientificas (CSIC), Pr L.M. Garcia-Segura, Madrid.

- **1999 : Maître de Conférences en Neurosciences (Université de Lille 1)**
  «Stress Gestationnel et Adaptation chez le rat: plasticité comportementale, neuroendocrine et cérébrale chez la mère et sa progéniture".

COMPETENCES TECHNIQUES

Neurobiologie et physiologie :

- **Microdialyse intracérébrale chez le rat libre de se mouvoir** : chromatographie liquide haute performance (HPLC) pour l'étude de l'Acétylcholine (fabrication des colonnes de chromatographie ; montage, démontage, maintenance du système complet comprenant l'HPLC et l'injecteur) ; préparations des tampons ; fabrication de canules transversales, implantations de canules à demeure et/ou guide canules pour microdialyse verticale.
- **Enregistrements électrocorticographiques chez l'animal libre de se mouvoir** : étude du cycle veille/sommeil (sommeil lent, sommeil paradoxal) ; implantation des électrodes d'enregistrement ; analyse du signal.
- **Chirurgie Stéréotaxique** : implantation de canules (injection, dialyse), implantation d'éléctrodes ECoG, lésions cérébrales (acide iboténique, kainate, électrolytiques), injections intracérébrales.
- **Microdissection chez le rat :** structures cérébrales (hippocampe, hypophyse, hypothalamus, cortex, striatum, cervelet, tronc cérébral) ; utilisation de matrice de dissection pour repérage stéréotaxique de petites structures (noyau accumbens, amygdale etc.).
- **Coupes tissus au vibratome, cryotome et microtome:** pour immunohistochimie, hybridation in situ et vérifications de coordonnées stéréotaxiques.
- **Immunohistochimie (c-fos ; BrdU ; GFAP…) :** perfusion pour fixation des tissus ; coupes ; réaction immunologiques ; montage des coupes ; comptage.
- **Injections :** intrapéritonéale, intraveineuse, sous-cutanées.
- **Prélèvements d'organes** : glandes mammaires, utérus, surrénales, foie…

Neuroendocrinologie :

- **Implantations intra-jugulaires pour injections et prélèvements chroniques chez l'animal libre de se mouvoir :** fabrication des canules, implantation intracardiaque, prélèvements et administrations intraveineuses.
- **Dosages hormonaux radioimmunologiques (RIA) et immunoenzymatique (ELISA) :** ACTH, corticostérone, estradiol, hormones thyroïdiennes (T3, T4)…
- **Scatchard :** caractérisation (affinité et liaison maximale) des récepteurs centraux aux corticostéroïdes et de la transcortine plasmatique (validation du dosage pour la détermination de la corticostérone libre).

Comportement :

Etudes comportementales et pharmacologie du comportement Réalisation d'éthogrammes.

- **Apprentissages et Mémoires :** Mesures des performances spatiales en mémoire de référence (labyrinthe radial, piscine de Morris), en mémoire de travail (labyrinthe radial). Evaluations des performances mnésiques dans des tâches de reconnaissance spontanée : épreuves spatiales allocentriques (labyrinthe aquatique, labyrinthe en Y), épreuve de reconnaissance de topographies (validation d'une nouvelle tâche mnésique chez le rat) ; reconnaissance visuelle (reconnaissance d'objet) ; reconnaissance olfactive (reconnaissance sociale).
- **Anxiété / émotivité :** Labyrinthe en croix surélevé, Open-Field, Test d'émergence, test de la nage forcée, interaction sociale, réactivité locomotrice à la nouveauté.
- **Motivation pour des renforçateurs naturels ou pharmacologiques :** Conditionnement opérant en cage de Skinner, préférence de place pour l'amphétamine, réponse locomotrice à la nouveauté, réponse locomotrice aux drogues.
- **Observation du comportement maternel et de la progéniture :** Construction du nid, allaitement (passif, actif), capacités de récupération des petits, tests d'agression maternelle. Comportement d'amassement de nourriture. **E**tude du développement psychomoteur du raton.
- **Stress Prénatal :** Gestion des reproductions, frottis, contentions, sevrage, sexage.

SOCIETES ET RESPONSABILITES ADMINISTRATIVES

- Membre de la Société des Neurosciences française.
- Membre de la Société française de Neuroendocrinologie (demande en cours).
- Représentant ACMO du laboratoire 2000-2005.

- Membre du Conseil de Gestion de l'Animalerie de l'UFR de Biologie depuis 2000.
- Membre élue du Conseil Scientifique de l'Université de Lille 1 2003-2007.
- Membre élue de la commission des spécialistes de l'Université de Lille 1 depuis 2004-2007 (Sciences de la Vie, section 66-69).
- Resp. L2, Cours de Neurophysiologie (Psychologie).
- Resp. L1, Cours de Biologie (MIASH)

RESPONSABILITES SCIENTIFIQUES

Financements obtenus et responsabilités de projets scientifiques

- **1994 : Bourse de DEA critères Universitaires.**
- **1999 : Bourse post-doctorale de l'Institut National de la Santé et de la Recherche Médicale (INSERM).** *« Neurostéroïdes et plasticité cérébrale ».*
- **2000 : Bonus Qualité Recherche (BQR) Université de Lille 1, « Installation nouveaux collègues ».**
- **2001-2002 : Programme Européen d'Action Intégrée (PAI) EGIDE, PICASSO.** *« Stress prénatal, vieillissement cérébral et cognitif »*
- **2002-2004 : Subvention Centre National de la Recherche Scientifique, programme « Sciences biomédicales, santé et société».** (co-responsable avec le Pr S. Maccari ; H. Louvart). *"Facteurs de vulnérabilité à l'état de stress post-traumatique : validation d'un modèle animal".*
- **2003-2005 : Subvention Mission Interministérielle de Lutte contre les Drogues et Toxicomanie/Institut National de la Santé et de la Recherche Médicale (MILDT/INSERM).** *"Influence du stress prénatal sur la vulnérabilité aux drogues pendant l'adolescence ".*
- **2004-2005 : Financement Ministériel pour l'accueil d'un chercheur post-doctorant étranger.** *"Influence du stress prénatal sur la vulnérabilité aux drogues pendant l'adolescence ".*
- **2005-2006 : Obtention d'un congés pour Recherche, Université de Lille 1.**
- **2006 : Bonus Qualité Recherche Université de Lille 1 « Aide à la mobilité doctorant ».** *« Stress prénatal et alcoolisation chronique : impact sur les récepteurs glutamatergiques métabotropiques dans l'hippocampe ». (V. Van Waes)*
- **2003-2007 : Subvention Institut de Recherche sur les Boissons (IREB).** *"Adolescence et Vulnérabilité à l'alcool dans le modèle du Stress Prénatal chez le rat".*
- **2004-2007 : Subvention FEDER-REGION, Action de Recherche Concertée d'Initiative Régionale (ARCIR).** PirCAD (Pôle Interdisciplinaire de Recherche

sur les Conduites Addictives) *Axe : " Stress périnatal, adolescence et conduites addictives : étude d'un modèle animal "*

- **2005-2007 : Subvention européenne « Programme Alfa » pour le développement d'un réseau d'échange eurocaraïbéen.** *« Formation d'étudiants en comportement niveau Master, Doctorat».*
- **2007 : Bonus Qualité Recherche Université de Lille 1 « Aide à la mobilité pour les orateurs invités ».**
- **2007 : Coopération Industrielle.** *« Effect of prenatal stress on crying, anxiety, quality of sleep and neurobiological markers in infant and adolescent rats »* (co-responsable avec le Pr S Maccari).
- **2008 : Obtention d'une Délégation auprès du CNRS (1 an à mi-temps).**

Encadrements de Pré-doctoral *(stages L3, M1, M2)*

- **2001-2003**, **F. Del Faverro :** "Neurogénèse hippocampique chez la femelle rat allaitant" / "Conséquences d'un stress durant la gestation sur le comportement maternel du rat". (Master 1 et 2 Psychologie) **1 article**
- **2001-2003, G. Bélizaire** : "Influence d'une sous-nutrition précoce sur le vieillissement cognitif, conatif et sur l'activité de l'axe corticotrope du rat mâle" / "Influences de l'IGF-1 sur le vieillissement des capacités cognitives, comportementales et sur l'activité de l'axe corticotrope du rat femelle stressé prénatalement ". (Master 1 et 2 Psychologie) **1 article**
- **2002 (2 mois), Olivier Pouyol :** « Le modèle du stress prénatal chez le rat » (Elève Ecole Vétérinaire de Lyon).
- **2002 (3 mois), Aurore Douchet :** « Effets d'une sous-nutrition périnatale sur le vieillissement cognitif du rat » (M1 Sciences de la Vie, Biologie et Physiologie Cellulaire)
- **2003-2004, Ludovic de France :** « Effet d'un stress intense sur la consommation spontanée d'alcool chez la femelle : modulation par le stress prénatal » (M1 Sciences de la Vie, Biologie et Physiologie Cellulaire) **1 article**
- **2005, Ndeye Aissatou Ndiaye :** « Stress prénatal et mémoire chez le rat » (M1 Sciences de la Vie, Biologie et Physiologie Cellulaire)
- **2004-2005, Céline Bossard :** « Effet d'un stress prénatal et d'une procédure de choc traumatique sur la mémoire » (TE Psychologie, L3)
- **2004-2005, Julie Berteloot :** « Stress prénatal chez le rat femelle : conséquences sur le plan mnésique suite à un stress intense » (TE Psychologie, L3)
- **2005-2006 (6 mois), José Miguel Laffita :** « Prenatal stress and brain aging in an animal model of PTSD » (Master Biologie Santé, Programme Alfa, CUBA)
- **2006-2007, Fatima Aitkhamoh :** « Stress prénatal et vulnérabilité à l'alcool chez le rat » (TE Psychologie, L3)
- **2006-2007, Marina Faucoeur:** « Stress prénatal et vulnérabilité à l'alcool chez le rat » (TE Psychologie, L3)

- **2006-2007, Enrique Talavera :** « Prenatal stress, alcohol and COX2 expression in the brain » (Master Biologie Santé, Programme Alfa, VENEZUELA)
- **2007 (3 mois), John Freddy Castro :** (Master Biologie Santé, Programme Alfa, COLOMBIE)

Encadrements de Thèses de Doctorat Biologie Santé, Option Neurosciences

1. **2000-2002 :** S. Morley-Fletcher. " Environnement Prénatal et Capacités Adaptatives chez le rongeur : troubles émotionnels et vulnérabilité aux drogues ". Encadrement : Dr M. Darnaudéry (50 %), Pr S. Maccari (50%). Financement : *Bourse de Fondation pour la Recherche Médicale.*
   5 articles
2. **2001-2005 :** H. Louvart. " Facteurs de vulnérabilité à l'état de stress post-traumatique : validation d'un modèle animal ". Encadrement : Dr M. Darnaudéry (80%), Pr S. Maccari (10%), Pr P. Thomas (10%). Financement : *Bourse de thèse de la Fondation pour la Recherche Médicale ; Subvention CNRS programme « Sciences biomédicales, santé et société».*
   5 articles
3. **2004- :** V. Van Waes. " Conséquences d'un stress prénatal sur la vulnérabilité à l'alcool lors de l'adolescence chez le rat ". Encadrement : Dr M. Darnaudéry (80%), Pr M. Lhermitte (10%), Pr S. Maccari (10%). Financement : *Bourse Conseil Régional Nord-Pas de Calais, Bourse du Président Université de Lille 2.*
   1 article

Encadrement Post-doctoral

- **2003-2007 :** Mihaela Enache. "Influence du stress prénatal sur la vulnérabilité aux drogues pendant l'adolescence". Encadrement : Dr Darnaudéry (80%) ; Pr Vieau, Dr I Dutriez (20%). Financement : *Bourse postdoctorale ministère recherche ; ARCIR (FEDER-REGION) PirCAD ; ATER postdoc étranger.* **3 articles**

- **2007 :** Charlotte Lalloux. "Ontogénèse des comportements anxieux chez les rats stressés en période prénatale ". Encadrement : Dr Darnaudéry (33%) ; Pr Maccari (33%) ; Dr Van Reeth (33%). Financement : *Industrie.*

Titulaire de l'Habilitation à Expérimenter chez l'animal vivant : (niveau I) et de l'Autorisation pour Chirurgie sur petit animal (# 5900 238)

Titulaire de la Prime d'Encadrement et de Recherche (PERD): 2004-2008

Collaborations

Nationales :

- **Equipe du Pr J.P. Dupouy puis du Pr D. Vieau:** Equipe de Neuroendocrinologie du Développement, Univ. de Lille 1.
- **Dr C. Rolando :** Lab. de Chimie Organique et Macromoléculaire, UMR CNRS 8009, Univ. de Lille 1.
- **Equipe du Pr M. Lhermitte :** Lab. de Toxicologie Analytique, Univ. de Lille 2.
- **Pr P. Thomas, Dr G. Vaiva, Dr F. Ducroq :** Service de Psychiatrie, CHU, Univ. de Lille 2.
- **Pr R. Garcia :** Université de Nice Sofia Antipolis.
- **Equipe du Dr Cador :** Neuropsychobiologie des systèmes addictifs, CNRS UMR 5227 - Université Bordeaux 2.

Internationales :

- **Pr L. M. Garcia-Segura :** CSIC, Institut Ramon y Cajal, Madrid, Espagne
- **Dr M. Perez-Martin :** Universidad de Malaga, Espagne.
- **Pr A Mathé :** Karolinska Institutet, Stockholm, Sweden.
- **Equipe du Pr A. Nicoletti:** Université La Sapienza, Rome.
- **Dr O. Berton :** Southwestern University, Dallas.

Expertises auprès de revues scientifiques

Behavioral and Brain Functions ; Behavioural Brain Research ; Brain Research ; Behavioural Neurosciences; Developmental Psychobiology ; Naunyn-Schmiedeberg's Archives of Pharmacology; European journal of Applied Psychology ; Physiology and Behavior; Psychoneuroendocrinology.......

Information scientifique et vulgarisation

- Participation au programme de « la semaine du cerveau » 2001.
- Participation au programme « Chercheurs à l'Ecole » 2005.
- Chairman à la 10ème journée LARC Neurosciences, Lille, 10 novembre, 2006.
- Participation à l'organisation du colloque « Conduites addictives chez les jeunes : de la prédiction à l'action », PIRCAD Pôle Interdisciplinaire de Recherche sur les Conduites Addictives 7-8 décembre 2006.
- Participation à la mise en place du programme de la formation des personnels à l'expérimentation animale pour l'aspect comportement (niveau I et II).
- Participation à l'élaboration d'un réseau de formation avancée d'étudiants entre Europe et Amérique du Sud (Colombie, Venezuela, Argentine, Cuba, Angleterre, Espagne, France) : programme Alfa (responsable global du réseau : Pr Lopera ; responsable France : M Darnaudéry).
- Tuteur dans le cadre du Programme Alfa formation d'étudiants en 3ème cycle : Europe-Amérique du Sud (2003-2007).

ENSEIGNEMENTS UNIVERSITAIRES

- **Etablissements :** Université des Sciences et Technologies de Lille 1, Université de Lille 3, Université de Lille 2.
- **UFR :** Biologie, Psychologie, MIASH, Formation continue, relations Internationales
- **Disciplines :** Neurosciences, Biologie, Neuropsychologie, Psychologie, Statistiques.
- **Nature (CM, TD, TP)** : 192 heures équivalents TD, dont : CM : 118 h, TD : 59 h, TP : 16h.
- **Niveaux** : 3ème cycle : cours ou TD en Masters Professionnels et Recherche ; 2ème cycle cours, TD, TP et 1^{ier} cycle : cours, TD.

Cours Magistraux (L1, L2, L3, M2 Psychologie ; L1 MIASH; L2, L3, M2 Biologie Cellulaire)

- Biochimie (L1 Psychologie)
- Neurotransmission et Neuromédiateurs (L2 Psychologie)
- Apport des modèles animaux (L2 Biologie Cellulaire)
- Adaptations physiologiques : comportements et stress (L3 Biologie Cellulaire)
- Psychopharmacologie (M1 Biologie Cellulaire, M2 Neuropsychologie)
- Plasticité cérébrale et Comportements (L3 Biologie Cellulaire, M2 Neuropsychologie)
- Neurobiologie des addictions (L3 Biologie Cellulaire)
- Stress Prénatal et Désadaptation (M2 Biologie Santé, Option Neurosciences)

Travaux Dirigés (L2, L3 Psychologie ; L3 Sciences de la Vie et de la Santé)

- Neuroanatomie humaine
- Neurochimie
- Comportement maternel et adaptation de la descendance
- Pathologies de la motricité : Parkinson et Huntington
- Modèles animaux de psychopathologies
- Drogues et toxicomanies
- Facteurs neurotrophiques et plasticité hippocampique
- Modèles animaux et psychopathologies

Travaux Pratiques (L2, L3 Psychologie)

- Etude des seuils absolus et différentiels, Vision- Audition
- Mesures EEG, EMG et ECG
- Conditionnement classique et instrumental chez le poisson rouge et chez le rat
- Mesure du comportement anxieux chez le rongeur
- Ontogénése des Encéphales de Vertébrés, Neuroanatomie humaine
- Temps de Réaction

TER de la M1 « Sciences de la Vie et de la Santé »

- 2000 : Initiation aux approches comportementales chez l'animal.
- 2002 : Evaluation des capacités de mémoire spatiale lors du vieillissement chez des rats ayant reçus un stress prénatal.
- 2003 : Stress prénatal et capacités d'adaptation : effets sur les capacités d'apprentissage et l'anxiété de la femelle rat.
- 2004 : Stress social et capacités d'apprentissage.

Formation Continue

- **Formateur Habilitation à l'Expérimentation Animale (niveaux 1 et 2),** Institut Pasteur de Lille, Univ. Lille 1 : Stress et Comportement, Plasticité Cérébrale et Comportement, Modèles animaux, Méthodes d'étude du comportement chez le rongeur.

International Summer School in Neuroscience

- **Summer School in Neuroscience "Brain Plasticity in Life Span",** Lille September 2-7 2006. http://www.neuroschool.univ-lille1.fr
- **Summer School in Neuroscience "Advance courses in Neuroplasticity"**, Rome September 5-11, 2007. http://www.neuroschoolrome.univ-lille1.fr

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1. Abrous DN, Montaron MF, Petry KG, Rougon G, **Darnaudéry M**, Le Moal M, Mayo W (1997) Decrease in highly polysialylated neuronal cell adhesion molecules and in spatial learning during ageing are not correlated. ***Brain Research*** 744: 285-292. **IF = 2.389**

2. M. Le Moal, M. Vallée, **M. Darnaudéry**, M. Pallarès, H. Simon and W. Mayo, (1997) Memory and cognition: A role for neurosteroids, ***European Neuropsychopharmacology*** 7 : 80-S81. **IF = 3.510**

3. Vallée M, Mayo W, **Darnaudéry M**, Corpechot C, Young J, Koehl M, Le Moal M, Baulieu EE, Robel P, Simon H (1997) Neurosteroids: deficient cognitive performance in aged rats depends on low pregnenolone sulfate levels in the hippocampus. ***Proceedings of the National Academy of Sciences of the United States of America (PNAS)*** 94: 14865-14870. **IF = 10.50**

4. Pallares M, **Darnaudéry M**, Day J, Le Moal M, Mayo W (1998) The neurosteroid pregnenolone sulfate infused into the nucleus basalis increases both acetylcholine release in the frontal cortex or amygdala and spatial memory. ***Neuroscience*** 87: 551-558. **IF = 3.456**

5. **Darnaudéry M**, Koehl M, Pallares M, Le Moal M, Mayo W (1998) The neurosteroid pregnenolone sulfate increases cortical acetylcholine release: a microdialysis study in freely moving rats. ***Journal of Neurochemistry*** 71: 2018-2022. **IF = 4.604**

6. **Darnaudéry M**, Bouyer JJ, Pallares M, Le Moal M, Mayo W (1999) The promnesic neurosteroid pregnenolone sulfate increases paradoxical sleep in rats. ***Brain Research*** 818: 492-498. **IF = 2.389**

7. **Darnaudéry M**, Pallares M, Bouyer JJ, Le Moal M, Mayo W (1999) Infusion of neurosteroids into the rat nucleus basalis affects paradoxical sleep in accordance with their memory modulating properties. ***Neuroscience*** 92: 583-588. **IF = 3.456**

8. Koehl M, **Darnaudéry M**, Dulluc J, Van Reeth O, Le Moal M, Maccari S (1999) Prenatal stress alters circadian activity of hypothalamo-pituitary-adrenal axis and hippocampal corticosteroid receptors in adult rats of both gender. ***Journal of Neurobiology*** 40: 302-315. **IF = 4.209**

9. **Darnaudéry M**, Koehl M, Piazza PV, Le Moal M, Mayo W (2000) Pregnenolone sulfate increases hippocampal acetylcholine release and spatial recognition. ***Brain Research*** 852: 173-179. **IF = 2.389**

10. Maccari S, **Darnaudéry M** and Van Reeth, O (2001) Hormonal and behavioral abnormalities induced by stress « in utero » : an animal model for depression. ***Stress*** 4: 169-181. **IF = 2.389**

11. Koehl M, Bouyer JJ, **Darnaudéry M**, Le Moal M, Mayo W (2002) The effect of restraint stress on paradoxical sleep is influenced by the circadian cycle. ***Brain Research*** 937: 45-50. **IF = 2.389**

12. Deroche-Gamonet V, **Darnaudéry M**, Bruins-Slot L, Piat F, Le Moal M, Piazza PV (2002) Study of the addictive potential of modafinil in naive and cocaine-experienced rats. ***Psychopharmacology (Berl)*** 161: 387-395. **IF = 3.994**

13. **Darnaudéry M**, Pallares M, Piazza PV, Le Moal M, Mayo W (2002) The neurosteroid pregnenolone sulfate infused into the medial septum nucleus increases hippocampal acetylcholine and spatial memory in rats. ***Brain Research*** 951: 237-242. **IF = 2.389**

14. Salome N, Viltart O, **Darnaudéry M**, Salchner P, Singewald N, Landgraf R, Sequeira H, Wigger A (2002) Reliability of high and low anxiety-related behaviour: influence of laboratory environment and multifactorial analysis. ***Behavioural Brain Research*** 136: 227-237. **IF=2.992**

15. Maccari S, **Darnaudéry M**, Morley-Fletcher S, Zuena AR, Cinque C, Van Reeth O (2003) Prenatal stress and long-term consequences: implications of glucocorticoid hormones. ***Neuroscience Biobehavioural Review*** 27: 119-127. **IF=8.293**

16. Morley-Fletcher S, **Darnaudéry M**, Koehl M, Casolini P, Van Reeth O, Maccari S (2003) Prenatal stress in rats predicts immobility behavior in the forced swim test. Effects of a chronic treatment with tianeptine. ***Brain Research*** 989: 246-251. **IF = 2.389**

17. Mayo W, George O, Darbra S, Bouyer JJ, Vallee M, **Darnaudéry M**, Pallares M, Lemaire-Mayo V, Le Moal M, Piazza PV, Abrous N (2003) Individual differences in cognitive aging: implication of pregnenolone sulfate. ***Progress in Neurobiology*** 71: 43-48. **IF = 11.933**

18. Lesage J, Del Favero F, Leonhardt M, Louvart H, Maccari S, Vieau D, **Darnaudéry M** (2004) Prenatal stress induces intrauterine growth restriction and programmes glucose intolerance and feeding behaviour disturbances in the aged rat. ***Journal of Endocrinology*** 181: 291-296. **IF = 3.059**

19. **Darnaudéry M**, Koehl M, Barbazanges A, Cabib S, Le Moal M, Maccari S (2004) Early and later adoptions differently modify mother-pup interactions. ***Behavioural Neuroscience*** 118: 590-596. **IF = 3.071**

20. **Darnaudéry M**, Dutriez I, Viltart O, Morley-Fletcher S, Maccari S (2004) Stress during gestation induces lasting effects on emotional reactivity of the dam rat. ***Behavioural Brain Research*** 153: 211-216. **IF = 2.992**

21. Morley-Fletcher S, **Darnaudéry M**, Mocaer E, Froger N, Lanfumey L, Laviola G, Casolini P, Zuena AR, Marzano L, Hamon M, Maccari S (2004) Chronic treatment with imipramine reverses immobility behaviour, hippocampal corticosteroid receptors and cortical 5-HT(1A) receptor mRNA in prenatally stressed rats. ***Neuropharmacology*** 47: 841-847. **IF = 3.860**

22. Louvart H, Maccari S, **Darnaudéry M** (2005) Prenatal stress affects behavioral reactivity to an intense stress in adult female rats. ***Brain Research*** 1031: 67-73. **IF = 2.389**

23. Louvart H, Maccari S, Ducrocq F, Thomas P, **Darnaudéry M** (2005) Long-term behavioural alterations in female rats after a single intense footshock followed by situational reminders. ***Psychoneuroendocrinology*** 30: 316-324. **IF = 4.850**

24. Louvart H, Maccari S, Lesage J, Leonhardt M, Dickes-Coopman A, **Darnaudéry M** (2006) Effects of a single footshock followed by situational reminders on HPA axis and behaviour in the aversive context in male and female rats. ***Psychoneuroendocrinology*** 31:92-99. **IF = 4.850**

25. **Darnaudéry M**, Perez-Martin M, Belizaire G, Maccari S, Garcia-Segura LM (2006) Insulin-like growth factor 1 reduces age-related disorders induced by prenatal stress in female rats. ***Neurobiology of Aging*** 27:119-127. **IF = 5.599**

26. Viltart O, Mairesse J, **Darnaudéry M**, Louvart H, Vanbesien-Mailliot C, Catalani A, Maccari S (2006) Prenatal stress alters Fos protein expression in hippocampus and locus coeruleus stress-related brain structures. ***Psychoneuroendocrinology*** 31:769-780. **IF = 4.850**

27. Van Waes V, Enache M, Dutriez I, Lesage J, Morley-Fletcher S, Vinner E, Lhermitte M, Vieau D, Maccari S, **Darnaudéry M** (2006). Hypo-response of the hypothalamic-pituitary-adrenocortical axis after an ethanol challenge in prenatally stressed adolescent male rats, ***European Journal of Neuroscience*** 24, 1193-1200. **IF = 3.949**

28. Canu MH, **Darnaudéry M**, Falempin M, Maccari S, Viltart O (2007) Effect of hindlimb unloading on motor activity in adult rats: impact of prenatal stress. ***Behavioural Neuroscience*** 121:177-185. **IF = 3.071**

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31. Garcia-Segura L.M., Diz-Chaves Y., Perez-Martin M. and **Darnaudéry M.** Estradiol, Insulin-like growth factor-I and brain aging. ***Psychoneuroendocrinology*** **32:S57-S61. IF = 4.850**

32. **Darnaudéry M,** Perez-Martin M, Del FaveroF, Gomez-Roldan C, Garcia-Segura LM, Maccari S (2007) Early motherhood in rats is associated with a modification of hippocampal function. ***Psychoneuroendocrinology*** 32:803-812. **IF = 4.850**

33. **Darnaudéry M**. and Maccari S., Programming of the stress response in male and female rats by prenatal restraint stress. (2007). ***Brain research review.*** Nov 28 **IF = 5.595**

34. Enache M., Van Waes V., Vinner E., Lhermitte M., Maccari S. and **Darnaudéry M**. (2008) Impact of an acute exposure to ethanol on the oxidative stress status in the hippocampus of prenatally stressed adolescent male rats. ***Brain Research***. 1191:55-62. **IF = 2.389.**

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1. **Darnaudéry M.**, Pallarès M., Koehl M., Le Moal M. et Mayo M., (1998) Le sulfate de prégnènolone stimule la libération d'acétylcholine corticale et améliore les performances dans une tâche de mémoire spatiale chez le rat. Dans *:* ***Actualités sur la maladie d'Alzheimer et les syndromes apparentés***. Solal (Eds) pp 159-166.

2. Mayo W., Vallée M., **Darnaudéry M.** and Le Moal M. (1999) Neurosteroids : behavioral studies. In : ***Neurosteroids : A new regulatory function in the Nervous System.*** Baulieu E.E, Robel P. and Schumacher M. (eds), The Humana Press Inc.

3. **Darnaudéry M**, Van Waes V, Enache M, Morley-Fletcher S, Dutriez-Casteloot I, Lesage J, Vinner E, Lhermitte M and Maccari S (2005). 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 : 35-41.

4. Van Waes V, Enache M , Vinner E , Lhermitte M , Maccari S, **Darnaudéry M** (2007) Impact du stress prénatal sur la vulnérabilité à l'éthanol chez le rat. ***Cahier de l'IREB.***

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- Vallée M., Maccari S., **Darnaudéry M.,** Dellu F., Le Moal M., Simon H. et Mayo W. Effets opposés du stress prénatal et de la manipulation postnatale sur les performances cognitives de l'animal âgé. 4ème colloque de la formation doctorale de Neurosciences et Pharmacologie des Universités de Bordeaux I et II, Arcachon (France), 4 mai 1995.

- Vallée M., Maccari S., **Darnaudéry M.,** Dellu F., Le Moal M., Simon H. and Mayo W. Perinatal environment and cognitive aging: a longitudinal study in rats. 25rd Annual Meeting Society for Neuroscience, San Diego (USA), 11-16 novembre 1995.

- Abrous D.N., Montaron M.F., Petry K.G., Rougon G., **Darnaudéry M,** Le Moal M, Simon H, Mayo W. Ageing related decrease in PSA-NCAM is not related to cognitive deficits. 25th Annual Meeting of the Society for Neuroscience, San Diego (USA), 11–16 novembre 1995.

- Montaron M.F., Petry K.G., Rodriguez J.J., Rougon G., Marinelli M., **Darnaudéry M.,** Premier S, Mayo W, Le Moal and Abrous DN, Influence du vieillissement sur l'expression de PSA-NCAM. Troisième colloque de la Société des Neurosciences, Bordeaux (France) 25-28 mai 1997.

- **Darnaudéry M.,** Mayo W., Le Moal M. and Piazza P.V. Validation d'un nouveau test de mémoire spatiale chez le rat. 4ème colloque de la formation doctorale de Neurosciences et Pharmacologie des Universités de Bordeaux I et II, Arcachon (France), 4 mai 1997.

- **Darnaudéry M.,** Pallares M., Koehl M., Le Moal M. and Mayo W. Le sulfate de prégnénolone stimule la libération d'acétylcholine corticale et améliore les performances dans une tâche de mémoire spatiale chez le rat. 4ème réunion francophone sur la maladie d'Alzheimer et les syndromes apparentés, Montpellier (France), sept 1997.

- **Darnaudéry M.,** Koehl M., Pallares M., Vallée M., Le Moal M. et Mayo W, Le sulfate de prégnénolone stimule la libération d'acétylcholine dans le cortex frontal et l'hippocampe: étude par microdialyse chez le rat éveillé. 3ème colloque de la société des Neurosciences, Bordeaux (France) 25-28 Mai 1997.

- Bouyer J.J., **Darnaudéry M.,** Déminère J.M., Le Moal M. and Mayo W., Le prégnénolone sulfate augmente le sommeil paradoxal chez le rat. 3ème colloque de la société des Neurosciences, Bordeaux (France) 25-28 Mai 1997.

- Pallares M., **Darnaudéry M.,** Day J., Vallée M., Le Moal M. and Mayo W., Infusions of pro-mnesic doses of pregnenolone sulfate into the nucleus basalis increase acetylcholine release in the frontal cortex and amygdala. 27rd Annual Meeting Society for Neuroscience, New-Orleans (USA), 25-30 october 1997.

- Bouyer J.J., **Darnaudéry M.,** Déminère J.M., Le Moal M. and Mayo W., Systemic administration of pregnenolone sulfate modifies the sleep-wakefulness cycle in rat. 27rd Annual Meeting Society for Neuroscience, New-Orleans (USA), 25-30 october 1997.

- **Darnaudéry M.,** Koehl M, Pallares M., Le Moal M. and Mayo W. Pregnenolone sulfate increases cortical and hippocampal acetylcholine release: a microdialysis study in behaving rats. 27rd Annual Meeting Society for Neuroscience, New-Orleans (USA), 25-30 october 1997.

- Le Moal M., Vallée M., **Darnaudéry M.,** Pallares M., Simon H. et Mayo W., Memory and cognition : a role for neurosteroids. 10th ECNP congress, Vienne (Autriche), 13-17 september 1997.

- **Darnaudéry M.,** Neurostéroides et mémoire : implication des systèmes cholinergiques centraux. 7ème colloque de la formation doctorale de Neurosciences et Pharmacologie des Universités de Bordeaux I et II, Arcachon (France), 29 avril 1998.

- **Darnaudéry M.,** Bouyer J.J., Pallarés M., Le Moal M. and Mayo W., The promnesic neurosteroid pregnenolone sulfate increase paradoxical sleep, Forum of European Neuroscience, Berlin (Germany), 27-30 june 1998.

- **Darnaudéry M.,** Bouyer J.J., Pallarés M., Le Moal M. and Mayo W., Neurosteroid infusions into the nucleus basalis affect paradoxical sleep in a way that is compatible with their memory properties in rat, 28rd Annual Meeting Society for Neuroscience, Los Angeles (USA), 7-12 november 1998.

- **Darnaudéry M.,** Pallarés M. , Piazza P.V., Le Moal M. and Mayo W., L'administration de sulfate de prégnènolone dans le septum médian améliore les performances de mémoire spatiale et augmente les concentrations extracellulaires d'acétylcholine dans l'hippocampe, 4ème colloque de la société des Neurosciences Marseille (France), 25-28 Mai 1999.

- Bouyer J.J., **Darnaudéry M.,** Pallarés M, Le Moal M. and Mayo W., Modulations opposées du sommeil paradoxal par deux neurostéroïdes, le sulfate de prégnènolone et l'alloprégnanolone. 4èmes Journées Internationales des Troubles du Sommeil, Bordeaux (France), 27-28 Mai 1999.

- **Darnaudéry M.,** Viltart O., Salome N., Maccari S. Emotional reactivity of mother rats is durably affected by chronic stress during pregnancy, Forum of European Neuroscience, Brighton (England), 24-28 june 2000.

- **Darnaudéry M.,** Viltart O., Salome N., Sequeira H., Maccari S. Emotional reactivity of mother rats is durably affected by chronic stress during pregnancy, VIth International Conference on Hormones, Brain and Behavior, Madrid (Spain), 5-9 august 2000.

- Maccari S., **Darnaudéry M.,** Dugovic C., Rettori M.C. and Van Rreeth O. Abnormalities induced by stress "*in utero*": an animal model for depression. 3rd World Congress on Stress, Dublin (Ireland), 24-27th september 2000.

- **Darnaudéry M.,** Viltart O., Salomé N., Sequeira H., Maccari S. Effet d'un stress chronique lors de la gestation sur la réactivité émotionnelle des mères chez le rat. 4ième journée LARC (Lille), 20 octobre 2000.

- Salomé N, Viltart O, Ohl F., Wigger A, **Darnaudéry M,** Landgraf R., Sequeira H Validation comportementale d'un modèle d'anxiété congétinale : les rats HAB/LAB. 4ième journée LARC (Lille), 20 octobre 2000.

- **Darnaudéry M.,** Buée L., Viltart O., H. Sequeira and Maccari S. Chronic stress during pregnancy increases anxiety of females rats, 30th Annual Meeting Society for Neuroscience, New Orleans (USA), 4-9 november 2000.

- Morley-Fletcher S, **Darnaudéry M.,** Mocaer E and Maccari S. Effet d'un traitement chronique d'imipramine dans le modèle du stress prénatal chez le rat. Toulouse (France) 25-28 mai 2001.

- **Darnaudéry M**., Del Faverro F., Maccari S. Effets d'un stress chronique lors de la gestation sur l'adaptation comportementale des mères chez le rat. 5ieme journée LARC (Rouen), 19 octobre 2001.

- **Darnaudéry M.,** Del-Favero F., Maccari S. Effects of restraint stress during pregnancy on coping strategies in lactating dams. IBNS, Capri (Italy), 19-23 june 2002.

- **Darnaudéry M.,** Del-Favero F., Maccari S.Chronic stress during pregnancy affects coping strategies of mother rats. FENS, Paris (France), 13-17 july 2002.

- Morley-Fletcher S., **Darnaudéry M.,** Mocaer E., Froger N., Laviola G., Casolini P., Maccari S. Prenatal stress and antidepressants: effects of a chronic treatment with imipramine in rats. 3rd Forum of European Neuroscience, Paris, 13-17 juillet 2002.

- Lesage J, **Darnaudéry M**, Léonhardt M, Bréant B, Matias I, Di Marzo V, Dupouy JP, Maccari S. Prenatal stress leads to the development of type 2

diabetes and disturbs the feeding behaviour in aged male rats. Forum of European Neurosciences, Juillet 2002, Paris.

- Bélizaire G., Perez M., Maccari S. , Garcia-Segura L.M., **Darnaudéry M.** Prenatal stress and cognitive ageing: effects of insulin-like factor-1 on spatial learning and neurogenesis in the hippocampus. 6ieme journée LARC (Caen), 18 octobre 2002.

- Louvart, H., Ducrocq F., Thomas, P., Maccari, S., **Darnaudéry, M**. Effets du stress prénatal sur la réponse à un stress intense chez la femelle rat : implications pour l'étude du stress post-traumatique. 6ème journée scientifique du réseau LARC, Caen, 18 octobre 2002.

- Morley-Fletcher S., **Darnaudéry M.**, Mocaer E., Froger N., Lanfumey L., Hamon M., Laviola G., Casolini P., Zuena A., Maccari S. Effects of a chronic treatment with imipramine on behavioural and neurochemical parameters in prenatally stressed rats.6ème Journée de LARC, Caen, 18th October 2002.

- **Darnaudéry M.,** Perez M., Bélizaire G., Maccari S. and Garcia-Segura L.M. Prenatal stress impairs memory of aged female rats: effects of chronic igf-1 treatment. 32nd Annual Meeting, Orlando, Florida (USA), 11–16 november 2002.

- **Darnaudéry M.,** Del Favero F., Defrance L., Maccari S., Garcia-Segura L.M., Perez-Martin M. Motherhood Affects Hippocampal Neurogenesis and Behaviour In Rats. Program N°212.12 – 2003 Abstract Viewer / Itinerary Planner. Washington, Dc : Society For Neuroscience.

- Louvart, H.,Maccari, S., Thomas, P., Ducrocq, F., **Darnaudéry, M.,** Stress précoce et réponse à un stress intense à l'âge adulte chez le rat femelle. Implications pour l'étude de l'Etat de Stress Post-Traumatique. 3ème journée André Verbert, colloque annuel des doctorants de l'Ecole Doctorale Biologie et Santé de Lille, Lille. Septembre 2003.

- Morley-Fletcher S., **Darnaudéry M.,** Enache M., Mathé A.A., Maccari S. Prenatal stress and forced ethanol consumption during adolescence in female rats: effects of an adult post-treatment with escitalopram. 4th Forum of European Neuroscience, Lisbon, 10th 14th July 2004.

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

- Maccari S., Louvart H., Lesage J., Léonhardt M., Dickes Coopman A., **Darnaudéry M.** Sex-differences in the effects of a single footshock followed by situational reminders in rats : implication for Post Traumatic Stress Disorder. 4th Forum of European Neuroscience, Lisbon, 10th 14th July 2004.

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- Van Waes V., Enache M., Morley-Fletcher S., Dutriez-Casteloot I., Lesage J., Vinner E., Lhermitte M., Vieau D., Maccari S. et **M. Darnaudéry.** 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. 8ème Journée Scientifique du réseau LARC-Neurosciences, Paris, France, 15 Octobre 2004,

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- Enache M., Van Waes V., Morley-Fletcher S., Vinner E., Lhermitte M., Dutriez-Casteloot I., Lesage J., Vieau D., Maccari S., **Darnaudéry M.** Effect 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, Lille, France, 17-20 Mai 2005.

- **Darnaudéry M.,** Enache M. and S. Maccari, Chronic stress during pregnancy affects the spatial memory of dam rats. 7ème colloque de la Société des Neurosciences, Lille, France, 17-20 Mai 2005.

- 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, Lille, France, 17-20 mai 2005.

- Morley-Fletcher S, **Darnaudéry M,** Mairesse J, Enache M, Galietta G, Mathé A, Maccari S. Prenatal stress and forced ethanol consumption during adolescence in female rats: effects of an adult post-treatment with escitalopram. 7ème colloque de la Société des Neurosciences, Lille, 2005.

- **Darnaudéry M.,** Louvart H., Defrance L., Maccari S. Effet d'un stress intense sur l'appétence spontanée pour l'éthanol chez des rats femelles : modulation par le stress prénatal, 9ème journées LARC Neurosciences, Amiens, France, 14 Octobre 2005.

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- **Darnaudéry M.,** Perez-Martin M., Garcia-Segura L.M., Maccari S. early motherhood affects hippocampal function Parental Brain Conference, Back Bay Hilton Hotel, Boston, USA, June 7-10, 2007.

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COMMUNICATIONS ORALES

- « Stress et Toxicomanie ». Semaine du Cerveau, Lille le 20 Mars 2001.

- « Implication des facteurs maternels dans les effets à long terme du stress prénatal ». Cinquième colloque de la Société des Neurosciences, Toulouse (France) 25-28 mai 2001.

- "Does prenatal stress causes depression offspring? An animal model." ISAD, Sicily, (Italy) 9-12 March 2002.

- « Comportement maternel et anxiété. » Conférence invitée dans le cadre d'un cycle de séminaires « Psychiatrie et Psychobiologie » organisé par le Pr J. Epelbaum à l'Hôpital Ste Anne (Paris) 16 mars 2003.

- « Conséquence d'un stress prénatal sur l'anxiété et l'activité de l'axe corticotrope après administration aigue d'alcool chez le rat adolescent. » 17ème colloque de l'IREB Iles des Embiez 1-2 décembre 2004.

- « Stress gestationel chez le rat : impact à court et à long terme chez le mère et sa descendance » Séminaire sur invitation du Pr G Simonet, Laboratoire "Homéostasie-Allostasie-Pathologie" EA 3666 (Bordeaux) 22 avril 2005.

- "Perinatal stress and the mechanism of programming of the brain for later life", Séminaire sur invitation du Dr G Bergonzelli Nestlé Research Center (Lausanne, Suisse) 12 avril 2006.

- « Stress Prénatal et Vulnérabilité à l'alcool chez le rat ». Colloque « Conduites addictives chez les jeunes : de la prédiction a l'action », PIRCAD Pôle Interdisciplinaire de Recherche sur les Conduites Addictives (Lille) 7-8 décembre 2006.

- "Long lasting effects of stress during pregnancy on HPA function and behaviour in mother and offspring rats". Conférence invitée lors du Symposium "Corticosteroid effects and stress", IV International Meeting on "Steroids in the Nervous System" (Turin, Italy) 17-19 February 2007.

- "Effet à long terme du stress lors de la gestation sur l'axe corticotrope et le comportement chez le rat" Séminaire sur invitation du Dr JM Devaud au Centre de Recherches sur la Cognition Animale, UMR 5169, UPS – CNRS, Pr M Giurfa, (Toulouse) le 7 mars 2007.

- « Stress et modèles animaux pour l'étude du PTSD » Journée de réflexion sur le réseau national sur le thème des psychotraumas, (Marseille), le 21 juin 2007.

ACTIVITES DE RECHERCHE

AVANT PROPOS

Lors de mon cursus de Psychologie, je me suis orientée vers la recherche en Psychologie Expérimentale et par la suite, du fait de la qualité de l'environnement bordelais dans le domaine des Neurosciences, vers la Psychobiologie. En 1992, alors en Maîtrise de Psychologie Expérimentale, j'ai effectué un stage de recherche d'une année,au sein du laboratoire dirigé par le Pr Robert Jaffard (UMR CNRS 5106, Université de Bordeaux 1). Dans cette équipe, j'ai appris les bases théoriques et expérimentales de la neurobiologie de la mémoire et le rôle crucial des cortex de transition entourant la formation hippocampique (cortex périrhinal et enthorinal) pour la mémoire à court terme (Meunier et coll., 1993 ; Jaffard et Meunier, 1993). Alors que le paradigme de la reconnaissance par non appariement différé (« delayed non matching to sample task », DNMST) était parfaitement connu chez le singe (Mishkin et Delacour, 1975), les bases neuroanatomiques sous-tendant cette mémoire de travail visuelle faisaient encore l'objet de débat chez le primate (Murray et Mihshkin 1984, Squire et Zola-Morgan, 1991) et cette épreuve non validée chez le rongeur. J'ai travaillé avec le Dr Martine Meunier et le Pr Claude Destrade à la mise au point de l'épreuve de reconnaissance différée par non appariement chez la souris et sur l'impact de la lésion des cortex de transition. En dépit de mes efforts réitérés, je n'ai jamais réussi à faire apprendre à des souris (C57BL6) à réaliser cette épreuve de mémoire de travail. Mumby publiera quelques mois plus tard, une étude chez le rat au titre assez évocateur : *« Assessing working memory for objects in rats: no one said it was easy»* (Mumby, 1995). A ma connaissance, à ce jour personne n'utilise chez la souris, le « *delayed non matching to sample task* », tel qu'il est décrit chez l'homme ou le singe. Depuis, je suis toujours réticente pour faire de la neurobiologie du comportement avec des souris, à mon grand regret, quand je pense à ce qui est possible d'obtenir en manipulant un escargot de mer (Kandel, 2000). Ce séjour dans un grand centre d'étude de la Mémoire, m'a cependant permis d'acquérir les méthodes fondamentales d'étude du comportement chez le rongeur.

Au cours de ma maîtrise de Psychologie Expérimentale, j'ai découvert lors d'un module de « Neurobiologie Intégrative », le laboratoire INSERM (U259) de Psychobiologie des Comportements Adaptatifs dirigée par le Pr Michel Le Moal. Fortement impressionnée par la grandeur technologique et scientifique de ce laboratoire et attirée par la problématique de la psychiatrie biologique, j'ai postulé pour réaliser mon DEA de Biologie Santé (option Neurosciences et Pharmacologie). J'ai d'abord travaillé dans l'équipe du Professeur Hervé Simon sur la problématique des addictions et de la sensibilité aux propriétés renforçantes de la nourriture, chez des rats ayant des différences de vulnérabilités aux psychostimulants (les « high responder » et « low responder » à la nouveauté, Piazza et coll., 1990). Puis, suite à des restructurations au sein du laboratoire, j'ai réalisé une thèse en Neurosciences et Pharmacologie dans une nouvelle équipe sur le thème des neurostéroïdes, sous la direction du Dr Willy Mayo et du Pr Michel Le Moal. Lors de ma thèse, j'ai étudié chez le rat, le rôle des neurostéroïdes sur la transmission cholinergique (Darnaudéry et coll., 1998, 2000, 2002), le sommeil paradoxal (Darnaudéry et coll., 1999a, b) et la fonction mnésique (Darnaudéry et coll., 1999, 2000). Les neurostéroïdes décrits par Etienne Emile Baulieu dans les années 80 (Baulieu et Robel, 1990), présentent la caractéristique d'être produits localement de *novo* par le système nerveux. Nos travaux ont permis de montrer, en utilisant la microdyalise in vivo chez l'animal libre de se mouvoir, que certains neurostéroïdes tels que le sulfate de prégnénolone et allopregnanolone pouvaient réguler la libération d'acétylcholine (ACh) dans le cerveau, et plus particulièrement dans des zones clefs dans la régulation des processus émotionnels et cognitifs (le cortex frontal, l'amygdale, l'hippocampe). Ainsi, des stéroïdes, classiquement décrit pour leur action relativement lente, via des récepteurs nucléaires, s'avéraient capables de réguler rapidement la libération de neuromédiateurs. Par ailleurs, grâce aux connaissances en électrophysiologie amenées au laboratoire par le Dr Jean Jacques Bouyer (Bouyer et coll., 1982, 1983), nous avons pu montrer que les neurostéroïdes modulaient aussi le cycle veille-sommeil et principalement le sommeil paradoxal, lui aussi central pour la fonction mnésique (Leconte et coll., 1973 ; Stickgold et coll., 2001). Ainsi, l'injection centrale d'un neurostéroïde « promnésiant » (le sulfate de prégnénolone) provoquait des augmentations des quantités de sommeil paradoxal et celle d'un neurostéroïde « amnésiant » (l'alloprégnanolone) induisait des baisses des quantités de sommeil paradoxal. De

façon intéressante, les doses de neurostéroïdes pharmacologiquement actives pour réguler la transmission cholinergique et le sommeil paradoxal, avaient également la propriété de moduler les performances dans une épreuve de reconnaissance spatiale. En dépit de l'intérêt incontestable de l'étude des effets pharmacologiques des neurostéroïdes, une question cruciale demeure toujours sans réponse, plus de 20 ans après la découverte princeps de Baulieu, celle du rôle physiologique des neurostéroïdes. Du fait de limitations technologiques considérables, en particulier dans la capacité à doser de manière précise et reproductible des lipides dans différentes structures du cerveau, les connaissances sur la physiologie des neurostéroïdes et de leurs dérivés sulfatés restent difficiles à compléter (Liere et coll., 2000, 2004 ; Ebner et coll., 2006). Même si mes travaux sur les neurostéroïdes se trouvent de ce fait mis à l'écart de mon projet scientifique actuel, la thématique « hormones stéroïdes – cerveau – comportement » demeure centrale dans mes recherches.

En 1999, grâce à une bourse post-doctorale INSERM, j'ai intégré le prestigieux Institut Santiago Ramon y Cajal à Madrid, l'un des berceaux de la neuroanatomie moderne. J'ai travaillé auprès du Professeur Luis Miguel Garcia-Segura et son équipe « Neuroactive Steroids », j'ai pu entrevoir l'incidence considérable des hormones et peptides sur la plasticité du cerveau adulte et ses capacités de restauration (Garcia-Segura et coll., 1999 ; Cardona-Gomez et coll., 2001 ; Garcia-Segura et coll., 2003) et acquérir la base des approches de biologie cellulaire et moléculaire. Lors de ce séjour, j'ai développé mes propres recherches et avec chance établit une collaboration avec Pr Luis Miguel Garcia Segura et son équipe qui se poursuit toujours. Dans le cadre d'un programme d'action intégré franco-espagnol, nous avons montré pour la première fois qu'un traitement chronique lors du vieillissement avec de l'Insulin-Growth-Factor 1 (IGF-1) pouvait atténuer les perturbations mnésiques et neuroendocriniennes induite par le stress prénatal et stimuler la neurogénèse hippocampique (Darnaudéry et al., 2006).

Attirée par l'enseignement, j'ai postulé, la même année, sur 2 postes de Maîtres de Conférences qui correspondaient à mon profil recherche, l'un à l'Université de Nice Sofia Antipolis dans l'équipe du Pr Claude Gottesman sur la thématique « Sommeil et Microdialyse Acétylcholine » et l'autre à l'Université de Lille 1, sur le thème du « Stress Périnatal ». J'ai eu la chance d'être classée 1^{ière} sur ces 2 postes et

j'ai fait le choix de rejoindre fin 1999, le Pr Stefania Maccari, dont je connaissais le dynamisme et la thématique de recherche passionnante sur la périnatalité (Maccari et coll., 1995 ; Barbazanges et coll., 1996a, b ; Vallée et coll., 1997 ; Day et coll., 1998 ; Dugovic et coll., 1999). A mon arrivée à l'Université de Lille 1, j'ai répondu à différents appels d'offres, pour permettre l'achat d'équipements pour le laboratoire et financer mes recherches. De part mon expertise en neurobiologie du comportement, une partie de mon activité a consisté à la mise en place d'une plate-forme permettant l'étude exhaustive chez le rongeur, des processus mnésiques (labyrinthes en Y, piscine de Morris, reconnaissance d'objet, reconnaissance de topographies, reconnaissance sociale), émotionnels (labyrinthe en croix surélevé, champs ouvert, comportement social, test de Porsolt) et motivationnels (activité locomotrice, test de préférence de place, cages d'autoadministration). Pour ce faire, j'ai fait les plans de pièces de comportements, lesquelles occupent actuellement presque un étage du bâtiment (6 pièces expérimentales) et une pièce spécialisée au sein de l'animalerie (systèmes de vidéotracking). J'ai validé ces tests au laboratoire et ces équipements ont permis la réalisation de différents projets de recherche et ont été utilisés dans le cadre de plusieurs thèses. Ils ont également permis d'établir des collaborations avec les équipes locales sur le site de Lille 1 et de Lille 2.

Parallèlement, je me suis intégrée dans la thématique du stress prénatal (induit par la contention maternelle chronique lors de la gestation) que Stefania Maccari développait sous l'impulsion de Michel Le Moal, depuis 1992 à Bordeaux et depuis 1998, à Lille. Mes activités de recherche, portent sur l'analyse des effets chez le rat, de l'exposition à des stress précoces (période périnatale) et tardifs (chez l'adulte), sur les capacités d'adaptations tout au long de la vie. Mes travaux récents ont contribué à mettre en évidence qu'un stress à des périodes critiques de la vie produisait une empreinte permanente sur l'individu. Ainsi, un stress chronique lors de la gestation a des conséquences durables sur les capacités d'adaptation de la descendance, mais également sur la mère. L'ensemble des études menées m'a permis d'être co-auteur de 24 publications dans des revues internationales depuis 2000. J'ai participé à l'encadrement de 3 étudiants en formation doctorale sur des projets de recherches financés (S. Morley-Fletcher, H. Louvart et V. Van Waes) et au recrutement de 2 chercheurs post-doctoraux (M. Enache et C. Laloux).

Dans la partie de mon dossier de candidature à l'Habilitation à diriger des Recherches, qui suit, je présenterai mes activités de recherche menées au cours de ces dernières années, elles portent sur l'analyse des liens entre « stress, gestation et allostasie chez le rat ». La première partie expose les orientations générales de mes recherches ; puis des exemples de travaux pour lesquels j'ai co-encadré des étudiants regroupés autour de 2 grands thèmes : 1) Stress précoces et surcharge allostasique chez la descendance ; et 2) Stress, gestation, allostasie maternelle. Enfin, dans une troisième partie, j'aborderai les grands axes de mes orientations futures.

I) ORIENTATIONS GENERALES DE LA RECHERCHE

A) Stress et allostasie : « Can't live with it, can't live without it »

Le milieu intérieur est le concept original de Claude Bernard (1855) selon lequel l'environnement interne est maintenu dans un équilibre constant même si les conditions du monde environnant changent. Précisant ce concept, Cannon proposa le terme d'homéostasie pour décrire la constance du milieu intérieur. Cannon fut le premier à étudier les variations des réponses physiologiques aux conditions environnementales menaçantes (Cannon, 1932). Cet auteur fonde sa proposition sur l'idée selon laquelle des états stables comme le taux de glucose, la température corporelle et l'équilibre acido-basique sont étroitement régulés. Cette stabilité nécessite que toute tendance au changement rencontre automatiquement des facteurs de résistance. Dans les années 1930, la théorie de l'homéostasie a conduit H. Selye à proposer le concept de stress : « réponse non spécifique que donne le corps à toute demande qui lui est faite » (Selye, 1956).

Le mot stress qui est très populaire auprès du grand public, constitue sur le plan scientifique une notion complexe, multidimensionnelle et de ce fait difficile à définir. Bien que le stress soit souvent cité comme cause de désordres psychologiques ou physiques (plus de 10000 revues de question consacrées au lien stress-maladie), il n'apparaît pas de manière évidente, justifié, de considérer la réponse de stress, comme étant inévitablement néfaste à l'organisme. En effet, le rôle fonctionnel crucial de la réponse de stress est trop souvent négligé. Le paradoxe du stress est lié principalement à la simultanéité de sa nature hautement adaptative et de ses possibles conséquences désadaptées (Korte et coll., 2005). Dans les années 90, un nouveau concept de réponse au stress a été développé (Mc Ewen and Stellar, 1993 ; Mc Ewen et Wingfield, 2003 ; Koob et Le Moal, 2001), il s'agit de la notion d' « allostasie » (*allostasy*), qui introduit une nouvelle terminologie évitant l'ambiguïté du mot « stress » (Figure 1). L'allostasie est définie comme le processus adaptatif permettant la stabilité par le changement (Sterling et Eyer, 1988).

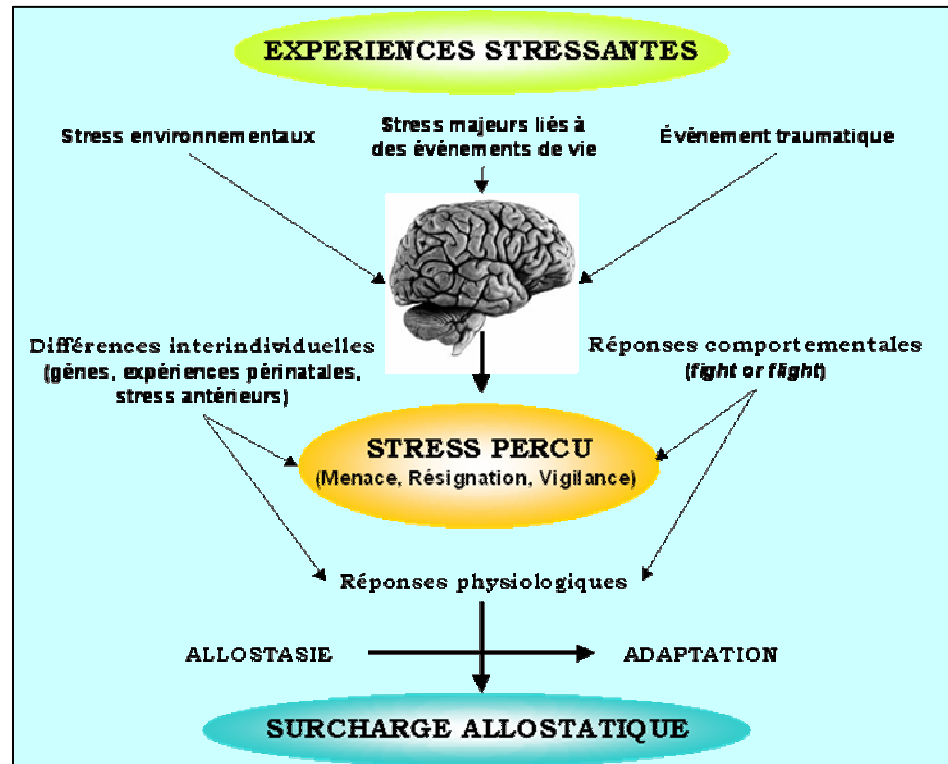

Figure 1 *: Suite à une expérience stressante (ou stressor), l'organisme « réagit » par une réponse complexe mettant en jeu l'ensemble du système nerveux central et périphérique, entraînant des réponses neuroendocrines et immunitaire qui activent des fonctions adaptatives de survie. Les conséquences de cette activation physiologique sont multiples : mobilisation d'énergie (acides gras libres, glycérol, glucose, acides aminés) issue des nutriments en stock (triglycérides, glycogène, protéines) et arrêt du stockage d'énergie, augmentation du tonus cardiovasculaire et pulmonaire pour faciliter l'alimentation en oxygène et en glucose, ralentissement des processus d'anabolisme, suppression de la digestion, de la croissance cellulaire, des fonctions reproductives, des réponses inflammatoires et immunitaires (Stein-Behrens et Sapolsky, 1992). Simultanément, la cognition est modifiée, avec une tendance à l'aiguisement des seuils sensoriels. Au même moment, des mécanismes de rétrocontrôles négatifs sont activés pour contrecarrer l'activation physiologique et réinstaller l'équilibre interne de l'organisme. Lorsque les rétrocontrôles réussissent, l'organisme sera capable de gérer la situation stressante, éliminer sa source et conduira le sujet à avoir des comportements adaptés : le stress est alors une réponse adaptative (allostasie) qui rend l'organisme capable de gérer les stimuli menaçants quotidiennement rencontrés. Les comportements adaptés consistent en un état accru d'éveil, d'alerte et de vigilance, ainsi qu'une augmentation des capacités cognitives. En revanche si la source de stress se prolonge et/ou est incontrôlable (augmentation de la charge allostasique), les mécanismes de rétrocontrôles échouent à restaurer l'équilibre (homéostasie) ou un nouvel équilibre (allostasie), et la réponse au stress deviendrait désadaptée voir pathogène (surcharge allostasique).*

Le cerveau joue un rôle crucial dans ce processus, car en contrôlant tous les mécanismes simultanément, il peut renforcer ses commandes et incorporer l'influence de facteurs comme l'expérience, l'anticipation et la réévaluation des besoins. L'allostasie est essentielle à la fois dans des situations imprévisibles (conflit social, compétition pour les ressources ou trauma…) et dans les situations prévisibles (rythme circadiens, changement de saisons…). Le maintien de l'équilibre par le changement s'illustre dans de nombreux exemples. Ainsi, les variations de la pression sanguine au cours de la journée : le matin la pression artérielle augmente lorsqu'on se lève et le flux sanguin est maintenu au cerveau lorsqu'on se trouve

debout, ce qui nous permet d'être conscient. Ce type d'allostasie aide au maintien de l'apport d'oxygène au cerveau (Sterling et Eyer, 1988). Dans un autre contexte, les profonds changements dans la prise alimentaire, les processus métaboliques et l'osmorégulation reflètent l'allostasie chez la femelle lors de la lactation (Bauman et Griinari, 2000). La réponse au stress comme elle est classiquement décrite s'intègre dans le concept plus large d'allostasie et constitue « l'état allostastique » (*allostatic state*) (Koob et Le Moal, 2001 ; McEwen et Wingfield, 2003). L'idée centrale du concept d'allostasie est qu'il y peut y avoir accumulation et finalement un coût pour l'organisme si les médiateurs de l'allostasie tels que les hormones surrénaliennes, les neuromédiateurs ou les cytokines etc. sont libérées de façon trop fréquentes ou régulées de manière inefficace. Ce coût constitue « la charge allostasique » (*allostatic load*) qui est le résultat des réponses auxquelles l'organisme a du faire face. On distingue également « la surcharge allostasique » (*allostatic overload*) qui est observée lorsque les capacités d'ajustement de l'organisme ont été dépassées. Bruce McEwen propose 2 exemples de surcharge allostasique. D'une part, celle qui se produit lorsque la demande énergétique, dépasse l'offre possible (en hiver par exemple), il en découle la mise en place de processus de survie pour réduire la surcharge allostasique (migration, hibernation, consommation de nourriture plus énergétique). D'autre part, la surcharge allostasique peut se produire, alors que l'offre énergétique est suffisante, voire en excès, mais qu'elle est associée à des conflits ou dysfonctions sociales. Cette dernière est propre aux sociétés humaines, mais s'observe également chez certains animaux en contact avec l'humain ou en captivité (McEwen et Wingfield, 2003). Lorsque la surcharge allostasique est chronique, les pathologies émergeraient.

Il est remarquable, que l'expérience de stress subjective est individuelle et ne reflète pas forcément l'activation hormonale des systèmes de stress. Les expériences de stress vont induire des charges allostasiques différentes selon les individus et leur milieu (Korte et coll., 2005). Le but de la sélection naturelle n'est pas le bien-être de l'individu mais de maximiser le succès reproductif. Sur le plan évolutif, il en découle que les individus vont développer différentes stratégies comportementales, afin de faire face aux événements stressants. Au cours de l'évolution il existe une certaine stabilité dans les différences de modes d'allostasie pourtant diamétralement opposés, et ceci à l'intérieur d'une même espèce. Un bon exemple est celui des stratégies du type «faucon versus colombe» reproduit dans la table 1 (Maynard Smith, 1982).

	Hawk	Dove
Behavioral strategy	Fight–flight	Freeze–hide
Coping style	Proactive	Reactive
Emotional state	Aggressive and bold	Non-aggressive and cautious
Biological role	Establish territory or defend existing territory	Adopt strategy to avoid danger within territory, e.g. immobility
Exploration	Fast and superficial	Cautious and thorough
Behavioral flexibility	Rigid and routine-like	Flexible
Energy metabolism	High energy consumption	Energy conservation
Body damage (e.g. wounds, blood loss)	High risk	Low risk
Advantage according to food availability	When stable and abundant	During food scarcity
Advantage according to population cycle	When density is high	When density is low

Table 1 : *Les différentes interactions gènes x environnement chez le faucon (Hawk) et la colombe (Dove) et les conséquences sur la santé dépendent fortement de la stratégie comportementale qu'ils choisissent d'adopter, du contexte environnemental, de la disponibilité de la nourriture.*

Les 2 stratégies s'avèrent hautement adaptatives en fonction du milieu. Mais, en cas de surcharge allostasique, le coût de l'adaptation s'exprimera de façon différentielle selon le type de stratégie adopté. Les stratégies de type « faucon » ou « colombe » sont largement présentes dans le règne animal, non seulement entre mâles et femelles, mais également au sein d'un même genre, à travers différentes espèces.

Une question cruciale, est de déterminer pourquoi chez certains individus, la charge allostasique aboutit à une surcharge, à haut risque de vulnérabilité à certaines pathologies. Chez l'humain la mise en relation d'une forte corrélation entre le statut socio-économique et le risque de pathologies chroniques, telles que l'hypertension, l'ostéoporose, l'obésité abdominale, suggère que la surcharge allostasique induite par un faible niveau de vie, s'accompagne d'une mortalité plus élevée (DeVesa et Silverman, 1980). Concernant les psychopathologies, les corrélations existent également, mais dans tous les cas les corrélations stress – maladies n'aboutissent pas à la mise en évidence d'un lien causal. En effet, l'émergence d'un phénotype vulnérable résulterait de l'interaction entre une vulnérabilité génétique et « l'histoire du sujet », au sein d'un environnement particulier (DeKloet et coll., 2005). Sur le plan biologique, l'axe corticotrope constitue un système fortement plastique, dont le mode de fonctionnement varie en fonction des gènes, de l'histoire de stress du sujet, mais aussi, en fonction de son histoire précoce néonatale, voire prénatale.

B) La machinerie de l'axe hippocampo-hypothalamo-hypophyso-corticosurrénalien

Dans la fin des années soixante, Bruce McEwen démontre que le cerveau est capable de reconnaître des hormones glucocorticoïdes, connues pour leur rôle dans la réponse endocrine au stress et que la région cérébrale présentant la densité la

plus élevée de récepteurs pour les glucocorticoides est l'hippocampe, une structure centrale dans la régulation des processus d'apprentissage et de mémoire (McEwen et coll., 1968 ; Gerlach et McEwen, 1972). L'axe hypothalamo-hypophyso-corticosurrénalien (ou axe corticotrope) constitue avec le système sympathique, l'un des principaux systèmes impliqué dans la réponse au stress (Figure 2). Le fonctionnement schématique de l'axe corticotrope et ses principales régulations neuronales sont résumés dans la figure 2.

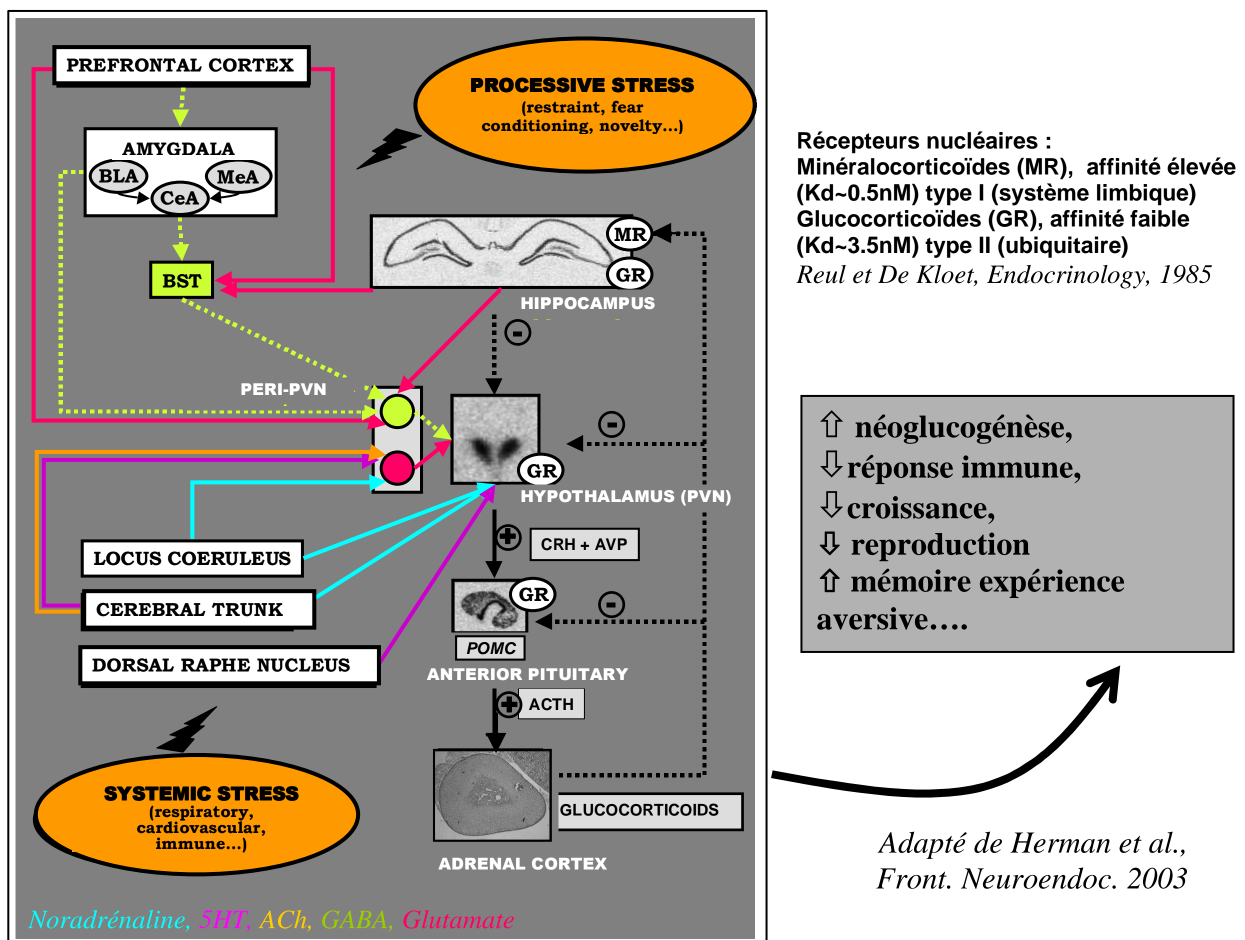

Figure 2 : *Résumé schématique du fonctionnement de l'axe corticotrope et de ses régulations neuronales. Le noyau paraventriculaire de l'hypothalamus (NPV) subit de multiples afférences qui l'informent notamment du fonctionnement viscéral (tronc cérébral) et de l'état émotionnel (régions limbiques). Les stress mettant en jeu la survie immédiate (stress respiratoire, cardiovasculaire, immunitaire) activent le circuit « systémique » qui constitue un relais direct entre les efférences viscérales et le NPV. Ce circuit implique des projections du tronc cérébral : noradrénergiques (NA) du locus coeruleus et sérotoninergiques (5HT) du noyau du raphé. Les stress psychogéniques (contention, peur conditionnée, exposition à un nouvel environnement) ont en commun la nécessité d'une intégration sensorielle supérieure. La stimulation d'un circuit « processif » impliquant des structures limbiques (cortex préfrontal, hippocampe, amygdale) permet de comparer la menace immédiate avec les expériences précédentes et de moduler la réponse de l'axe corticotrope. Des afférences limbiques glutamatergiques (hippocampe et cortex préfrontal) et GABAergiques (amygdale) font relais au niveau des neurones GABAergiques du noyau de la strie terminale, de l'aire préoptique et de différents noyaux hypothalamiques, afin d'inhiber ou de stimuler l'activité du NPV. Parmi les structures limbiques influençant l'axe corticotrope, l'hippocampe est le plus étudié. Des lésions hippocampiques entraînent une augmentation de la sécrétion de glucocorticoïdes en réponse à un stress et de l'expression génique de la CRH et de l'AVP dans le NPV. Inversement, sa stimulation conduit à une diminution de l'activité de l'axe corticotrope chez le rat comme*

chez l'homme. Selon les agents stressants, d'autres facteurs tels que l'angiotensine II, diverses cytokines et des lipides inflammatoires sont secrétés et agissent sur l'hypothalamus, l'hypophyse et/ou le cortex des glandes surrénales, potentialisant leurs activités (Bornstein, 1999). Au niveau central, les neurones parvocellulaires du NPV intègrent les informations issues de diverses afférences des centres supérieurs et en réponse, libèrent la corticolibérine (CRH : corticotropin-releasing hormone) et l'arginine vasopressine (AVP) au niveau de l'éminence médiane. La CRH et l'AVP sont ensuite transportées, par l'intermédiaire du système porte hypophysaire, vers l'adénohypophyse. La CRH se fixe sur deux types de récepteurs couplés aux protéines G: CRH-R1 et CRH-R2, respectivement impliqués dans l'initiation et l'atténuation de la réponse au stress. L'AVP exerce ces effets via l'activation des récepteurs V1b. La CRH stimule, via les récepteurs CRH-R$_1$ adénohypophysaires, l'expression du gène de la proopiomélanocortine (POMC) et la libération des peptides qui en dérivent : les lipotropines, la β–endorphine et la corticotropine (ACTH : adenocorticotropic hormone). L'ACTH passe dans la circulation générale, jusqu'aux glandes surrénales et agit au niveau des cellules de la couche réticulo-fasciculée du cortex surrénalien sur des récepteurs membranaires de la famille des mélanocortines (MC2-R) couplés positivement à l'adénylate cyclase pour stimuler la synthèse et la libération de glucocorticoïdes. Par ailleurs, l'innervation sympathique de la glande surrénale est également impliquée dans la stimulation du cortex surrénalien.

Les glucocorticoïdes (majoritairement le cortisol chez l'humain et la corticostérone chez le rat) sont des hormones stéroïdes dérivant du cholestérol. 90% des glucocorticoïdes circulent dans le plasma, sous une forme liée à des protéines (albumine et transcortine). Leur liposolubilité leur permet de traverser facilement les barrières biologiques, sous forme libre ou liée à l'albumine. Les glucocorticoïdes agissent par l'intermédiaire de deux types de récepteurs intracellulaires : les récepteurs aux minéralocorticoïdes (MR ou de type I) et les récepteurs aux glucocorticoïdes (GR ou de type II), qui présentent des affinités différentes pour la corticostérone (forte pour les MRs et faible pour les GR, Figure 2). Les récepteurs MR sont surtout présents dans le système limbique, alors que les récepteurs GR ont une distribution ubiquitaire dans le cerveau, mais majoritaire au niveau hypophysaire et limbique. L'activation des récepteurs MR reflète plutôt l'action tonique des glucocorticoïdes et serait mise en jeu dans le cadre de fluctuations circadiennes de la corticostérone et dans la détermination de la sensibilité des réponses au stress. Quant aux récepteurs GR, leur activation intervient essentiellement pour contenir l'hyperactivité phasique en réponse à des stimulations environnementales par rétroaction sur les centres supérieurs. La sécrétion de CRH et d'AVP suit un rythme circadien dont la synchronisation et l'amplitude augmentent lors d'un stress aigu, entraînant une libération accrue d'ACTH et de corticostérone. Les glucocorticoïdes accroissent les métabolites énergétiques utilisables pour l'organisme par une action hyperglycémiante et entraînent des réactions anti-inflammatoires, anti-allergiques et immunosuppressives. Ces effets sont divisés en deux catégories : les effets préparateurs et les effets modulateurs. Les effets préparateurs servent à moduler la réponse à un stress futur. Les effets modulateurs sont répartis en trois composantes : l'action permissive, suppressive et stimulatrice. Lors de la phase initiale, l'action

permissive prépare les mécanismes de défense de l'organisme. Une heure après le début du stress, l'action stimulatrice a pour but d'augmenter les effets des hormones libérées alors que l'action suppressive protège l'organisme des conséquences négatives d'une réponse excessive. Les glucocorticoïdes exercent donc un rétrocontrôle négatif sur l'axe corticotrope qui met en jeu les GR aux niveaux hypophysaire et hypothalamique et les deux types de récepteurs au niveau hippocampique. Sur le plan moléculaire, les glucocorticoïdes liés à leurs récepteurs sont transportés vers le noyau et se fixent sous la forme d'un dimère sur des séquences d'ADN nommées GRE (Glucocorticoid Responsive Element) situées dans la région promotrice des gènes qu'ils régulent. Ce mécanisme va aboutir à la transactivation ou à la transrépression de la transcription. Enfin les glucocorticoïdes peuvent aussi agir à des étapes post-transcriptionnelles ou via des récepteurs membranaires, mécanismes qui pourraient être impliqués dans le rétrocontrôle immédiat de la sécrétion d'ACTH.

La perturbation du système du stress produit chez l'homme comme chez l'animal, de profondes altérations émotionnelles et cognitives. Ainsi, l'hypercortisolémie associée au traitement chronique avec des glucocorticoïdes de synthèse ou celle des patients présentant le syndrome de Cushing (désordre hormonal consécutif à l'exposition prolongée à des haut niveaux de cortisol du fait d'une tumeur des glandes surrénales ou de l'hypophyse antérieure), serait la cause de désordres comportementaux émotionnels et cognitifs. A ce titre, à la fois dans le syndrome de Cushing et dans la dépression on observe une diminution du volume de l'hippocampe (Sheline et coll., 1999) Par ailleurs, des dysfonctionnements de l'axe corticotrope sont fréquemment rapportés dans plusieurs psychopathologies. Trois désordres comportementaux sont associés de façon récurrentes à des dysfonctionnements de l'axe corticotrope : la dépression, l'état de stress post-traumatique (PTSD, post-traumatic stress disorder) et le vieillissement cognitif (Lupien et Lepage, 2001 ; McEwen, 2002a, b ; DeKloet et coll., 2005). De façon remarquable, même si ces désordres présentent des symptômes commun (humeur déprimée en particulier et troubles mnésiques) ; sur le plan neuroendocrine des différences notables sont observées (Figure 3). Si la dépression, tout comme le PTSD semblent associés à une augmentation des taux de CRH centraux, les changements dynamiques de l'axe corticotrope diffèrent fortement.

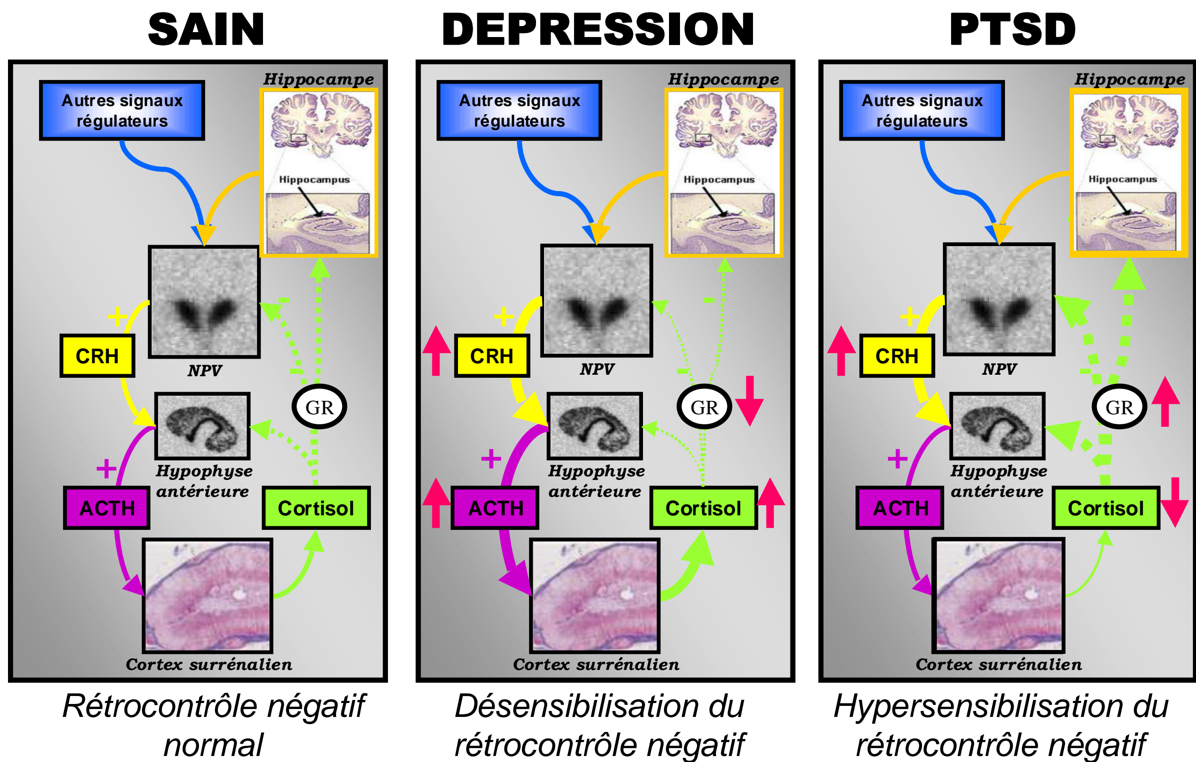

Figure 3 : *Altérations de l'axe corticotrope dans la dépression et le PTSD (d'après Yehuda, 2002).*

Ainsi, après stimulation pharmacologique ou environnementale, le cortisol serait élevé de manière durable chez certains sujets dépressifs, du fait d'une désensibilisation du rétrocontrôle négatif ; alors que chez les sujets PTSD, on observerait une hypersensibilisation du rétrocontrôle négatif et donc une hypocortisolémie (Yehuda, 2002). Bien qu'associées au stress et à des dysfonctions de l'axe corticotrope, ces pathologies sont multifactorielles et reflètent l'interaction de facteurs de vulnérabilité génétiques et environnementaux.

C) Environnement maternel et adaptation.

Chez les mammifères, les processus développementaux qui aboutissent à la transformation d'une seule cellule embryonnaire à un organisme possédant par exemple quelques 10^9 neurones dans le cortex cérébral après 40 semaines chez l'humain sont excessivement complexe. Le développement se fait sous la double influence des gènes et de l'environnement utérin qui l'entoure. Des stress lors de la période prénatale peuvent avoir des conséquences sur le développement et inscrire une empreinte durable sur l'individu, voir sur les générations suivantes (Newbold et coll., 2006 ; Goss et coll., 2007). L'environnement maternel constitue à la fois une protection contre les agents stressant, tels que les agressions externes (variations de

température, prédateurs,…), mais également, via le placenta un puissant système de « conduction », qui relie le futur individu à l'environnement qui sera le sien à la naissance (Figure 4). Ainsi, l'état physique de la mère, par exemple son statut nutritionnel, détermine fortement le développement des fœtus qu'elle porte (Moles et coll., 2003). Des stressors de type systémique peuvent atteindre l'embryon via le passage placentaire et avoir des effets irrémédiables, c'est le cas par exemple : des drogues d'abus, des médicaments, des virus, des toxines microbiennes, ou encore des rayons X. Il existe également des perturbations de l'environnement immédiat de l'embryon, dont les conséquences restent mal connues. Ainsi, les conséquences du stress embryonnaire associé aux technologies de la procréation médicalement assistée (Testard, 1986 ; 1992), comme la fécondation in vitro (« FIV ») et transferts d'embryons obtenus par FIV (« Fivète »), ou les traitements hormonaux aboutissant à une « superovulation » et donc à la présence d'embryons multiples dans l'utérus, restent à explorer.

Des données épidémiologiques récurrentes montrent une forte association entre la maltraitance durant l'enfance et un risque de développer des troubles psychiatriques (dépression, anxiété, stress post-traumatique) à l'âge adulte (Holmes et Robins, 1987 ; Heim et coll., 1997, 1999). La plupart du temps, le stress postnatal, auquel est exposé l'enfant (agression, malnutrition, …), fait suite à un environnement prénatal également défavorable (malnutrition, anxiété maternelle…), de sorte que les incidences des stress pré- et post- nataux sont difficilement dissociables. La croyance que l'état psychologique de la mère est capable d'influencer son enfant en développement est présente dans la plupart des sociétés. Depuis plus de 60 ans, de nombreux auteurs ont évoqué la possibilité d'effets néfastes de stress lors de la gestation sur la progéniture et sur sa vulnérabilité future aux troubles physiques et psychiques (pour revue voir Wadha, 2005 et Talge et coll. 2007). Cependant, sur le plan épidémiologique la démonstration d'un lien demeure difficile, du fait de la lourdeur méthodologique des études prospectives et du risque de subjectivité des études rétrospectives. Dans les années 90, Barker propose l'idée d'une programmation précoce des maladies de l'adulte. En effet, en utilisant des mesures simples et facile d'accès rétrospectivement : le poids de naissance et la taille de naissance, il observe que le retard de croissance intra-utérin est corrélé à une augmentation du risque de pathologies cardiovasculaires et métaboliques à l'âge adulte (Barker, 2002, 2004, 2005 ; Barker et Hanson, 2004).

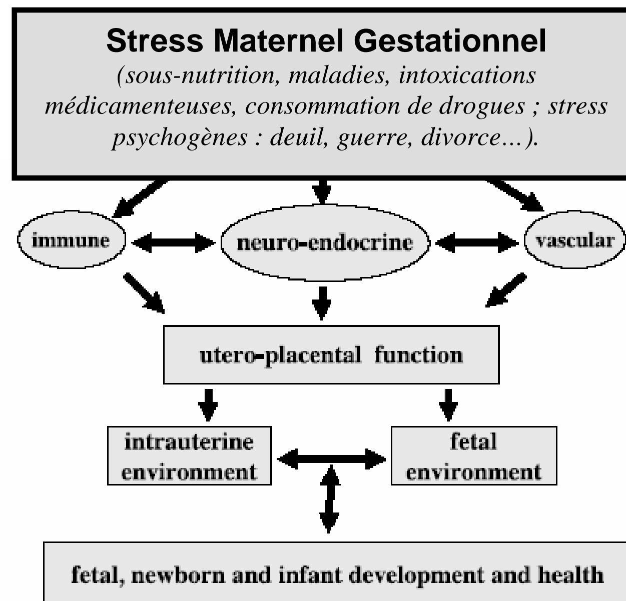

Figure 4 : *Modèle hypothétique de médiation des effets du stress maternel lors de la gestation sur le fœtus en développement (adapté de Wadhwa 2005).*

Même, si les origines du retard de croissance intra-utérin peuvent être multiples, les travaux de Barker inaugurent le concept de « programmation fœtale » (Barker et coll. 1993, 1995, 2006 ; Feldt et coll., 2007). Depuis de nombreuses années, les équipes de Pathik Wadhwa aux Etats-Unis et de Vivette Glover en Grande Bretagne, mènent des travaux sur l'incidence du stress maternel lors de la gestation sur le devenir de l'enfant. Dans une revue récente, Talge et coll. (2007) soulignent qu'une dizaine d'étude prospectives indiquent que l'exposition de la femme à des stress psychologiques lors de la gestation, conduit à des troubles émotionnels et cognitifs chez l'enfant, incluant, un risque plus élevé de troubles de l'attention et d'hyperactivité, une augmentation de l'anxiété et un retard de langage. De façon très intéressante, ces effets sembleraient indépendants de causes postnatales telles que la dépression maternelle. Outre la question de la mise en évidence des périodes de vulnérabilité prénatale, une question cruciale dans les études sur les liens entre stress gestationnel et vulnérabilité chez la descendance, est de déterminer par quels mécanismes le stress maternel affecte la descendance. Etant donné, les liens entre axe corticotrope et stress d'une part, et l'importance de ce système dans de nombreuses fonctions de l'organisme, d'autre part ; les hypothèses principales se sont focalisées sur une implication des glucocorticoïdes dans l'émergence des altérations chez la progéniture des stress subit par la mère gestante (pour revue, voir Seckl et Meaney, 2004, 2006 ; Wadhwa, 2005 ; Matthews, 2004 ; Maccari et coll.,

2003). Les données obtenues chez l'animal, en particulier, chez le rat ont offert la possibilité de valider cette hypothèse.

D) Le stress « périnatal » chez le rat, comme modèle de surcharge allostasique.

Chez certains mammifères comme le rat, dont les petits sont totalement dépendants à la naissance (espèces *« altriciales»),* on note une extrême immaturité à la naissance. Cette immaturité, en particulier du cerveau, rend l'individu très sensible à l'environnement qui l'entoure. Comme pour le rôle des gènes, la croyance que l'environnement parental détermine profondément le type d'adulte que deviendra l'enfant, est inscrite, que ce soit dans le domaine du sens commun, de la psychiatrie ou encore des politiques sur la criminalité. Il s'agit d'une question fondamentale en psychobiologie du développement, qui met en jeu des questions déontologiques importantes. Il existe à ce jour, un consensus assez large pour reconnaître l'importance d'un environnement précoce défavorable sur l'individu en développement. Chez l'animal, de nombreuses études menées depuis plus de cinquante ans, suggèrent que les événements de vie précoces ayant lieu *après* la naissance, mais également *avant* peuvent exercer un effet profond et persistant sur le fonctionnement du cerveau et les comportements du future de l'individu, voire sur les générations suivantes. Les travaux sur princeps menés par Levine (1957, 1959) ; Joffe (1965, 1969), et Ward (1972, 1980) ont permis de poser les bases de l'étude des périodes critiques pour les effets du stress et pour l'incidence de facteurs périnataux sur le comportement chez le rongeur. Par exemple, dès 1965, Joffe démontre un effet significatif sur le comportement de la descendance, d'une interaction entre le génotype et les effets de stress maternels lors de la gestation et même avant la conception (Joffe, 1965). Il montre également que les effets du stress précoce sont différents chez les descendants élevés, par des mères biologiques ou par leur mère adoptive (Joffe, 1965). Les manipulations périnatales principales utilisées dans la littérature sont de 4 types. Le stress prénatal consiste en un stress gestationnel appliqué à la mère, celui-ci peut être de différents types : processifs, comme la contention (Joffe, 1965 ; Ward, 1984 ; Maccari et coll., 1995) ou systémique comme l'inoculation d'une infection par exemple (Meyer et coll., 2006). Les études sur le stress prénatal chez le rongeur ont été inaugurées par les travaux princeps de Thompson, Joffe et de Ward dans les années 60 (Thompson, 1957 ;

Joffe, 1965 ; 1969 ; Ward, 1972 ; Ward et Weisz, 1980). Ces derniers ont été parmi les premiers, à faire la démonstration que le comportement de la descendance pouvait être modulé par des changements de l'environnement hormonal intra-utérin (Meisel et Ward, 1981), et par les expériences de la mère lors de la gestation, voire même avant la conception (Thompson, 1957 ; Joffe, 1965 ; Smith et Joffe, 1975 ; Meisel et coll., 1979). Depuis, une littérature abondante s'est portée sur le stress gestationnel chez le rongeur et son incidence sur la descendance (pour revue voir en autres : Chapillon et coll., 2002 ; Fenoglio et coll., 2006 ; Koehl et coll. 2001, 2002 ; Kofman, 2002 ; Maccari et coll., 2001 ; 2003 ; Weinstock, 2001, 2002). De nombreux types de stress émotionnels différents ont été décrits, à titre d'exemples, on peut citer : le conditionnement d'évitement (Thompson, 1957) ; la contention (Ward, 1972) ; le « surpeuplement » (Dahlof et coll., 1978) ; les piqûres de NaCl (Peters, 1982) ; le bruit (Fride et Weinstock, 1984) ; la suspension par la queue (Alonso et coll., 1991) ; les chocs électriques (Takahashi et Kalin., 1991) ; l'exposition à un chat (Lordi et coll., 1997) ; ou le stress chronique imprévisible (Rangon et coll., 2007). Les procédures varient principalement en fonction du type de stressor (douloureux ou non) ; sa durée (aigu ou chronique) ; la période au cours de la gestation durant laquelle il est appliqué (toute la gestation ou en fin de gestation) ; la nature (éthologique ou non) et sa prédictibilité (faible ou forte).

Le modèle sur lequel nous travaillons a été développé dans les années 70 par Ingeborg Ward, puis adapté par l'équipe de Stefania Maccari dans le laboratoire de Michel Le Moal à Bordeaux (Figure 5).

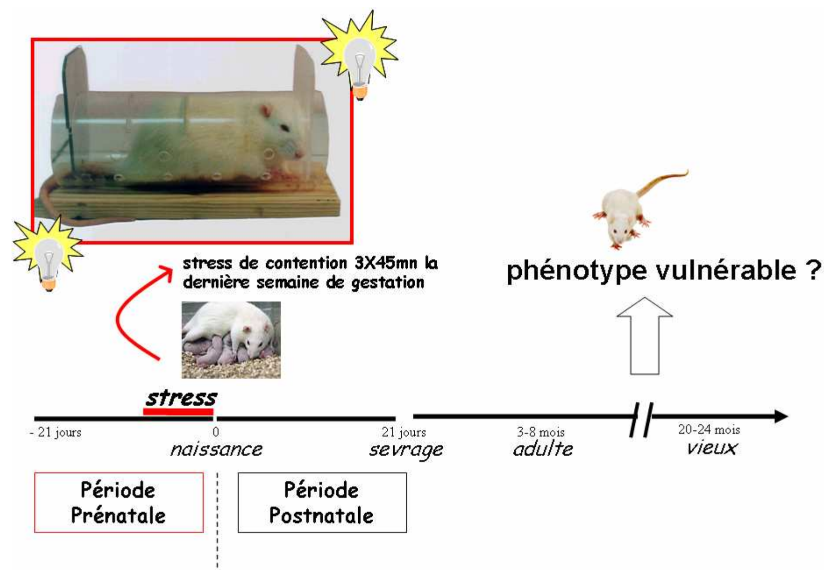

Figure 5 : *Procédure de stress prénatal (d'après Maccari et coll., 1995). De façon générale, le stress gestationnel consiste à placer la mère gestante (Sprague Dawley, Wistar ou Long-Evans) 3 fois par jour (matin,*

midi, fin de journée) dans un boîte de contention (7.5 cm de diamètre, 19 cm de long) sous lumière vive (650 lux), à partir du jour 11 de gestation et ceci durant la période diurne du cycle. A la naissance, les portées ne sont pas manipulées et les mères biologiques élèvent leur progéniture.

Nos études, menées sur le modèle du stress maternel gestationnel, sont vraisemblablement associées à des effets maternels postnataux. En effet, notre protocole implique que la mère biologique (ayant donc une histoire de stress) élève ses petits, ce qui n'est pas trivial si l'on considère les effets marqués de l'adoption sur la mère et sa progéniture (Maccari et coll., 1995 ; Barbazanges et coll., 1996 ; Darnaudery et coll., 2004). Dans cette optique, des travaux récents ont démontré que des manipulations postnatales précoces réduisent certaines altérations provoquées par le stress prénatal (Koo et coll., 2003 ; Lemaire et coll., 2006) ; par ailleurs, le stress gestationnel peut perturber le comportement de soin maternel envers la progéniture et cette perturbation peut se transmettre par des mécanismes non-génomiques aux futures générations (Champagne et Meaney, 2006). Les procédures de stress postnatal, de façon classique consistent en des séparations mères-petits de plus ou moins longues durées lors de la période de lactation (D'Amato et coll., 2005). Les séparations de courtes durées (« handling ») ou de longues durées pendant plusieurs heures ont généralement des effets opposés sur la descendance (Hofer, 1970 ; Meaney et coll., 1991 ; Vallée et coll., 1997 ; Lippmann et coll. 2007). Finalement, l'une des stratégies les plus élégante pour tenter de dissocier les effets pré et post-nataux consiste à faire des adoptions croisées par des mères ayant de génotypes différents et/ou avec des stress gestationnels ou pas (Joffe, 1965 ; Maccari et coll., 1995). Enfin, des études ont également été réalisées en France par Gilles Le Pape, Jean-Michel Lassalle et leurs collaborateurs montrant dès les années 80, l'existence d'une transmission non-génomique du comportement maternel et le rôle de l'environnement utérin dans l'épigenèse (Le Pape et coll., 1981, 1983, 1986). Depuis, des travaux considérables menés par l'équipe de Michael Meaney à McGill ont permis de montrer l'incidence du comportement maternel sur les capacités d'adaptations de la descendance, et la transmission non génomique du « style maternel » sur plusieurs générations (Champagne et Meaney, 2001 ; Meaney, 2001).

Bien que des effets relativement différents puissent être observés, selon les procédures utilisées, la littérature indique globalement que le stress gestationnel exerce des effets à long terme sur la descendance. Les observations qui ont été réalisées concernent principalement l'axe corticotrope et les comportements émotionnels. La plupart des travaux menés, suggèrent que la réponse de l'axe

corticotrope de la descendance stressée est affectée à court et à long terme après exposition à différents stressors. Ainsi, le stress gestationnel provoque sur la descendance une hyper-réactivité de l'axe corticotrope associées à une altération du rétrocontrôle négatif de l'axe corticostérone suite à des stress psychogènes (table 2 : effets du stress gestationnel par contention ; pour revue dans d'autres modèles voir Weinstock 2001). Sur le plan comportemental, les stress maternel lors de la gestation s'accompagne chez la descendance mâle de nombreuses altérations connues pour être reliée à la fonction de l'axe corticotrope (voir table 2, et pour revue, Koehl et coll., 2001).

Ainsi, chez la descendance de mères stressée, on observe une augmentation des comportements anxieux dans différentes épreuves, une augmentation des épisodes de sommeil paradoxal. Le stress prénatal induit également une augmentation de la vulnérabilité aux psychostimulants. Au cours du vieillissement, les animaux exposés à un stress in utero présentent parallèlement à un vieillissement accéléré de l'axe corticotrope, une augmentation des perturbations de la mémoire spatiale liées à l'âge. De façon intéressante, certaines altérations reportées chez les animaux exposés à un stress prénatal, rappellent des perturbations présentent dans la dépression. Ainsi, l'hyperactivité de l'axe corticostérone et le déficit de rétrocontrôle de l'axe sont observés chez certains patients déprimés (Then Bergh et coll., 1999) ; les perturbations du cycle veille-sommeil, précisément les augmentations des épisodes de sommeil paradoxal sont des marqueurs importants de la symptomatologie dépressives (Reynolds et Kupfer 1987).

Axe corticotrope :

Avant le sevrage :

- *suppression de la période d'hyporéponse au stress de l'axe HPA classiquement observés lors des premières semaines de vie avant le sevrage (Henry et coll. 1994).*

Après le sevrage :

- *🡇 du rétrocontrôle négatif de l'axe corticotrope suite à des stress de nouveauté et de contention (Henry et coll., 1994 ; Maccari et coll., 1995 ; Barbazanges et coll., 1996 ; Vallée et coll., 1997, 1999).*
- *🡇 du nombre (Bmax) de récepteurs aux corticostéroïdes (type I et II) dans l'hippocampe,l'effet le plus robuste étant observé pour les récepteurs Minéralocorticoïdes, c'est-à-dire le type I (Henry et coll., 1994 ; Maccari et coll. 1995 ; Koehl et coll., 1999)*
- *Perturbation du rythme circadien de la corticostérone : 🡅 des taux à la fin de la période diurne chez les mâles et 🡅 sur l'ensemble du cycle jour-nuit chez les femelles (Koehl et coll., 1997, 1999).*
- *Hypertrophie des surrénales (Ward et coll., 2000 ; Lemaire et coll., 2000)*

Lors du vieillissement :

- *Accélération du vieillissement de l'axe corticotrope : 🡅 des taux de bases diurne à l'âge de 16 mois (Vallée et coll., 1997)*
- *Les animaux stressés âgés de 16 mois présentent le profil de sécrétion de la corticostérone suite à des stress de contention semblable à celui d'animaux témoins de 24 mois : 🡇 du rétrocontrôle négatif de l'axe corticotrope (Vallée et coll., 1997)*

Comportement :

« Anxiété-dépression » : 🡇 de l'exploration des bras ouverts du labyrinthe en croix surélevé ; hyper-réactivité à la nouveauté (Vallée et coll., 1997) ; 🡅 des épisodes de sommeil paradoxal (Dugovic et coll., 1999).

Vulnérabilité aux psychostimulants : 🡅 autoadministration d'amphétamine ; 🡅 motivation pour la cocaïne, 🡅 des réponses locomotrice à l'amphétamine, et à la nicotine ; 🡅 perturbations motrices induites par le MDMA (ecstasy) (Déminière, et coll., 1992 ; Henry et coll., 1995 ; Koehl et coll., 2000 ; Morley-Fletcher et coll., 2003 ; Kippin et coll., 2007).

Mémoire : 🡅 des perturbations de mémoire spatiale liées à l'âge (Vallée et coll., 1999).

Table 2 : *Quelques exemples de conséquences du stress gestationnel de contention observées sur la descendance mâles (d'après Maccari et coll., 2001, 2003 ; Koehl et coll., 2001, 2002).*

E) Situation des thèmes et questions posées

De nombreux travaux suggèrent que l'adaptation de l'individu à des événements stressant a un coût pour l'individu et qu'une charge allostasique répétée peut aboutir, chez certains individus vulnérables, à une augmentation du risque de développer des désordres biologiques et comportementaux. Par ailleurs, l'exposition de l'embryon à un environnement maternel délétère peut influencer durablement l'axe corticotrope,

l'un des principaux médiateurs la réponse allostasique. Les travaux menés sur l'animal, montrent qu'un stress maternel lors de la gestation s'accompagne de profonds bouleversements de l'axe corticotrope chez la descendance.

Mes activités de recherche portent sur l'analyse des conséquences chez le rat, de l'exposition à des stress lors de périodes critiques de la vie (période périnatale ; gestation) sur les capacités d'adaptations.

➔ Thème « Stress précoce et surcharge allostasique chez la descendance ».

- *Le stress prénatal peut-il constituer un modèle pertinent pour l'étude des liens entre axe corticotrope et pathologies comportementales (dépression, du stress post-traumatique, vieillissement cognitif et la vulnérabilité aux drogues) ?*
- *Les altérations observées dans ce modèle peuvent-elles être diminuées par des stratégies pharmacologiques ?*

➔ Thème «Stress, Gestation et charge allostasique chez la mère ».

- *Quelles sont les origines des désadaptations de la descendance ? l'unité mère-fœtus ? la période post-natale ?*
- *La gestation constitue-t-elle un événement critique pour l'adaptation maternelle ?*

II) RESULTATS

J'ai co-encadré les travaux présentés dans cette section. Ils ont fait l'objet d'une partie de la thèse de Biologie Santé de Sara Morley-Fletcher (Stress prénatal et « dépression » : sensibilité aux antidépresseurs); des mémoires du Master Recherche en Psychologie (M1 et M2) de Guylaine Bélizaire (Stress prénatal et vieillissement : sensibilité aux facteurs neurotrophiques) ; de la thèse de Biologie Santé d'Hélène Louvart (Etude d'un modèle animal de l'état de stress post-traumatique); de la thèse de Biologie Santé de Vincent Van Waes et du séjour post-doctoral de Mihaela Enache (Vulnérabilté à l'alcool chez le rat adolescent et adulte : impact du stress prénatal) et des mémoires de recherche du Master Recherche en Psychologie (M1 et M2) de Fabien del Favero (Stress, Gestation et charge allostasique chez la mère) .

A) Stress précoces et surcharge allostasique chez la descendance

1) Dépression et Vieillissement Cognitif

a) Stress prénatal et « dépression » : sensibilité aux antidépresseurs

Un modèle animal avec toutes les limites que cela implique doit satisfaire trois critères de validité : similarité dans l'étiologie (*construct validity*), la similitude dans la symptomatologie (*face validity*) et dans la réponse aux traitements pharmacologiques (*predictive validity*). Les travaux antérieurs suggéraient que le stress prénatal pourrait constituer un modèle valide pour la dépression. Sur le plan de la validité « théorique », il existe de nombreux travaux reliant stress et dépression, de plus, des études chez l'homme soulignent le rôle d'un environnement précoce délétère sur la vulnérabilité à la dépression chez l'adulte (Heim et Nemeroff, 1999). Les altérations de l'axe corticotrope ainsi que les désordres circadiens et du cycle veille-sommeil (Koehl et coll., 1997 ; Dugovic et coll., 1999) indiquent que le stress prénatal possèdent certaines similitudes avec la symptomatologie dépressive.

Le but de ces études menées en collaboration avec les laboratoires Servier, était d'évaluer la validité « prédictive » de ce modèle.

Dans l'épreuve de la nage forcée validée par Porsolt, en traitements aigus avec des antidépresseurs, on observe classiquement une diminution de la latence et du temps passé en immobilité lors de la 2ème exposition à l'épreuve (Bourrin et coll., 2004). Cependant, les données de la clinique indiquent que l'efficacité thérapeutique des antidépresseurs n'apparaît qu'au bout de plusieurs semaines (Wagstaff et coll., 2001). Pour ce faire, nous avons traité de façon chronique les animaux témoins et stressés en période prénatale avec des antidépresseurs. Les résultats indiquent que le stress prénatal augmente le comportement d'immobilité dans l'épreuve de la nage forcée, et que ce comportement est corrélé avec l'hyperactivité de l'axe corticotrope après un stress (table 3). On observe une réduction du temps passé en immobilité après des traitements chroniques (10 mg/kg, intrapéritonéal, i.p. durant 21 jours) avec des antidépresseurs très différents comme la tianeptine (augmente la recapture de la sérotonine), ou l'imipramine (tricyclique qui inhibe la recapture des monoamines), chez les animaux stressés en période prénatale. En revanche, le traitement chronique aux antidépresseurs ne modifie pas le comportement des animaux témoins. La table 3, résume les effets pharmacologiques que nous avons obtenu avec des antidépresseurs chez les animaux exposés à un stress prénatal (texte en rouge).

⬆ Activité de l'axe corticotrope (Maccari et coll., 1995, Vallée et al. 1997) : ⬇ nombre de récepteurs aux corticostéroïdes dans l'hippocampe atténués par l'administration chronique d'imipramine (Morley-Fletcher et coll., 2004)
⬆ Désordre rythmes circadiens (Koehl et coll., 1997, 1999)
⬆ Sommeil paradoxal (Dugovic et coll., 1999)
⬆ Immobilité dans le test de la nage forcée (Morley-Fletcher et coll., 2003, 2004) : ⬇ de l'immobilité après l'administration chronique de tianeptine et d'imipramine (Morley-Fletcher et coll., 2003, 2004)
⬇ Neurogénèse hippocampique (Lemaire et coll., 2000)
⬆ Nombre de récepteurs 5HT1A : ⬇ après l'administration chronique d'imipramine (Morley-Fletcher et coll., 2004)

Table 3 : *Stress Prénatal et « Dépression » : validité prédictive. Principales altérations rappelant la symptomatologie « dépressive » observées chez les animaux stressés en période prénatale. En rouge, apparaissent les principaux résultats que nous avons obtenus avec les antidépresseurs.*

Les travaux menés dans le cadre de la thèse de Sara Morley Fletcher ont permis de montrer que le stress prénatal possédait une bonne validité prédictive. Cet axe se

poursuit actuellement dans le cadre du projet de recherche de Sara Morley-Flechter « Stress prénatal et dépression ». Dans la continuité de ses travaux, d'autres études ont été conduites montrant en particulier le rôle important des désordres hippocampiques (Viltart et coll., 2006) et de la neurogénése dans les altérations observées (Morley-Fletcher et coll., 2007).

Publications : ***Maccari et coll., 2003 ; Morley-Fletcher, et coll., 2003; Morley-Fletcher et coll., 2004.***

b) Stress prénatal et vieillissement : sensibilité aux facteurs neurotrophiques

Chez l'homme, comme chez l'animal, il existe de fortes différences interindividuelles dans le vieillissement cognitif. En effet, certains sujets âgés présentent des troubles mnésiques sévères alors que d'autres ont des performances identiques à celles de sujets jeunes (Gage et coll., 1984 ; Rapp et Amaral, 1992). Parmi les facteurs à l'origine de cette vulnérabilité, le stress pourrait participer à l'émergence de désordres cognitifs avec l'âge (pour revue voir Sapolsky, 1999). Ainsi, Vallée et coll. (1999) ont montré dans une étude longitudinale que les animaux mâles stressés prénatalement présentaient une accélération du processus d'hyperactivité de l'axe corticotrope classiquement observé lors du vieillissement. Parallèlement il a pu être mis en évidence que ces animaux présentaient une accélération du vieillissement cognitif (Vallée et coll., 1999). De plus, des travaux récents indiquent que chez les rats mâles, la neurogénèse hippocampique est altérée tout au long de la vie, ce qui pourrait participer aux déficits cognitifs (Lemaire et coll., 2000). Malgré la littérature considérable reliant l'hyperactivité de l'axe corticotrope et le vieillissement cognitif, très peu de travaux existent sur l'étude du vieillissement chez l'animal stressé prénatal et aucun chez la femelle âgée.

Le but de ces études menées en collaboration avec le Prof LM GARCIA-SEGURA et Dr M. PEREZ-MARTIN de l'Institut Cajal de Madrid était d'évaluer si le stress prénatal induisait des perturbations cognitives chez les femelles au cours du vieillissement et de déterminer si les altérations liées à l'âge pouvaient être atténuées par un traitement chronique avec un facteur neurotrophique, l'IGF-1. En effet, ce facteur diminue avec l'âge et semblerait moduler la neurogénèse hippocampique.

La table 4 résume les principaux effets du stress prénatal sur le vieillissement cognitif, en rouge apparaissent les principaux résultats que nous avons obtenus. Nos résultats montrent que le stress prénatal augmente les altérations liées à l'âge chez

les rats femelles. En revanche, après un traitement chronique avec un facteur neurotrophique (IGF-1), on observe une atténuation de l'hyperactivité de l'axe corticotrope, une augmentation des taux d'estradiol plasmatique et une augmentation de la prolifération cellulaire dans le gyrus dendatus. Ces effets de l'IGF-1 s'accompagnent d'une réduction des altérations de mémoire spatiale chez les femelles âgées ayant subi un stress in utero.

⬆ Activité de l'axe corticotrope chez les rats mâles et femelles (Maccari et coll., 1995, Vallée et coll. 1997 ; 1999 ; Lemaire et coll., 2000 ; Darnaudéry et coll., 2006) : ⬇ l'hyperactivité observées après un stress chez les femelles âgées,⬇ l'hypertrophie des surrénales après traitement chronique avec de l'IGF-1 (Darnaudery et coll., 2006).

⬆ des taux d'oestradiol plasmatique chez les femelles âgées ayant été soumises à un stress prénatal traitées chroniquement avec de l'IGF-1 (Darnaudery et coll., 2006).

⬆ Perturbations de mémoire spatiale liées à l'âge (Vallée et al ; 1999 ; Darnaudery et coll., 2006) : ⬇ des perturbations dans le test de la piscine de Morris avec traitement chronique à l'IGF-1 chez des femelles âgées ayant subi un stress prénatal (Darnaudery et coll., 2006)

⬇ Neurogénèse hippocampique (Lemaire et coll., 2000) : il n'y pas de diminution de la neurogénése hippocampique chez les femelles âgées, en revanche les effets promnésique de l'IGF-1 sont associés à une augmentation de la neurogénèse hippocampique (Darnaudery et coll., 2006).

Table 4 : *Stress Prénatal et Vieillissement : impact de l'IGF-1. Principales altérations observées au cours du vieillissement chez les animaux stressés en période prénatale. En rouge, apparaissent les principaux résultats que nous avons obtenus chez les femelles âgées et après traitement intracérébroventriculaire avec l'IGF-1.*

***Publications :* Darnaudéry et coll. 2006 ; Garcia-segura et coll., 2007.**

2) Etat de stress post-traumatique

Chez l'humain, lorsqu'un individu est confronté à une situation traumatique imprévisible qui dépasse ses capacités adaptatives (attentats, accidents de la circulation…), il peut développer au cours des mois suivants le trauma, un syndrome de choc psycho-traumatique ou " Etat de stress post-traumatique " (ESPT, ou PTSD pour *post-traumatic stress disorder*, American Psychiatric Association 1994). Cependant, tous les individus exposés à ces situations de stress intenses ne développent pas la pathologie. Ainsi, des sujets exposés à des stress précoces présentent une augmentation du risque de développer cette pathologie. Par ailleurs, des troubles préexistants tels que des troubles dépressifs augmentent également fortement la probabilité de développer un ESPT (Heim et coll., 1997). De plus le facteur sexe semble aussi constituer un facteur de vulnérabilité dans la mesure où les femmes ont un risque beaucoup plus marqué de développer un ESPT suite à un trauma. L'ESPT se caractérise sur le plan neuroendocrinien, par une augmentation

du rétrocontrôle négatif de l'axe corticotrope (Yehuda, 2000 ; 2004a,b) et non par une réduction de ce rétrocontrôle comme on l'observe pour un grand nombre de cas de dépression. Sur le plan comportemental, on observe entre autre une hyperanxiété et de l'hyper-vigilance (American Psychiatric Association 1994).

Le but de ces études menées en collaboration avec des cliniciens spécialisés dans le psychotrauma (Pr P. Thomas, Dr G Vaiva et Dr F Ducros, Hôpital Fontan, CHU, Lille) était de valider un modèle animal de PTSD et de déterminer l'incidence d'un stress précoce sur les altérations observées.

a) Validation d'un modèle animal de Stress post-traumatique

Afin de mieux appréhender les facteurs de vulnérabilité associés à l'EPST, nous avons développé une procédure traumatique chez le rat, d'après un protocole décrit par Pynoos et coll. (1996), consistant en un unique choc électrique intense suivi de rappels contextuels de ce choc (Figure 6).

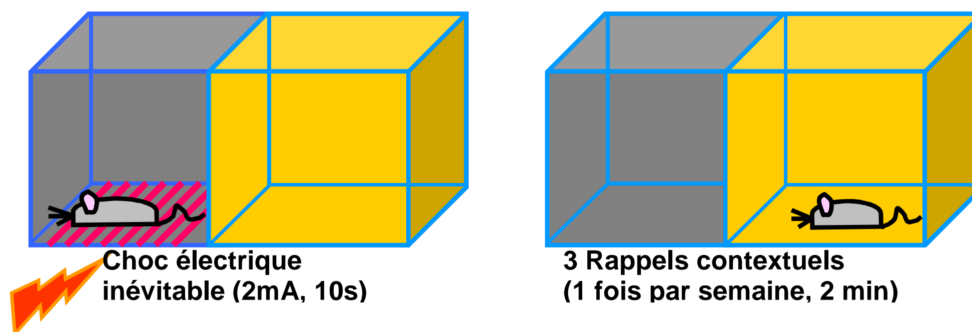

Figure 6 : *Procédure de stress traumatique (d'après Pynoos et coll., 1996). L'animal est placé dans le compartiment éclairé de la cage, après un court délai, la porte s'ouvre et l'animal se réfugie dans le compartiment sombre dans lequel il est enfermé et il reçoit un bref choc électrique intense. Chaque semaine, l'animal est ensuite exposé à un rappel contextuel consistant en une exposition de 2 min dans le compartiment clair La porte permettant d'accéder au compartiment sombre est fermée afin d'éviter la mise en place d'une procédure d'extinction .*

Nos résultats indiquent que cette procédure induit à long terme (plus de 48 jours après le choc) de profondes perturbations comportementales et endocriniennes (Louvart et coll., 2005b). Ainsi, des femelles exposées à un stress intense duquel elles ne peuvent se soustraire, présentent une réponse d'évitement généralisé dans les contexte rappelant le choc électrique et ceci plus d'un mois après le choc électrique. Par ailleurs les animaux choqués présentent une hyperanxiété et une diminution du comportement social à long terme (Figure 7).

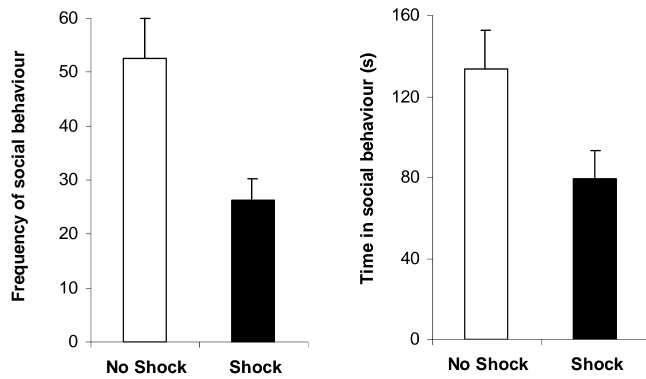

Figure 7 : *Les femelles ayant reçus un choc électrique intense dans un compartiment sombre, présentent une diminution de leur comportement social à long terme.*

Nous avons observé une augmentation du rétrocontrôle négatif de l'axe corticotrope chez des femelles soumises à la procédure traumatique, lorsqu'elles sont à nouveau exposées à une situation de stress, en revanche cette altération n'est pas observée chez les rats mâles (figure 8).

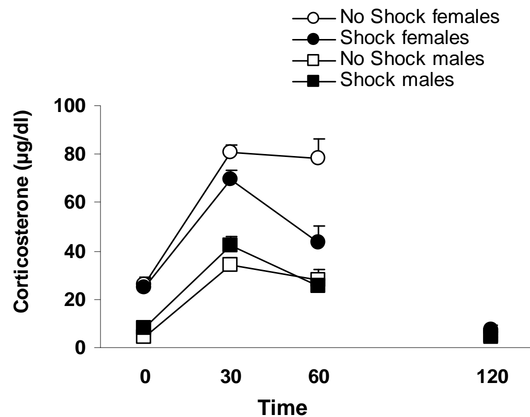

Figure 8: *Les femelles ayant reçus un choc électrique intense présentent une diminution de leur réponse corticotrope lorsqu'elles sont exposées à un nouveau stress.*

b) Impact du stress prénatal sur la réponse à une procédure de stress post-traumatique

Les événements stressant précoces constituent chez l'humain des facteurs de vulnérabilité au développement d'un ESPT suite à la confrontation à un trauma (Heim et coll. 1997).

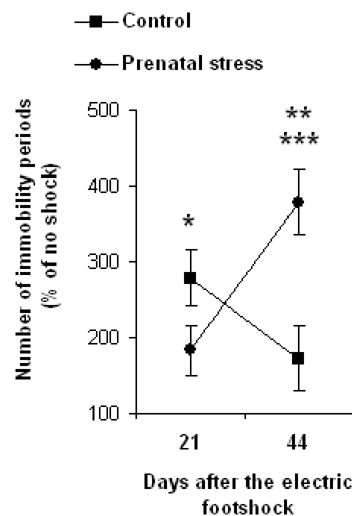

Figure 9 :
Le stress prénatal induit une sensibilisation du comportement de peur après la procédure traumatique. Ainsi, l'immobilité dans le contexte traumatique augmente au cours du temps chez les stressés prénataux.

Dans ce cadre, nous avons évalué si le stress prénatal pouvait influencer la réponse à la procédure traumatique (Louvart et coll., 2005a). Nos résultats indiquent que le stress prénatal augmente le comportement de peur suite à l'exposition au choc intense. Ainsi, lors des rappels contextuels, la majorité des animaux témoins ayant reçu la procédure traumatique ne présentent plus une immobilité totale importante (comportement de " freezing "). En revanche, la plupart des animaux stressés prénatalement continuent à présenter une immobilité très importante 44 jours après le choc (Figure 9).

Par ailleurs, nos travaux récents suggèrent que le stress prénatal accentue également les déficits de l'axe corticotrope induits par la procédure traumatique. Ainsi, plusieurs semaines après le choc électrique, lorsque les animaux sont soumis à un nouveau stress (stress de contention), nous avons observé une hyporéponse et/ou une hypersensibilisation du rétrocontrôle négatif de l'axe corticotrope chez les animaux stressés en période prénatale (Louvart et coll., en préparation).

Enfin, nous avons récemment mis en évidence que la procédure traumatique modifiait le comportement de prise d'alcool (Darnaudery et coll., 2007). Ainsi, après exposition au choc électrique intense des rats stressés en période prénatale préalablement caractérisés comme gros buveurs " maintiennent leur consommation à un niveau élevé alors que les animaux témoins " gros buveurs " présentent une diminution de leur appétence pour l'alcool (Figure 10).

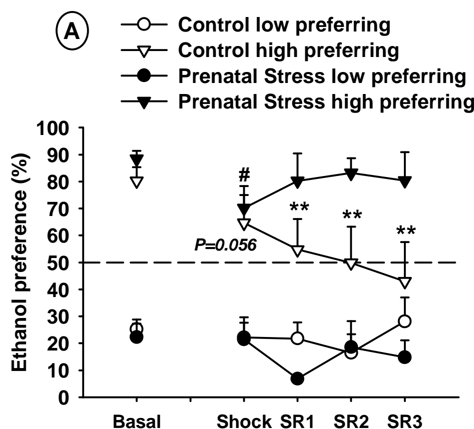

Figure 10 :
Suite à la procédure traumatique, les animaux contrôles diminuent leur consommation d'alcool, en revanche les animaux exposés à un stress prénatal maintiennent une prise élevée.

En conclusion, ces études nous ont permis de valider un modèle animal d' ESPT chez le rat femelle et de montrer que le stress prénatal pouvait moduler la réponse à une procédure traumatique chez l'adulte.

Publications : *Louvart et coll. 2005a, b ; Louvart et coll., 2006 ; Darnaudéry et coll., 2007.*

3) Vulnérabilité au drogues

Les données cliniques indiquent clairement que tous les individus ne présentent pas la même vulnérabilité aux drogues d'abus et à l'alcool (Vega et coll., 2002 ; Koob et le Moal, 2001). Cette vulnérabilité dépend d'un très grand nombre de facteurs (pour revue voir Koob et Le Moal 2005 ; Le Moal et Koob 2007), à la fois génétiques et environnementaux (Piazza et Le Moal, 1996, 1997). De nombreuses données de la littérature mettent en relation stress et vulnérabilité aux drogues, ainsi, les facteurs qui augmentent la charge allostasique favoriseraient la vulnérabilité et la rechute (Kreek et Koob, 1998 ; Le Moal et Koob, 2007). Le stress prénatal semble influencer la vulnérabilité aux psychostimulants (voir table 5 ci-dessous, pour revue voir Koehl et coll., 2001). En revanche l'impact du stress prénatal sur la vulnérabilité à l'alcool reste mal connu. De plus, en dépit des liens stress – axe corticotrope – vulnérabilité aux drogues, aucunes données n'est disponible sur l'effet des drogues sur la réponse neuroendocrine des animaux exposés à un stress in utero. De nombreuses études ont mis en évidence l'intérêt d'utiliser des rongeurs adolescents pour explorer la vulnérabilité aux drogues (pour revue voir Laviola et coll., 1999 ; Spear, 2004). Le modèle du rongeur adolescent reste cependant encore peu utilisé.

Le but de nos études conduites en collaboration avec l'équipe du Pr Lhermitte (Laboratoire de Toxicologie Analytique) est d'analyser les conséquences d'un stress prénatal sur la vulnérabilité aux effets centraux, endocrines et comportementaux de l'alcool, et de déterminer si le stress prénatal module l'appétence spontanée pour l'alcool chez le rat adolescent et adulte.

Trois aspects ont été analysés : les effets d'une exposition aigue à l'éthanol, les effets d'une exposition chronique à l'éthanol et la propension des animaux à consommer spontanément de l'éthanol. Nous nous sommes principalement focalisés sur différents paramètres hormonaux, neurobiologiques et comportementaux connus pour être modulés par l'éthanol et/ou par le stress prénatal. Nos principaux résultats indiquent que le stress prénatal modifie la réponse de l'axe corticotrope à une injection d'une dose modérée d'alcool lors de l'adolescence chez des rats mâles (Van Waes, 2006). La table 5, ci-dessous illustre les connaissances sur les liens entre stress prénatal et vulnérabilité aux drogues ; en rouge sont résumés nos principaux résultats.

Table 5 : ***Impact du stress prénatal sur la vulnérabilité aux drogues et les systèmes biologiques associés aux effets des drogues.***

Effets sur l'axe corticotrope *:*

⬇ du nombre (Bmax) de récepteurs aux minéralocorticoïdes et/ou aux glucocorticoïdes dans l'hippocampe (Maccari et coll., 1995 ; Koehl et coll., 1999) ; ⬇ des quantités d'ARNm des récepteurs aux minéralocorticoïdes et glucocorticoïdes dans l'hippocampe (effet maximal observé dans le CA3).

Hyporéponse de l'axe corticotrope à l'administration aiguë d'une dose modérée d'alcool (1.5 g/kg) chez des rats adolescent (ACTH, corticostérone, POMC dans l'adénohypophyse, CRH dans le noyau paraventriculaire de l'hypothalamus) (Van Waes et coll., 2006).

Effets comportementaux :

⬆ l'auto-administration d'amphétamine chez le rat mâle adulte (Déminière et coll., 1992)

⬆ la réponse locomotrice à l'amphétamine (Déminière et coll., 1992 ; Henry et coll., 1995), à la nicotine (Koehl et coll., 2000) et à la cocaïne (Kippin et coll., 2007) chez le rat mâle adulte.

⬇ l'élimination du MDMA (ecstasy) ; ⬆ des déficits moteurs induit par le MDMA chez le rat adolescent (Morley-Fletcher et coll., 2004).

⬆ de la résistance à l'extinction et la rechute dans un conditionnement opérant pour la cocaïne (Kippin et coll., 2007).

Maintien d'une consommation élevée d'alcool après choc électrique intense chez femelles stressées en période prénatale et caractérisée comme « gros buveurs » (Darnaudéry et coll., 2007)

Pas de changement sur l'appétence spontanée d'alcool (en libre choix : eau versus alcool 2.5, 5,10%) chez les rats mâles adolescents ou chez les adultes, que ce soit chez des rats naïfs ou ayant expérimenté l'alcool de façon chronique

⬆ des perturbations de mémoire spatiale après alcoolisation chronique, chez le rat adolescent.

⬇ des altérations mnésiques (reconnaissance spatiale spontanée) liées à l'âge après alcoolisation chronique sans période de sevrage chez les animaux âgés de 8 mois (van Waes et coll., en préparation).

Effets neurobiologiques :

⬆ de la densité des récepteurs D2 et ⬇ des D3 dans le noyau accumbens (Henry et coll., 1995).

⬆ des taux extracellulaires de dopamine dans le cortex frontal chez des rats mâles ayant une histoire d'autoadministration (Kippin, 2007).

⬆ des taux extracellulaires de dopamine et de glutamate dans le noyau accumbens après injection intrapéritonéale de cocaïne chez des rats mâles naïfs (Kippin, 2007).

⬆ dose dépendante de l'accumulation d'un facteur de transcription, le deltaFosB, dans le noyau accumbens après alcoolisation chronique (5% et 10% par voie orale pendant 10 mois (Van Waes et coll., en préparation).

⬇ des quantités de récepteurs métabotropiques au glutamate (mGluR1, mGluR5, mGluR2/3) dans l'hippocampe chez les rats naïfs ; après alcoolisation chronique (10 mois) modulation différentielle des quantités de récepteurs métabotropiques dans l'hippocampe : ⬇ des mGlur5 et mGluR2/3 chez

les animaux témoins. ⬆ *des mGluR1 chez les animaux exposés au stress prénatal (Van Waes et coll., en préparation).*

Modulation différentielle de la réponse de stress oxydatif dans l'hippocampe suite à une injection aiguë d'alcool (1.5 g/kg, i.p.) : ⬆ *de l'activité de la catalase et de la glutathion peroxydase sélenium dépendante chez les stressés prénataux adolescents ;* ⬇ *de la superoxyde dismutase chez les témoins (Enache et coll., soumis).*

a) Le stress prénatal modifie la réponse de l'axe corticotrope à une injection d'une dose modérée d'alcool lors de l'adolescence chez des rats mâles

Des modifications expérimentales de l'activité de cet axe corticotrope sont associées à des modulations de la consommation spontanée d'éthanol chez le rat (Lamblin et deWitte, 1996; Koenig et Olive, 2004). Une atténuation de la réponse de l'axe corticotrope après une administration d'éthanol a été décrite dans des populations humaines comportant un haut risque de développer des comportements d'abus vis-à-vis de l'éthanol (Schuckit et coll., 1987). Dans ce cadre, nous avons exploré la réponse le l'axe corticotrope d'animaux adolescents à une dose modérée d'alcool (1.5 g/kg, i.p.). Nos résultats indiquent que l'alcool induit une puissante activation de l'axe corticotrope. Cependant, le stress prénatal atténue la réponse de l'axe corticotrope à l'alcool (table 5).

Dans une deuxième étude nous avons cherché à déterminer si le stress prénatal pouvait moduler la réponse oxydative induite par l'alcool au niveau de l'hippocampe. Pour faire face à l'agression cellulaire pouvant générer une peroxidation lipidique toxique pour les tissus, des systèmes antioxydant sont activés, parmi eux on peut citer des agents enzymatiques tels que la superoxyde dismutase (SOD), la catalase (CAT) et le gluthation peroxidase ; mais également des agents non enzymatiques tels que le gluthation réduit (GSH). Nos résultats montrent que le stress prénatal modifie la réponse oxydative après une administration d'une dose modérée d'alcool (Figure 11). L'ensemble de ces données indique que le stress prénatal atténue la réponse hormonale de l'axe corticotrope et induit dans l'hippocampe une réponse de stress oxydatif différentielle après l'administration aigue d'une dose modérée d'alcool.

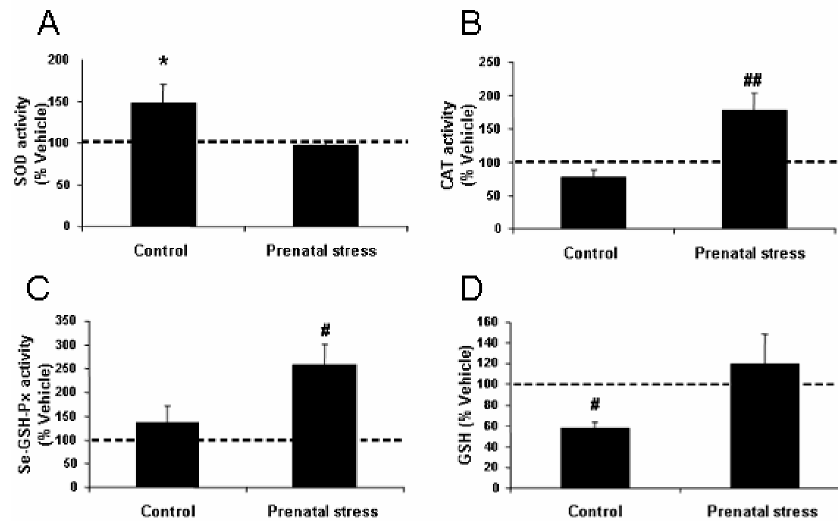

Figure 11: *L'activité de la SOD était augmentée dans l'hippocampe après administration d'alcool chez les rats adolescents témoins. En revanche, l'alcool induit spécifiquement chez les animaux stressés en période prénatale une augmentation de l'activité hippocampique de la CAT et de la GSHP sélénium dépendante.*

b) Le stress prénatal n'influence pas l'appétence pour l'alcool mais augmente les quantités de deltaFosB dans le noyau accumbens après alcoolisation chronique

L'observation d'une hyporéponse de l'axe corticotrope suite à l'administration d'alcool chez les animaux exposés à un stress précoce, nous a conduit à évaluer si le stress prénatal pouvait influencer l'appétence spontanée pour l'éthanol, lors de l'adolescence chez les rats mâles. Par ailleurs, nous avons examiné l'accumulation d'un facteur de transcription le deltaFosB dans le noyau accumbens. En effet, ce facteur a été proposé comme un des facteurs contribuant aux changements à long terme de la plasticité des systèmes dopaminergiques lors de la prise répétée de drogues d'abus (Nestler et coll., 2001). Nos résultats indiquent que le stress prénatal n'influence pas l'appétence pour l'alcool, que se soit chez des animaux adolescents naïfs, ou chez des adultes, après une consommation chronique d'alcool. En revanche nous avons constaté qu'en interaction avec le stress prénatal, l'alcoolisation chronique des animaux durant plusieurs mois (alcool comme seule source à la boisson : 2.5, 5, 10 %) conduisait à une augmentation dose dépendante de deltafosB dans le noyau accumbens (Figure 12).

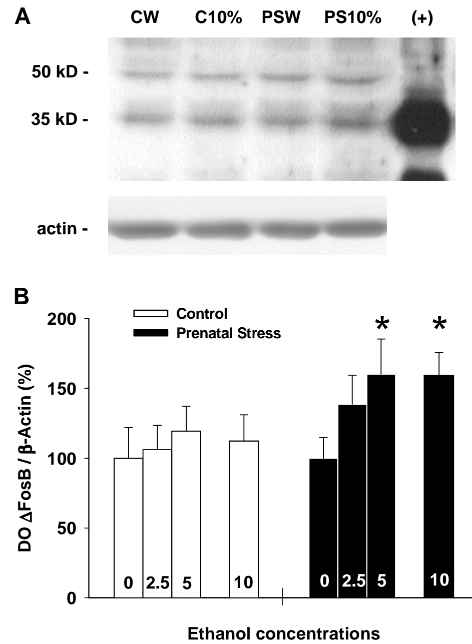

Figure 12 : *L'alcoolisation chronique provoque une accumulation du facteur deltaFosB dans le noyau accumbens dose dépendante chez les animaux ayant subi un stress in utero.*

c) Conséquences d'une alcoolisation chronique sur les performances mnésiques et les récepteurs mGluR hippocampiques chez les animaux ayant subis un stress prénatal.

La consommation d'alcool chronique est associée à des désordres cognitifs. Cependant, les effets peuvent varier considérablement en fonction de la dose, de la durée du traitement et de la présence ou non de période de sevrage. A l'âge de 8 mois, les rats stressés en période prénatale présentent des déficits de reconnaissance spatiale dans l'épreuve du labyrinthe en Y. Après une alcoolisation de plusieurs mois (10%), les animaux stressés présentent une amélioration de leur performances, tandis que l'alcool a tendance à diminuer les capacités mnésiques des animaux témoins. Par ailleurs, l'alcool augmente les quantités de récepteurs mGluR hippocampiques chez les rats exposés au stress prénatal (Figure 13).

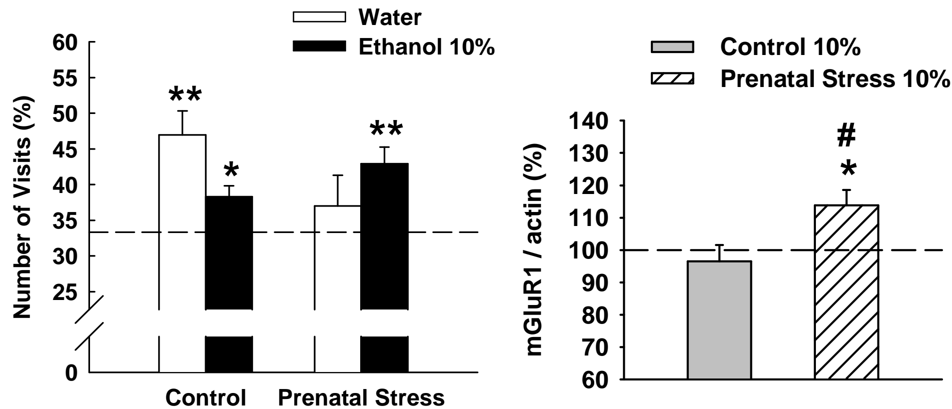

Figure 13: *A l'âge de 8 mois, l'alcoolisation chronique est associée à une atténuation des déficits de reconnaissance spatiale spontanée observées chez les rats exposés à un stress in utero. Chez ces mêmes animaux on observe également une augmentation des quantités de récepteurs mGluR1 dans l'hippocampe.*

L'ensemble de nos données indique que le stress prénatal modifie les réponses hormonales, neurobiologiques et comportementales, suite à des alcoolisations aiguës et chroniques. Par ailleurs, bien que le stress prénatal module la réponse de l'axe corticotrope à l'alcool, ainsi que l'accumulation du deltaFosB du noyau accumbens, la consommation spontanée d'alcool ne semble pas affectée dans des protocoles de libre choix.

Publications : ***Van Waes et coll. 2006 ; Enache et coll., soumis.***

B) Stress, Gestation et allostasie maternelle

Les mécanismes par lesquels, un stress maternel lors de la gestation influence les capacités adaptatives futures de la progéniture, restent mal connus. De plus, bien que de très nombreux travaux, y compris les nôtres, aient mis en évidence un impact du stress maternel sur la descendance adulte sur les sphères émotionnelles, cognitives et motivationnelles ; très peu de travaux se sont intéressés à l'évaluation de l'impact du stress lors de la gestation sur la mère elle-même (Maestripieri et coll., 1991 ; Meek et coll, 2001 ; Lemaire et coll. 2005). **Le but de ces études est d'analyser l'impact du stress gestationnel sur l'environnement maternel lors de la gestation et en période post-natale. Par ailleurs, nous analysons en quoi la maternité en elle-même constitue une période critique pour l'allostasie de la femelle.**

1) L'unité mère-fœtus

En période prénatale, les glucocorticoïdes sécrétés par la mère lors des épisodes de stress peuvent atteindre le fœtus en développement et jouer un rôle dans

l'émergence de certaines altérations chez la descendance (Barbazanges et coll., 1996 ; Zagron et Weinstock, 2006). Cependant, un dysfonctionnement placentaire pourrait également contribuer à ces déficits. Nous avons observé que le stress gestationnel induisait une hyperactivité de l'axe corticotrope chez la mère, en revanche, chez la progéniture on observe une hypoactivité de l'axe à la naissance (Lesage et coll., 2004). Par ailleurs, l'activité de l'enzyme 11β-HSD2 qui joue normalement un rôle de « barrière » au niveau du placenta contre les élévations des glucocorticoïdes maternels, s'est avérée fortement réduite chez les fœtus stressés (Mairesse et coll., 2007). Les principaux résultas obtenus dans cette étude sont résumé de facon schématique dans la figure 14.

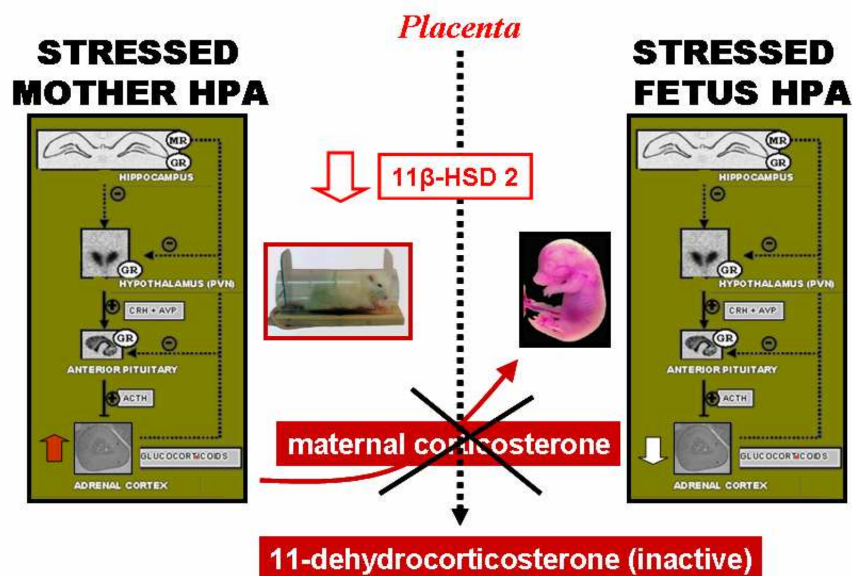

Figure 14 : *En conditions normales, le fœtus est protégé par l'enzyme 11βHSD2. Le stress gestationnel diminue les quantités d'ARNm et l'activité de cette enzyme.*

2) Le Stress gestationnel provoque des altérations à long terme de la physiologie et du comportement des mères

En période postnatale, des altérations du comportement de la mère pourraient également contribuer aux déficits observés chez la descendance (Maccari et coll., 1995). Des travaux antérieurs démontrent que l'adoption précoce atténue l'hyperactivité de l'axe corticotrope des animaux stressés prénatalement (Maccari *et coll.*, 1995). Or les procédures d'adoptions précoces, contrairement aux adoptions tardives, augmentent le comportement de maternage, chez des mères adoptives (Darnaudéry *et coll.,* 2004b). L'étude des capacités adaptatives des mères stressées lors de la gestation révèle chez ces femelles une hyperanxiété lors de la lactation, associée à une réduction du comportement agressif vis à vis d'un intrus exposé à la

progéniture, ainsi qu'une réduction de la qualité de l'environnement maternel (diminution de la qualité du nid, baisse du poids lors de la gestation, diminution du comportement d'amassement de nourriture ; Figure 15).

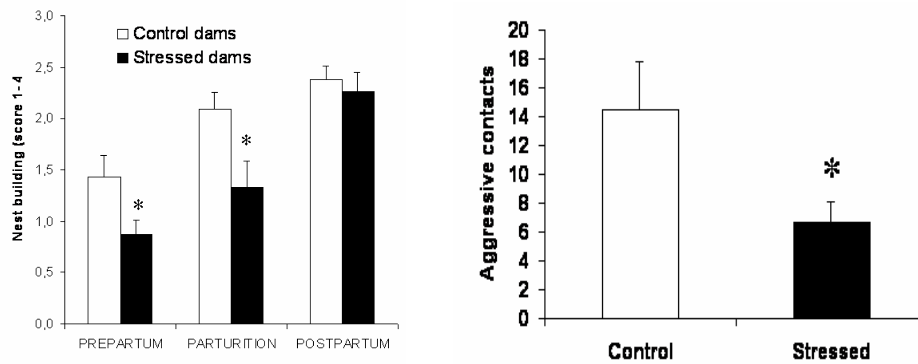

Figure 15: *Le stress chronique lors de la gestation retarde la mise en place du nid (panneau de gauche) et diminue le comportement d'agression de la mère envers un mâle intrus placé dans sa cage lors de la lactation (panneau de droite). *P<0.05.*

Nous avons, dans une première étude, démontré que le stress gestationnel augmentait les comportements anxieux des femelles bien au-delà de la période de stress (Darnaudéry et coll., 2004a). Nous avons également observé que le stress maternel affectait les capacités mnésiques des mères, bien au-delà de la période de lactation. Nos observations confirment des travaux récents (Lemaire et coll., 2006) démontrant que le stress gestationnel supprime les augmentations des performances mnésiques qui se produisent avec l'âge chez les femelles primipares. Bien que l'expérience de maternité ait été décrite comme bénéfique sur le plan cognitif à long terme (Kinsley et coll., 1999), peu de travaux ont été consacrés à la fonction hippocampique lors de l'expérience de maternité.

3) La maternité : un événement critique pour l'adaptation

Comme chez toutes les espèces où la progéniture naît immature, le comportement maternel chez le rongeur joue un rôle fondamental dans la mise en place du système nerveux et endocrinien des petits (Francis et coll., 1999 a,b; Maccari et coll. 1995). En retour cette expérience modifie durablement la psychobiologie des mères. Peu de travaux se sont jusqu'à lors intéressés aux variations des comportements, autres que ceux, directement liés à la fonction de reproduction. Dans ce cadre nous avons formulé l'hypothèse selon laquelle l'initiation de la maternité constitue une période critique de vulnérabilité chez la femelle.

Dans une étude récente, nous avons montré que les femelles présentaient en début de lactation des altérations d'apprentissage spatial comparé aux performances de femelles nullipares (Figure 16, panneau de gauche). En revanche, de façon surprenante les capacités de rétention de cette information sont augmentées chez les mères après 10 jours de rétention (figure16, panneau de droite).

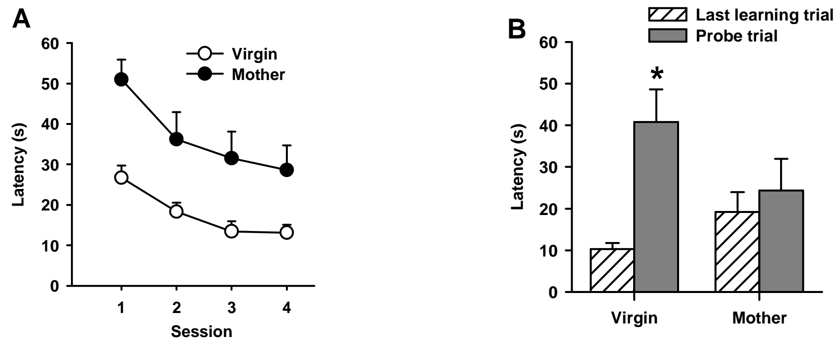

Figure 16 : *24h après l'accouchement, les mères présentent des altérations des capacités d'apprentissage spatial en comparaison à des femelles vierges (panneau de gauche). Après 10 jours de rétention, les vierges ont un déficit pour retrouver la zone de la plateforme ce qui n'est pas observé chez les mères. *P<0.05.*

Par ailleurs, dans l'hippocampe, nous avons observé que la prolifération cellulaire était réduite lors des premiers jours post-partum sans modification marquée de la survie cellulaire (Figure 17).

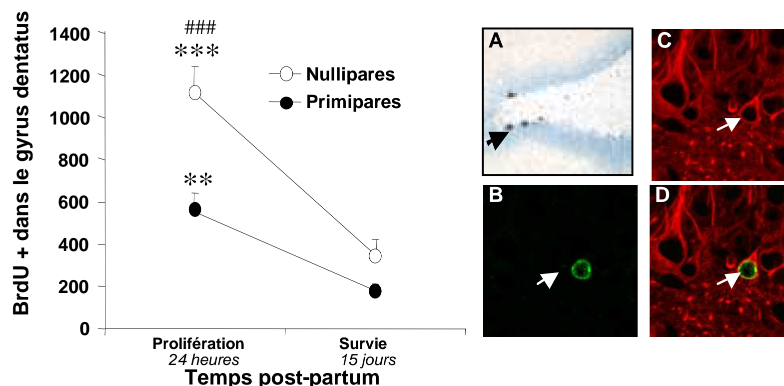

Figure 17 : *Prolifération cellulaire dans le gyrus dendatus de l'hippocampe chez des femelles allaitant nullipares et primipares, 2h et 15 jours après l'injection de BrdU; A) exemple de marquage anti-BrdU dans le gyrus dendatus, B,C,D Identification au microscope confocal du phénotype cellulaire dans la couche granulaire du gyrus dendatus. ***P<0.001, **P<0.01 prolifération versus survie; ### nullipares versus primipares.*

Pour déterminer les mécanismes sous-jacents, nous avons mesuré les variations hormonales après l'accouchement. En particulier, les taux d'estradiol et de corticostérone (Figure 18). En effet, ces deux hormones sont connues pour moduler la fonction hippocampique. Nos résultats montrent que 24 h après l'accouchement, les taux d'estradiol sont diminués, alors que les taux de corticostérone sont très élevés. Par ailleurs les mères présentent une hypertrophie des surrénales en

comparaison aux femelles nullipares suggérant une hyperactivité chronique de l'axe corticotrope. Ces altérations neuroendocrines s'atténuent au cours de la lactation, puisque 15 jours après l'accouchement les variations hormonales de diffèrent plus de celles des femelles.

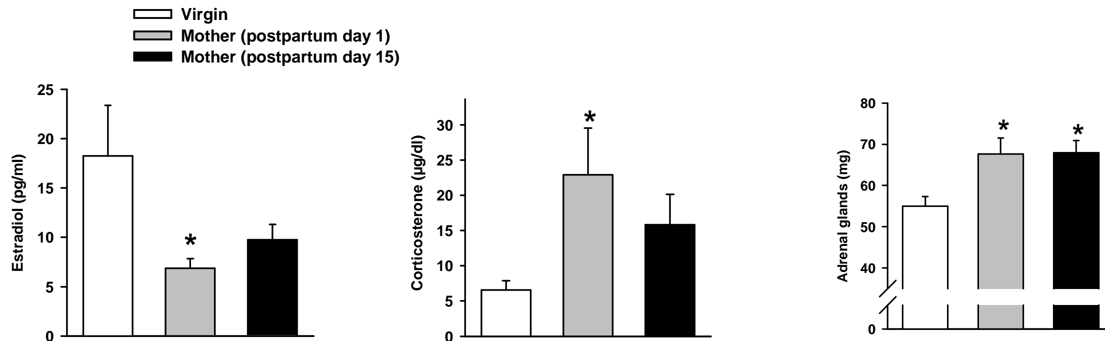

Figure 18 : *Variation des taux d'estradiol, de corticostérone et du poids des surrénales chez des femelles nullipares et primipares (24h et 15 jours post-partum). *P<0.05 comparé aux nullipares.*

Nos données suggèrent que l'initiation de la maternité diminue la fonction hippocampique (baisse des capacités d'apprentissage, baisse de la neurogénèse). Par ailleurs, ces altérations pourraient être sous-tendues par les changements hormonaux drastiques qui accompagnent l'accouchement et la lactation. Ces résultats soulignent combien la maternité constitue une période critique et un modèle naturel remarquable pour l'étude de la plasticité comportementale et cérébrale.

***Publications :* Lesage et coll. 2004 ; Mairesse et coll., 2007 ; Darnaudery et coll., 2004 ; Darnaudéry et coll., 2007.**

III) CONCLUSIONS ET PERSPECTIVES

Mes travaux ont contribué à mettre en évidence que l'organisme (la mère ou son petit) a une « mémoire » de l'expérience de stress et les réponses allostasiques se trouvent modifiées durablement. Ronald de Kloet (2000) souligne: *« …the important stress hormone retained in the hippocampus, an area with a critical function in cognition and affect. That could not be a coincidence!".* A ce titre, on peut noter que l'hippocampe constitue une structure qui de façon récurrente a été démontrée comme étant modifiée par le stress précoce. Cette zone du cerveau fait aussi l'objet d'une forte modulation épigenétique (Levenson et Sweat, 2005, Figure 19) ; tout comme le système hypothalamo-hypophyso-corticosurrénalien (Meaney et coll., 2007).

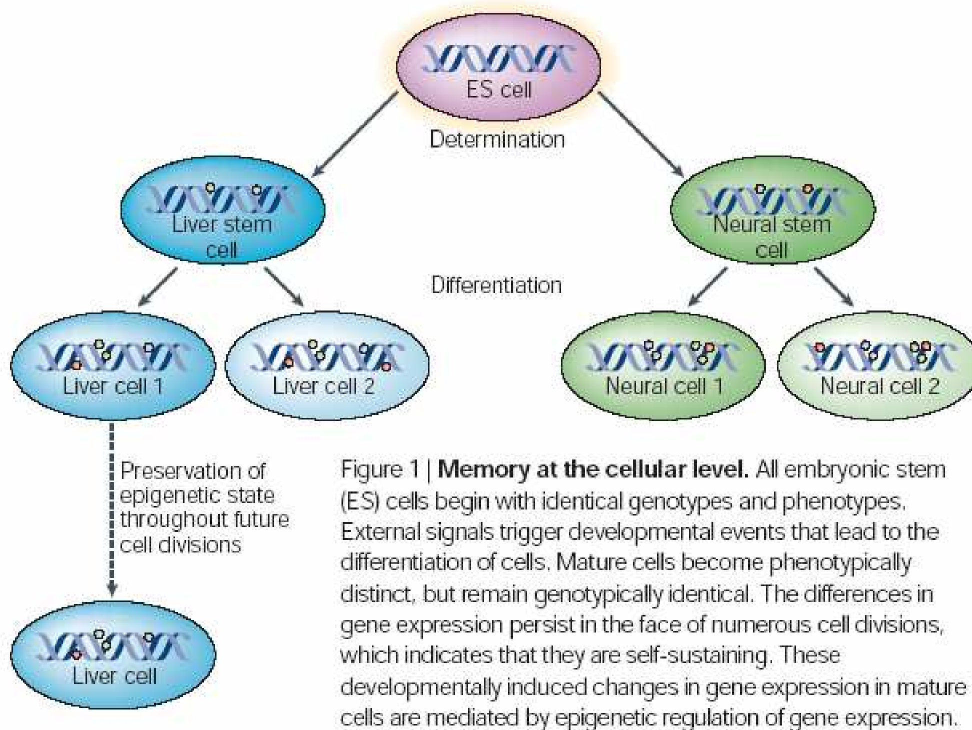

Figure 19 : *L'épigenèse aboutit à des cellules différentes sur le plan phénotypique, bien qu'elles restent génétiquement identiques. (Levenson et Sweatt, 2005).*

Mes recherches renforcent l'hypothèse selon laquelle une expérience très précoce, voire anténatale est capable d'exercer une empreinte durable sur l'individu. Des changements neuroendocriniens (axe corticotrope) ; neurobiologiques (système hippocampique) et comportementaux (mémoire, émotion) ont été observés. Ces travaux renforcent les théories de l'allostasie et soulignent combien l'adaptation se

fait par le changement. Il est fondamental de souligner que ces altérations sont rarement visibles à l'état « basal », mais se révèlent lorsque l'animal est soumis à un nouveau « challenge » adaptatif, ou après exposition à différents stressors au cours de sa vie. Ainsi, nous avons observé des effets importants lorsque les animaux sont âgés, soit plus de 2 ans après un stress de contention appliqué à leur mère (voir aussi, les effets sur la consommation d'alcool les observés chez femelles après combinaison du stress prénatal et de l'administration d'un stress intense inévitable). Pourtant, sur le plan de la charge allostasique, notre procédure de stress de contention ne constitue pas un stressor très fort pour le rat, au regard d'autres procédures telles que le stress de défaite sociale ou l'exposition à un prédateur ; ce qui renforce combien cette période périnatale est *critique* pour l'allostasie à la fois de la mère et pour celle de sa descendance.

Mon projet de recherche vise à mieux comprendre la surcharge allostasique induite par le stress maternel sur la descendance et la mère. Les principaux objectifs sont résumés ci-dessous.

A) Stress prénatal et modèle animal de PTSD

- Evaluations lors du vieillissement des perturbations allostasiques associées au stress traumatique.
- Caractérisation des mécanismes neurobiologiques sous-tendant les perturbations neurobiologiques associées au stress intense (étude du rôle de l'hippocampe, de l'axe corticotrope, des facteurs de transcription et neuropeptides).
- Evaluation de stratégies thérapeutiques : la tianeptine, les glucocorticoïdes et la stimulation haute fréquence de l'hippocampe.

Ce projet s'inscrit dans le cadre de la mise en place d'un **réseau national INSERM « Biologie et Approches cliniques des psychotraumas » (coordinateur : Pr René Garcia).** Le réseau « Biologie et Approches cliniques des psychotraumas », rassemblant des chercheurs de divers horizons (psychiatres, psychologues et neurobiologistes), a pour mission majeure de développer des interactions fortes entre ces deux formes de recherche. Le réseau se compose actuellement de 5 équipes de neurobiologistes et de cliniciens, basées dans 4 villes (Lille : 2 équipes, Montpellier, Nice, Toulouse). L'idée privilégiée étant que les études cliniques fourniront des informations précieuses au développement de modèles animaux et l'expérimentation

animale, en retour, livrera des informations pour le développement de nouveaux modèles de prévention et de traitement des psychotraumas.

Résumé :

Nos recherches portent sur l'étude chez le rat de l'impact à long terme de stress précoces (pré- et postnataux) sur les capacités d'adaptations comportementales (mémoire, émotion, motivation), neuroendocriniennes (axe corticotrope) et neurobiologiques. Récemment, nous avons exploré le rôle de différents facteurs de vulnérabilité sur un modèle animal d'état de stress post-traumatique (PTSD), proposé par Pynoos (1996). Nos études nous ont permis de mettre en évidence qu'une exposition à un stress intense inévitable (choc électrique) suivie de rappels situationnels produisait des perturbations comportementales et endocrines de l'axe corticotrope à très long terme. Cependant, nous avons observé que les effets comportementaux et neuroendocriniens diffèrent entre les rats mâles et les femelles. Par ailleurs, des animaux ayant subi un stress prénatal (stress chronique de la mère lors de la gestation) présentent également une réponse différentielle à la procédure traumatique que ce soit sur le plan comportemental ou hormonal. L'objectif de nos travaux est d'explorer la temporalité des effets observés au cours du vieillissement et d'étudier les systèmes neurobiologiques pouvant sous-tendre ces altérations. Par ailleurs, nous évaluerons des stratégies de restaurations (pharmacologiques et/ou électrophysiologiques). Des travaux récents indiquent que la manipulation de l'axe corticotrope peut moduler la peur phobique chez l'humain (Soravia et coll., 2006). Nous évaluerons donc la possibilité de réduire les altérations comportementales suite à des injections de corticostérone avant l'exposition à la procédure aversive dans le modèle utilisé. Parallèlement, l'efficacité de la Tianeptine, un antidépresseur connu pour réguler l'axe corticotrope sera déterminée en collaboration avec l'équipe du Professeur Vaiva (CHU de Lille). En collaboration avec l'équipe du Professeur René Garcia (Université de Nice), nous examinerons si les changements comportementaux et endocrines de l'axe corticotrope observés chez les animaux ayant subi un stress prénatal puis une procédure aversive à l'âge adulte (modèle animal de PTSD de Pynoos) peuvent être atténués par une stimulation à haute fréquence de l'hippocampe dorsal.

La réalisation de ce projet pourra se faire dans le cadre d'une thèse en co-tutelle entre différentes équipes du réseau.

B) Stress prénatal et vulnérabilité à l'alcool : étude de l'environnement précoce sur les perturbations des comportements de consommation

- Evaluation de la motivation pour l'alcool (procédures de conditionnement opérant)
- Evaluation de l'influence de l'exposition précoce lors de la période post-natale et de l'adolescence à des renforçateurs naturels et pharmacologiques
- Recherche des mécanismes neurobiologiques (axe corticotrope, facteurs de transcription, récepteurs mGluR, BDNF dans l'hippocampe et système mésolimbique, voie des kynurénines)

Ce projet s'inscrit dans la continuité du ***Pôle Interdisciplinaire de Recherche sur les Conduites Addictives*** (PIRCAd, coordinateurs: Pr. Michel LHERMITTE & Dr Jean VIGNAU – Laboratoire de Toxicologie Analytique CHRU Lille – (EA 2679) – Université de Lille 2) auquel je participe depuis sa création en 2004 (financement dans le cadre de l'ARCIR et du FEDER). Ce pôle régional a permis la mutualisation de moyens matériels et humains et l'organisation d'un colloque transdisciplinaire en décembre 2006 à destination des professionnels du champ de l'Addictologie. Une partie de mon projet s'inscrit dans le cadre d'une **délégation CNRS au sein de l'équipe du Dr Martine CADOR " Neuropsychobiologie des conduites de consommation pathologiques " (Laboratoire CNRS UMR 5227, « Mouvement, Adaptation, Cognition », Pr Cazalet).**

Résumé

De nombreux travaux de la littérature mettent en relation stress et vulnérabilité à l'alcool. Des données récentes obtenues par notre équipe suggèrent que le stress prénatal module la réponse aiguë à l'alcool en diminuant l'activation de l'axe corticotrope induite par l'éthanol (Van Waes et coll., 2006). Nous avons par ailleurs montré chez des rats femelles, que le stress prénatal interagissait avec les effets d'un stress intense à l'âge adulte sur la prise spontanée d'alcool (Darnaudéry et coll., 2007). Chez l'humain, certains travaux suggèrent qu'une hyporéponse de l'axe corticotrope à l'alcool est observée dans des populations plus vulnérables au développement de conduites d'abus vis à vis de l'alcool (Schuckit et coll., 1987). Par ailleurs, les données de la littérature, suggèrent qu'il existe une homologie dans les

processus et/ou les systèmes sous-tendant les désordres des conduites alimentaires et la vulnérabilité aux drogues d'abus (Kelley et Berridge, 2002 ; Barbano et coll., 2006). Cependant, l'importance des effets de l'exposition précoce à des agents renforçateurs naturels ou pharmacologiques sur la vulnérabilité future aux drogues d'abus et/ou aux désordres alimentaires est peu connue. De même les liens entre troubles des conduites alimentaires et vulnérabilité aux drogues restent à déterminer.

L'objectif de notre projet est d'une part d'évaluer la motivation pour l'alcool chez des animaux ayant une histoire de stress en période prénatale et/ou une histoire de postnatale d'exposition précoce à des renforçateurs naturels comme le sucre ou pharmacologique comme l'alcool (en collaboration avec le Dr CADOR). La motivation sera évaluée par des procédures de conditionnement opérant. Par ailleurs, sur le plan neurobiologiques, nous mesurerons dans le système mésocorticolimbique et/ou l'hippocampe : l'accumulation du facteur de transcription deltaFosB (Nestler et coll., 2001), les variations des taux de récepteurs métabotropiques glutamatergiques et les quantités de BDNF. Sur le plan endocrine, nous explorerons l'axe corticotrope, impliqué dans la réponse au stress et la vulnérabilité aux drogues (Piazza et Le Moal, 1998), ainsi que la leptine, du fait de son implication dans la régulation de la voie mesolimbique (Fulton et coll., 2006). Enfin, sur le plan enzymatique, nous explorerons une nouvelle piste, celle du métabolisme du tryptophane et en particulier la voie des kinurénines en collaboration avec le Dr ALLORGE (Equipe du Pr Lhermitte, Université de Lille 2). En effet, des dérégulations de ce système pourraient à la fois jouer sur la réponse au stress, l'appétence aux drogues, mais aussi expliquer certaines réponses différentielles aux drogues observées chez les animaux ayant subi un stress prénatal.

La réalisation de ce projet nécessitera le recrutement d'un chercheur postdoctoral dans le cadre du PIRCAD.

C) Maternité et plasticité neuro-comportementale : modulation par le stress gestationnel

- Evaluation des facteurs maternels précoces (épigenèse) dans l'ontogenèse du comportement anxieux chez la descendance.
- Etude de l'impact du stress gestationnel sur les changements de plasticité cérébrale associés à la maternité (hippocampe et bulbes olfactifs)
- Recherche des mécanismes neurobiologiques sous tendant les changements des comportements émotionnels et de la fonction mnésique lors de la maternité.

Ce projet s'inscrit dans la continuité de la collaboration établie avec le Pr Luis Miguel Garcia-Segura (CSIC, Madrid) et le Dr Margarita Perez-Martin (Université de Malagà).

<u>Résumé :</u>

L'accouchement et le début de l'expérience maternelle constituent une expérience à haute charge allostasique pour la mère primipare (Rosenblatt et Snowdon, 1996). La qualité du comportement maternel joue un rôle décisif pour la survie de la descendance et détermine en partie l'épigenèse de son axe du stress (Champagne et Meaney, 2006). Par ailleurs, de profonds changements des systèmes hypothalamique, mésolimbique et olfactifs ont été décrits chez la mère (Fleming et coll., 1999 ; Shingo et coll., 2003, figure 20). Bien que cette plasticité joue un rôle clefs dans le comportement maternel, elle a aussi des conséquences importantes sur d'autres fonctions, liées à ces systèmes, comme la mémoire et la motivation. Nous avons démontré récemment que l'initiation de la maternité constituait une période critique pour la fonction hippocampique (Darnaudéry et coll., 2007). Nos travaux antérieurs ont permis de mettre en évidence que le stress lors de la gestation a des conséquences durable sur la physiologie et le comportement des femelles (Darnaudéry et coll., 2004). Le but de notre projet est d'explorer le rôle des facteurs maternels lors de la lactation dans l'influence du stress maternel sur l'adaptation future de la descendance. Par ailleurs, nous examinerons les changements de la plasticité cérébrale au cours de la gestation ; et les mécanismes neurobiologiques sous-tendant les changements comportementaux associés à la maternité. Pour ce faire, nous caractériserons les changements comportementaux associés non seulement aux fonctions reproductives, mais également aux fonctions

cognitives, émotionnelles et motivationnelles chez des femelles soumises à un stress gestationnel et nous étudierons les mécanismes moléculaires et cellulaires sous-jacents, en particulier par l'étude de la plasticité cérébrale (facteurs de transcription constitutifs et inductible dans l'hippocampe et les régions mésolimbiques).

La réalisation de ce projet pourra se faire grâce au recrutement d'un étudiant en thèse.

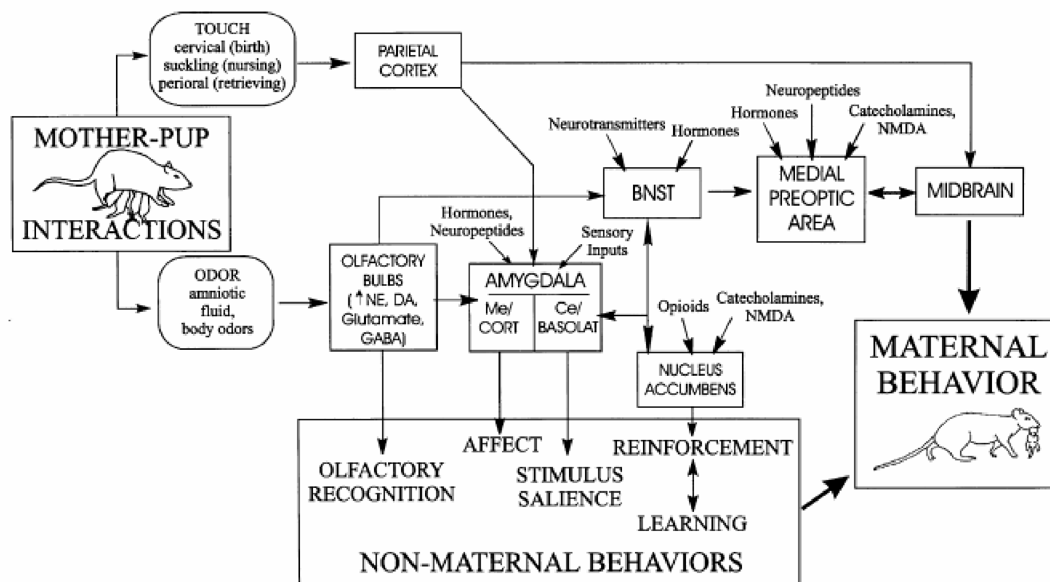

Figure 20: *Neuroanatomie fonctionnelle du comportement maternel (Fleming et coll. 1999).*

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I) Stress précoces et surcharge allostasique chez la descendance

A) Anxiété, dépression et vieillissement Cognitif

1. Maccari S, **Darnaudéry M**, Morley-Fletcher S, Zuena AR, Cinque C, Van Reeth O (2003) Prenatal stress and long-term consequences: implications of glucocorticoid hormones. ***Neuroscience Biobehavioural Review*** 27: 119-127.

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A) L'unité mère-foetus

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Prenatal stress and long-term consequences: implications of glucocorticoid hormones

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Abstract

We have shown that prenatal restraint stress (PNRS) induces higher levels of anxiety, greater vulnerability to drugs, a phase advance in the circadian rhythm of locomotor activity and an increase in the paradoxical sleep in adult rats. These behavioral effects result from permanent modifications to the functioning of the brain, particularly in the feedback mechanisms of the hypothalamic-pituitary–adrenal (HPA) axis: the secretion of corticosterone is prolonged after stress and the number of the central glucocorticoid receptors is reduced. These abnormalities are associated with modifications in the synthesis and/or release of certain neurotransmitters. Dysfunction of the HPA axis is due, in part, to stress-induced maternal increase of glucocorticoids, which influences fetal brain development. Some biological abnormalities in depression can be related to those found in PNRS rats reinforcing the idea of the usefulness of PNRS rats as an appropriate animal model to study new pharmacological approaches.

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Keywords: Prenatal restraint stress; Corticosterone; Maternal behavior; Circadian rhythms; Antidepressants

1. Perinatal events in humans

There is increasing evidence that variations in prenatal environment can influence the responses of the new-born. Barker [1] has emphasized how adult vulnerability to cardiovascular disease may be programmed during the fetal period. Indeed, non-genetic factors that could act early in life to organize or imprint permanently physiological systems are known as *perinatal programming* [2,3]. It can be speculated that prenatal plasticity of physiological systems allows environmental factors, acting on the mother and/or the fetus, to alter the differentiate functions of an organ or tissue system to prepare the unborn animal optimally for the environmental conditions ex utero. However, in extreme conditions like stress and/or under-nutrition, offspring of stressed mothers during pregnancy displayed short and long-term physiological and behavioral abnormalities such as reduced birth weight, increased infant morbidity, locomotion and cognition retardation, increased anxiety or sleep disturbances [4–6].

The fetus is sensitive to maternal environment and it has been shown that anxious pregnant women, who present an altered blood flow in the uterine arteries [7], can influence the development of the fetus she carries [1,8,9]. Similar results are also observed later, indeed there is an evidence showing that adverse environmental experiences early in life predispose individuals to the development of affective and anxiety disorders in adulthood [10].

Glucocorticoids may underlie the association between low birth weight and adult stress-related cardiovascular, metabolic and neuroendocrine disorders such as hypertension, type 2 diabetes, ischaemic heart disease and affective disorders [11]. These intriguing findings have spawned the *fetal origins hypothesis* of adult disease [1]. The brain is very sensitive to prenatal programming and glucocorticoids in particular have powerful brain-programming properties [11]. One of the most intensively systems studied is the hypothalamic-pituitary–adrenal (HPA) axis. Substantial evidence suggests that prenatal stress programs the HPA

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axis, and that plasticity of developing brain monoamine systems underlies, in part, these changes. Because an important feature of the stress response is the secretion of high levels of glucocorticoids, this steroid has become an obvious candidate for the role of *programming factor* in the prenatal stress paradigm. Thus, in human cohorts, it has been shown associations between low birth weight and adult hyperactivity of the HPA axis [12–14].

2. An animal model characterization

In order to better understand mechanisms involved in the long-term effects of such early experiences and considering the obvious difficulties inherent to human research in this particular field, different kind of prenatal stress animal models have been developed. Pregnant rats have been subjected to various types of stressors: conditioned avoidance training [15], tail suspension [16], crowding [17], repeated electric shocks [18], noise [19] or saline injections [20]. During the last years we have studied the influences of prenatal restraint stress (PNRS) in a rat animal model according to a revised model of Ward and Weisz [21]. The prenatal stress procedure we have used consisted in restraining the mothers. Adult virgin Sprague-Dawley female rats (Iffa Credo, France) were group-housed (10 per cage size 60×80 cm^2) for at least 10 days after arrival, to eliminate stress resulting from shipping and to coordinate their estrous cycle. Animals were then individually housed in the presence of a sexually experienced male Sprague-Dawley rat. Pregnant females were then randomly assigned to prenatal stress or control groups, and individually housed in plastic breeding cages. For all experiments, animals were allowed ad libitum access to food and water, and maintained on a regular light–dark cycle (lights-on 07:00–19:00 h) with constant temperature (23 °C) and humidity (60%). Prenatal stress was started between the days 11–15 of pregnancy until delivery at 21 days according to a revised model of Ward and Weisz: pregnant females were individually placed in plastic transparent cylinders (7 cm diameter, 19 cm long) and exposed to bright light for 45 min. Animals were daily submitted to three stress sessions starting at 09:00, 12:00 and 17:00 h, whereas control pregnant females were left undisturbed in their home cages. Male and female offspring were weaned 21 days after birth, and only offspring from litters containing 10–14 pups with similar numbers of males and females were used in the experiments. A maximum of two male pups were taken from each litter to remove any 'litter effects' [22,23].

2.1. Behavioral long-term consequences

It is clear from animal studies that the behavior of the adult offspring can be altered by PNRS. In rats, PNRS can exert profound influences on offspring's development, inducing abnormalities which extend from early to later life [27]. Adult PNRS rats (4–7 months) exhibit increased 'anxiety' (Fig. 1) [28,29], drug addiction [30] 'emotionality' [15,31,32] or depressive-like behaviors [16, 33–35]. We also reported in PNRS rats enhanced age-related (16–22 months) recognition memory impairment in the Y-maze compared to controls, and altered working memory in the radial-maze [27]. Furthermore, our data provide evidence of a long-term effect of a prenatal stressful procedure on the circadian system. We have shown significant phase advances in the circadian rhythms of locomotor activity relative to the entraining light–dark cycle in both male and female stressed rats [36]. When subjected to an abrupt shift in the light–dark cycle, male and female PNRS rats resynchronized their activity rhythm to the new light–dark cycle slower than control rats [37,38]. Those results raise the possibility that the circadian clock in the hypothalamic suprachiasmatic nuclei (SCN) [39,40] of those animals has been altered by prenatal stressful events. The altered phase-relationship between the circadian clock and lights-off could be due to a change in the underlying period of the circadian clock during entrained conditions. In order to test this hypothesis, we analyzed the free-running period of locomotor activity in temporal isolation in constant darkness. The free-running period was significantly shorter in PNRS rats compared to control rats.

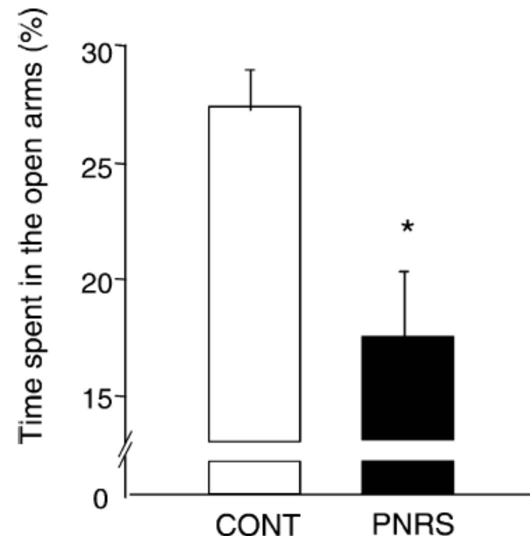

Fig. 1. The anxiety in the elevated plus maze. The time spent in the open arms was significantly shorter in males PNRS rats compared to control rats ($p < 0.05$). Error bars show SEM.

2.2. Neurobiological long-term consequences

The HPA axis has been shown to be affected by PNRS (Fig. 2), showing increased responsiveness to a novel stimulus [41,42]. Levels of both glucocorticoid type I and type II receptors were reduced in

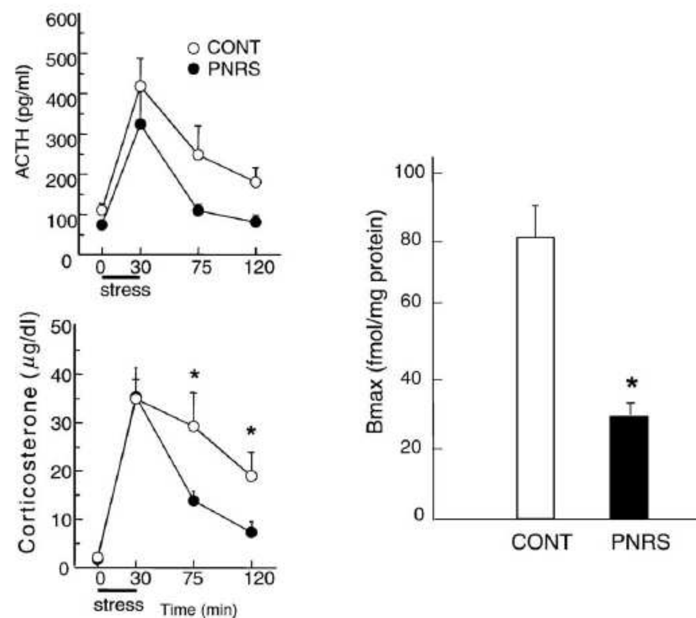

Fig. 2. In the bottom, corticosterone secretion at 4 months in control (CONT) and PNRS animals. Time-course of the secretion of corticosterone is represented at different times following a 30 min restraint stress (black line of abscissa). The poststress secretion of corticosterone, at T75 and T120 min, is increased in PNRS rats in comparison to CONT. In the top, ACTH secretion after 30 min of restraint stress. PNRS rats show a tendency to have a prolonged ACTH secretion after stress. In the right, hippocampal type I corticosteroid receptors. PNRS rats present decreased type I receptors (PNRS vs CONT, $*p < 0.05$). Error bars show SEM.

the hippocampus at 90 days, showing a possible mechanism for the long lasting effects on the HPA axis [24,41]. Associated with HPA axis hyperactivity, PNRS rats also displayed hyperglycemia [43]. Moreover, prenatal dexamethasone exposure, a synthetic glucocorticoid, has recently been implicated in the development of adult hyperglycaemia and hypertension as well as behavioral changes and HPA activation [44–46]. PNRS accelerated the age-related HPA axis dysfunction. Indeed, the HPA axis period of hyporesponsiveness was abolished in new-born PNRS rats [24] and circulating glucocorticoid levels of PNRS middle-aged animals were similar to those found in old control ones [27].

We have shown that PNRS altered circadian rhythms. More precisely, PNRS induced higher levels of total and free corticosterone secretion at the end of the light period in both males and females, and hypercorticism over the entire diurnal cycle in females (Fig. 3) [47]. Those effects could be mediated, at least in part, by a reduction in hippocampal corticosteroid receptors at specific times of day [42]. PNRS induces a reduction of type I glucocorticoid receptors both at the beginning of the light period, which is in agreement with previous data [41] and at the end of the light period at a time when total corticosterone levels are increased in PNRS rats.

Furthermore, prenatal noise stress rats also showed a decrease in benzodiazepine receptors in the hippocampus [48], which could explain the profile of anxiety observed in those rats. Prenatal stress is likely to influence not only the HPA axis but also other endocrinological factors such as sexual hormones in the mother and in the fetus [21]. Recently, the effect of prenatal stress was investigated on the sympathoadrenal response to novelty and footshock by measuring the time course of the changes in circulating catecholamines and their metabolites. Plasma NA levels were significantly higher in prenatal stress than in control rats immediately after footshock, indicating a greater activation of the sympathetic nervous system in prenatal stress rats. The findings demonstrate for the first time that prenatal stress can induce long-term changes in the sensitivity of the sympathoadrenal system to stress [49].

PNRS has been reported to affect neurotransmission. Adult PNRS offspring shows increased 5-hydroxytryptamine (5-HT) contents in several brain regions, e.g. the hypothalamus and the cortex [50–53]. Prenatal dexamethasone exposure mimics prenatal stress procedure, and induces a reduction of 5-HT turnover in the hypothalamus, the hippocampus and neocortex in the offspring moreover at 3 weeks of age [54]. This change in 5-HT function could be involved in alterations of behavioral and hormonal

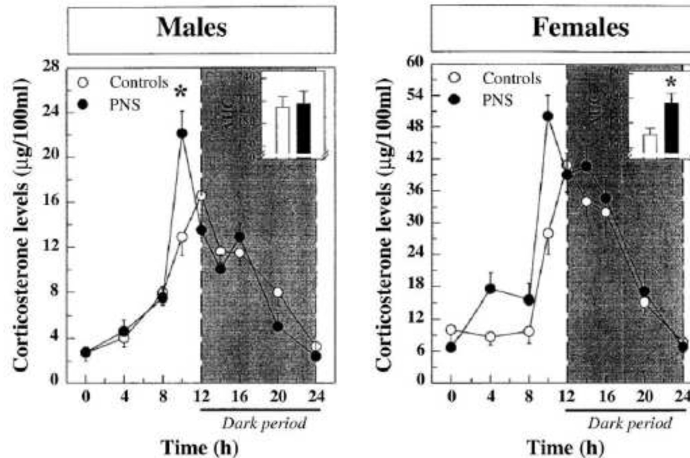

Fig. 3. Circadian fluctuations of plasma corticosterone levels at various points over the light–dark cycle in CONT and PNRS male (left panel) and CONT and PNRS female (right panel) adult rats. Prenatal stress induces in both males and females higher levels of the corticosterone during the light-phase, and only in females an hypercorticism over the entire light–dark cycle. $*p < 0.05$. Error bars show SEM.

responses to environmental stimuli, including the HPA axis given that serotonin is a major modulator of the HPA axis [55]. Furthermore, corticosterone regulates the activity of serotonergic system, including tryptophan hydroxylase in the raphe nuclei [56]. Prenatal stressed rats also show reduced noradrenaline contents, and increased noradrenaline turnover in the hippocampus and neocortex [54,57,58] and reduced dopamine turnover in the hypothalamus [54, 59]. Finally, PNRS has long-term effects on the forebrain cholinergic systems inducing an increased hippocampal acetylcholine release after a mild stress and CRH injection (Fig. 4) [60]. A higher release of acetylcholine in the hippocampal area in PNRS rats could be responsible for the decreased corticosterone feedback mechanisms, normally observed in those animals, by reducing glucocorticoid receptors.

This early life experience, the prenatal stress, seems to significantly influence the development of the brain. In this regard, prenatal stress induces structural abnormalities in the hippocampal formation and recently, it has been shown that PNRS produces learning deficits associated with an inhibition of neurogenesis in the hippocampus [61]. Given that glucocorticoids inhibit hippocampal cell proliferation [62] the increased HPA axis activity of prenatally stressed animals [41] could explain their reduced neurogenesis.

3. Parallel between prenatal stress effects and biological abnormalities found in depression

Taken together, our results indicate that prenatal stress induces an increased stress response and abnormal circadian and sleep function in adult rats, suggesting an underlying dysfunction of their circadian clock and a global bad adaptation to challenges. These biological alterations indicate that PNRS adult rats have similar biological correlates of depressed patients [63,64]. Thus, like depressed patients, PNRS rats do escape from the feedback inhibition responsible for returning corticosterone secretion to basal levels after stress [41]. Like in depressed patients in which cholinergic hyperactivity is described [65], PNS rats exhibit cholinergic hypersensitivity after a CRH challenge [60].

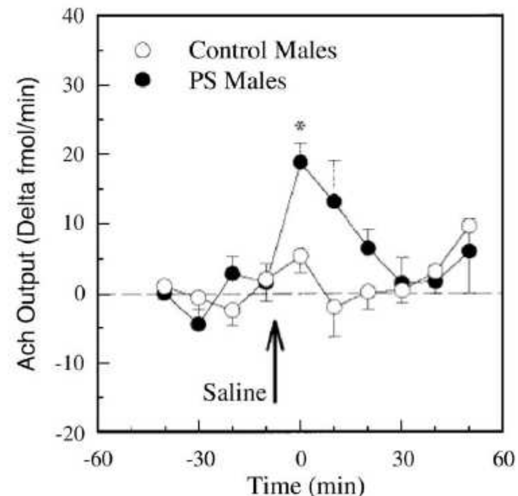

Fig. 4. Effects of prenatal stress on saline-stimulated hippocampal ACh output (Delta fmol/min) in males. Prenatal manipulation enhanced the transient increase of ACh release in males ($*p = 0.004$).

Various clinical observations in humans suggest a possible pathophysiological link between depression and disturbances in circadian rhythmicity, including rhythms of body temperature, various peripheral hormone concentrations and urinary levels of neurotransmitter metabolites [66]. Circadian abnormalities include changes in free-running period, amplitude, cohesion, and entrainment to photic and/or social 'zeitgebers' [67]. One of the current hypothesis on the neuroendocrinology of depression involves a flattened (and advanced) circadian cortisol rhythm with hypercortisolism, possibly due to an increased sensitivity of the adrenal cortex [68] thought to normalize pituitary ACTH release in spite of an enhanced drive from the hypothalamic CRH neurons [68,69]. Those hormonal features of depression can be related to those found in PNRS rats. Previous reports on the long-term effects of prenatal stress have already suggested its usefulness as an animal model to develop new pharmacological approaches of depression.

One of the hallmarks of human depression is an alteration in the sleep–wake cycle including shortened rapid eye movement (REM) sleep latency, an increase in the amount and frequency of REM sleep during the first part of the night, an increased sleep fragmentation, and a decrease in the amount of slow wave sleep [70]. Significant correlations between sleep abnormalities and dysfunctions of the HPA axis have been shown in depressed patients [71,72], and may result from a stress component [67,73]. In view of those data, we investigated the effects of PNRS on the sleep–wake cycle in adult rats. PNRS rats exhibited a significant increase in the amount of paradoxical sleep (PS) over the 24 h recording session that is positively correlated to plasma corticosterone levels (Fig. 5). Other changes include increased sleep fragmentation, total light slow wave sleep time, and a slight decrease in the percentage of deep slow wave sleep relative to total sleep time [74]. Although there are reports of abnormal 'sleep-like behaviors' in PNS monkeys [75] and PNS humans [5], our data provide a polygraphic demonstration of long-term effects of PNRS on the sleep–wake cycle when the animals reach adulthood. These results are also in agreement with the work of Poland group's [76].

Another important point is that the persistence of REM alterations in PNRS rats dramatically contrasts with the reversibility of these sleep effects in other chronic stress animal models. For example, in both the learned helplessness [77] and the intermittent foot shock paradigms [78], REM was increased only during the first day of recovery. In rats exposed to chronic mild stress, an increase in REM sleep was only observed during the first day of stress recovery [79]. Interestingly, we have noted that even at 6 months of age sleep–wake differences between PNRS and control rats were still present (unpublished data).

Added to our previous findings in PNRS rats of high anxiety and emotionality, dysfunction of the HPA axis and circadian timing abnormalities, the observation of long-term changes in their sleep organization supports the validity of the 'prenatal stress' model to develop new pharmacological approaches of depression.

4. Predictive validity

The PNRS seems to be an interesting animal model because of the permanent disturbances it produces in the long-term. We determined whether PNRS rats are sensitive to a chronic treatment with antidepressants. There is one single report showing efficacy of tricyclic antidepressants in prenatally stressed female rats [80].

We investigated the behavioral response of PNRS rats in the forced swimming test (Porsolt test), a behavioral test classically used to study antidepressant efficacy. The procedure of the forced swimming test consisted in plunging rats into a glass cylinder (height 59.5 cm; diameter 24.6 cm) containing 36 cm of water maintained at 24 °C for 15 min. A second trial was performed 24 h later for 5 min. PNRS

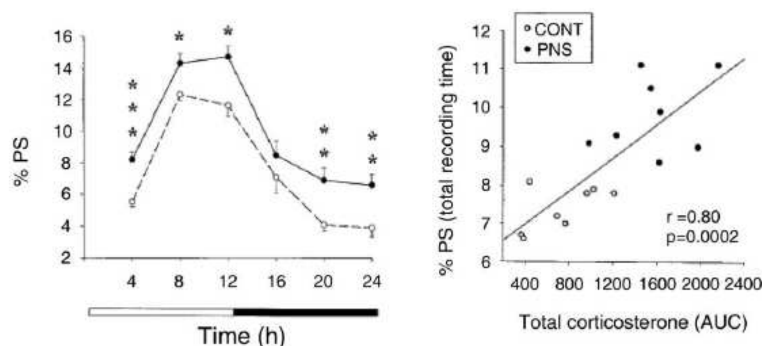

Fig. 5. Distribution per 4 h intervals of vigilance states in CONT and PNRS rats under baseline conditions. PS are expressed as percentage of recording time. PNRS rats show an increased PS over 24 h. ($*p < 0.05$, $**p < 0.01$). Positive correlations between individual stress-induced plasma corticosterone AUC values and amounts of PS expressed as percentage of total recording time in CONT and PNRS rats. *r*, coefficient of Pearson's correlation analysis.

rats showed increased immobility and decreased swimming in the Porsolt test. In another study we assessed, by means of various behavioral tests, the effects of a chronic treatment (once daily in the morning for 21 days) with imipramine, a classical tricyclic antidepressant (10 mg/kg i.p.). Chronic imipramine reversed the immobility behavior, increased hippocampal corticosteroid receptors and reduced cortical 5-HT1A receptor mRNA in PNRS rats. Levels of 5-HT_{1A} receptor mRNA in the cortex were also measured and imipramine decreased levels of these cortical 5-HT_{1A} mRNA in PNRS rats. In order to extend this study we examined the effects of the antidepressant on anxiety related parameters such as the elevated plus maze test and the social test. Interestingly, although showing as expected an increased profile of anxiety such as reduced exploration of the open arm and marked self-grooming behavior, PNRS rats were not affected by the antidepressant treatment in these behavioral parameters [81]. Overall, these results indicate PNRS animals as more sensitive than controls to the effects of antidepressant treatment, and reinforce the idea of the usefulness of this animal's model as an interesting tool for the design and testing of new pharmacological drugs.

5. Role of maternal factors

In order to understand the pathophysiological mechanisms by which stress in the mother reaches the fetus and influences its development, we studied the effects of blocking maternal corticosterone secretion during PNRS on stress-induced corticosterone secretion and hippocampal corticosteroid receptors in adult offspring [82]. Dams were adrenalectomized, at 13 days of pregnancy to block the increased in corticosterone secretion induced by restraint stress. Adrenalectomized dams were implanted with a corticosterone substitutive treatment (100 mg corticosterone pellet containing 50% corticosterone 21-hemisuccinate and 50% cholesterol). These adrenalectomized dams were submitted to a repeated restraint stress during the last 10 days of pregnancy. In a second experiment, we also studied the effects of a corticosterone injection (3 mg/kg) given concomitantly with the restraint stress to the adrenalectomized mothers implanted with a corticosterone pellet. The corticosterone injections in adrenalectomized pregnant rats elicited plasma corticosterone levels approximating those found in intact mothers in response to stress. The results show that hyperactivity of HPA axis in adult offspring induced by prenatal stress is related to the high levels of maternal corticosterone secretion during restraint stress. In fact, blocking stress-induced corticosterone secretion by adrenalectomy with corticosterone substitutive treatment suppresses the prolonged stress-induced corticosterone response and the reduction in hippocampal corticosteroid receptors observed in PNRS rats at 3 months of age (Fig. 6). Furthermore, administration of bolus injections of corticosterone reinstated the effects of prenatal stress [82].

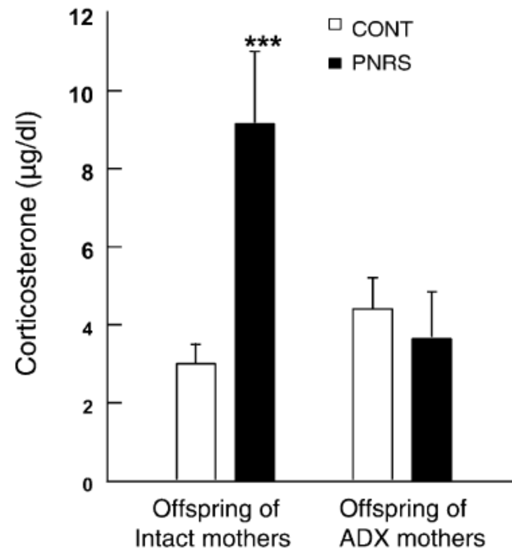

Fig. 6. Plasma corticosterone secretion after restraint stress in CONT and PNRS offspring of mothers with intact or blocked (ADX) stress-induced corticosterone secretion; 120 min after stress, prenatally stressed animals whose mothers were in the intact group had higher corticosterone levels than controls. Prenatally stressed rats whose mothers' corticosterone secretion was blocked did not differ from controls. $***p < 0.001$. Error bars represent SEM.

Results also show that restraint stress increased the basal corticosterone secretion only in the evening in pregnant females [42]. The setting of the circadian rhythmicity of the HPA axis has been shown to depend on maternal factors both during the pre and the postnatal periods [83,84]. The abnormal corticosterone secretion in pregnant females submitted to prenatal stress may then perturb the development of the fetal circadian clock through an action on glucocorticoid receptors, which are notably present in the SCN during early development [85]. It has also been clearly demonstrated that the prenatal dexamethasone exposure was a potent factor that could directly influence the development of the central monoaminergic systems, e.g. noradrenergic, dopaminergic, and serotonergic systems [54].

Taken together, those results suggest that disruption of the normal hormonal response to stress observed in PNRS individuals and the developmental alterations in brain monoamine metabolism depend on stress-induced increase in maternal glucocorticoids during pregnancy. Those findings are in agreement with data showing that exposure of pregnant rats to alcohol (i.e. a procedure that stimulates maternal glucocorticoid secretion) results in a hyperactive HPA axis in the offspring [86,87].

Similarly, non-abortive maternal infections, which increase maternal glucocorticoids [88,89], compromise the development of the fetal brain and alter HPA axis

functioning in the adult [90]. Thus, maternal hormones seem to be good candidates for communication between the dam and developing fetus [91,92]. Interestingly, those data in rats have been confirmed by recent human studies showing that a correlation exists between maternal and fetal plasma cortisol [93]. It should be noted that neuroactive substances other than glucocorticoids could be involved in these long-term prenatal stress effects, including catecholamines, ACTH or beta-endorphin [94,95].

Maternal factors other than hormones may contribute to the long-term changes in HPA reactivity in the offspring. For example, we have shown that chronic restraint stress during pregnancy can persistently affect mother's behavior. After several days of restraint stress the reactivity of the pregnant mothers was altered with an increased of their anxiety measured in the white/dark box test. One month after the end of stress at weaning time, stressed mothers seemed to be more anxious as shown by their reduced time spent in the open arms in the elevated plus maze test [96]. Such behavioral disturbances may affect maternal care during the lactating period and finally contribute to the long-term effects of PNRS on offspring. Indeed, we have also previously shown that early adoption, which increases maternal behavior, prevents PNRS-induced impairments in glucocorticoid feedback [41] (Fig. 7). Furthermore, handling, a postnatal manipulation, results in changes in maternal behavior [97,98] and contributes to improve behavioral performances in handled offspring.

In conclusion, we have shown that PNRS can cause long-term deficits of different biological systems, reproducing alterations of biological parameters that occur in human depression. So the PNRS rats, especially for what concern neuroendocrinological aspects, may represent an appropriate animal model to evaluate pharmacological interventions to the depression.

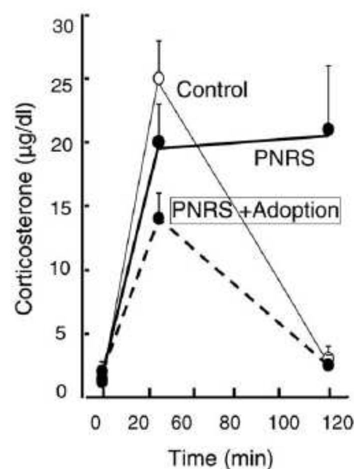

Fig. 7. Plasma corticosterone secretion after novelty exposure in adult CONT, PNRS, and adopted prenatally stressed (PNRS + Adoption). Prenatally stressed adult animals displayed higher corticosterone levels than those of control rats after 120 min of novelty exposure. Adult animals that were both stressed and adopted did not differ from controls. $^{*}p < 0.05$. Error bars shows SEM.

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Erratum :

Maccari S, **Darnaudéry M,** Morley-Fletcher S, Zuena AR, Cinque C, Van Reeth O (2003) Prenatal stress and long-term consequences: implications of glucocorticoid hormones. *Neuroscience Biobehavioural Review* 27: 119-127.

Page 121, Figure 2. The legends of the figure have been inverted. On the left panels, white circle indicated prenatally stressed group (PNRS) and black circle control group (CONT). On the right panel, white bar indicated PNRS animals and black bar CONT rats.

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Short communication

Prenatal stress in rats predicts immobility behavior in the forced swim test
Effects of a chronic treatment with tianeptine

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Abstract

Prenatally-stressed (PS) rats are characterized by a general impairment of the hypothalamo-pituitary-adrenal (HPA) axis and sleep disturbances indicating that this model has face validity with some clinical features observed in a subpopulation of depressed patients. The prolonged corticosterone secretion shown by PS rats in response to stress was positively correlated with an increased immobility behavior in the forced swim test. To investigate the predictive validity of this model, a separate group of animals was chronically treated with the antidepressant tianeptine (10 mg/kg i.p. for 21 days). Such chronic treatment reduced in PS rats immobility time in the forced swim test. These findings suggest that the PS rat is an interesting animal model for the evaluation of antidepressant treatment.
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Theme: Neural basis of behavior

Topic: Stress

Keywords: Stress in utero; Animal model; Corticosterone; Forced swim test; Depression; Tianeptine

In human beings, increasing attention has been shown to the fact that stress exposure in early life predisposes individuals to the development of affective and anxiety disorders in adulthood [16,17,39,46]. Depression, one of the most common human psychological diseases, is characterized by a general lack of motivation and anhedonia. Enhanced activity of the hypothalamo-pituitary–adrenal (HPA) axis [19,37], together with well reported dysfunction of the central serotonergic system [32], constitutes one of the core biological features of depression, at least in a subpopulation of depressed subjects. There is also growing evidence that neuropeptides as CRF [34] and NPY [30] play a role in the pathophysiology of depression, both through a direct action and by interacting with classical neurotransmitters. In addition, cholinergic hyperactivity is observed [20]. Alterations in the sleep–wake cycle [24] and in a variety of circadian rhythms including cortisol rhythm [43], also constitute one of the hallmarks of depressive illness.

In rats, we have previously shown that prenatal stress (PS) can cause a number of alterations that are stable throughout life span, as they can be observed at early [18] as well as at later stages of development [41]. Many of these abnormalities parallel those found in human depressed patients, suggesting that PS rats could have high face validity for an animal model of depression. PS rats present impairment in the feedback inhibition of HPA axis activity [5,15,27,41] display acetylcholine hypersensitivity to stress and CRH challenge [10], and exhibit a phase shift in circadian activity of corticosterone secretion [22,23].

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Finally, we have shown that PS has persistent effects on sleep–wake parameters inducing increased amounts and increased number of episodes of paradoxical sleep at adulthood [13]. More importantly, there is evidence of established correlation between HPA axis disturbances and sleep [13] and behavioral abnormalities in PS rats [42], that leads to suppose that HPA axis dysfunction can constitute one of the mechanism underlying those behavioral disturbances. In the present study, to further validate the PS rat as an animal model for depression, we investigated possible correlation between corticosterone response and behavior in the forced swim test, classically used to evaluate antidepressant efficacy [35]. Then, a chronic treatment with the antidepressant tianeptine, was conducted to assess normalization of the behavioral response.

Many experimental and clinical studies have shown that tianeptine presents a therapeutic efficacy and delay of action similar to that known for common antidepressants, but is devoid of the important side effects characterizing tricyclics [44]. Tianeptine reduces both basal and stress-evoked activity in the HPA axis [7,11]. More recently, tianeptine has been shown to increase neuroplasticity [9,28,31,45] in the hippocampus thus indicating a new mode of action for this drug. These points led us to test this antidepressant in PS rats that present impairment of HPA axis [18,41].

Adult virgin Sprague–Dawley female rats (Iffa Credo, France) were individually housed in the presence of a sexually experienced male for a whole estrous cycle. Pregnant females were then individually housed in plastic cages and randomly assigned to PS or control (C) group. For all experiments, animals were allowed ad libitum access to food and water, and maintained on a regular light/dark cycle (lights on 07:00–19:00 h) with constant temperature (23 °C) and humidity (60%). Prenatal stress was conducted as previously described [5,27]: from day 14 of pregnancy until delivery pregnant female rats were subjected daily to three stress sessions starting at 09:00, 12:00 and 17:00 h, during which they were placed in plastic transparent cylinders (d=7 cm; l=19 cm) and exposed to bright light for 45 min. Control pregnant females were left undisturbed in their home cages.

After weaning, male rats from each experimental group were collectively housed. At 3 months of age, C and PS male rats (n=13 per group) were tested for plasma corticosterone response to a 20-min restraint stress in the morning. Rats were moved to an adjacent room and individually placed in a transparent restraint tube. Blood samples were collected via the tail vein in heparinized tubes as previously described [5,27]. Plasma corticosterone levels were measured using a RIA kit (ICN Biomedicals) with a highly specific corticosterone antibody and a detection threshold of 0.1 μg/100 ml. The intra- and inter-assay coefficients of variation were 5 and 11%, respectively.

After 3 weeks, rats were assessed in the forced swim test. An adapted version of the Porsolt test was used [35]. On pre-test day animals were individually plunged for 15 min into a glass cylinder (d=25 cm; h=59 cm) containing 36 cm of water maintained at 25 °C. They were then removed from the water and allowed to dry in a heated room before returning to their home cages. Then 24 h later (test day), rats were put back into the cylinder for 5 min and latency and duration of immobility behavior (floating in the water with only movements necessary to keep the head above water) were measured. Since pharmacologically diverse antidepressant drugs affect different patterns of active behavior in the forced swim test [26], climbing (active movements with forepaws usually directed towards the walls) and swimming (active swimming motions around cylinder) behavior was also scored.

In a second set of animals, C and PS male rats (n=7 for each treatment group) were injected daily for 21 days either with saline or with 10 mg/kg tianeptine (Institut de Recherches Internationales SERVIER, France). Animals underwent the forced swim procedure on the last 2 days of chronic treatment.

All experiments were conducted in accordance with the principles of laboratory animal care (NIH Publication No.

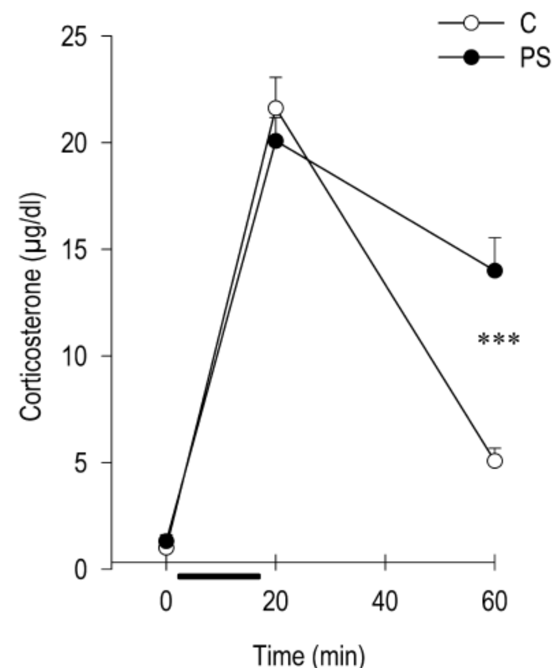

Fig. 1. Effects of prenatal stress on stress-induced corticosterone secretion. Corticosterone secretion induced by 20 min restraint stress (black bar) in C and PS male rats. *** $P<0.001$ Results are expressed as mean±S.E.M.

85-23, revised 1996 or European Communities Council Directive of 1986, 86/609/EEC).

For the first experiment, ANOVA with two levels of group (control vs. prenatal stress) and three levels of time (basal, peak, return to basal level), was used to compare values of corticosterone levels, whereas unpaired Student's t-test was used to analyze data of the forced swim test. Relationships between behavior and stress-induced corticosterone levels were assessed by Pearson's correlation analysis. For the second experiment, ANOVA with two levels of group and two levels of treatment (saline vs. tianeptine) was used followed by Student's t-test when appropriate. Significance level was set at $P<0.05$.

Fig. 1 shows that PS induced, as expected, increased stress-induced corticosterone levels (ANOVA, group by time interaction, $F_{2,48}=19.31$, $P<0.001$). Specifically, prolonged corticosterone values were observed in PS rats 60 min after the initiation of stress procedure (Student's t-test: $t=5.40$, $P<0.001$).

Fig. 2 shows that in the forced swim test PS rats displayed significantly higher amount of immobility ($t=3.04$, $P<0.01$) whereas it decreased duration of swimming behavior ($t=3.62$, $P<0.001$). Climbing behavior was not affected. Also latency to immobility was significantly reduced in PS rats with respect to C group ($t=2.40$, $P<0.05$).

A significant correlation between plasma corticosterone levels and the behavioral scores in this test was observed (see Fig. 2, right panel). Namely, corticosterone levels measured 60 min after stress were positively correlated with duration of immobility ($r=0.52$, $P<0.01$) and negatively with duration of swimming ($r=-0.50$, $P<0.01$).

Fig. 3 shows that chronic treatment with tianeptine significantly affected immobility behavior in the forced swim test (ANOVA group by treatment interaction $F_{1,24}=16.33$, $P<0.001$, and $F_{1,24}=3.96$, $P<0.05$ for duration and latency, respectively). As expected, PS–VEH animals showed increased duration of immobility ($t=2.38$, $P<0.05$) and reduced latency ($t=2.09$, $P<0.05$) with respect to the corresponding control group. Tianeptine markedly

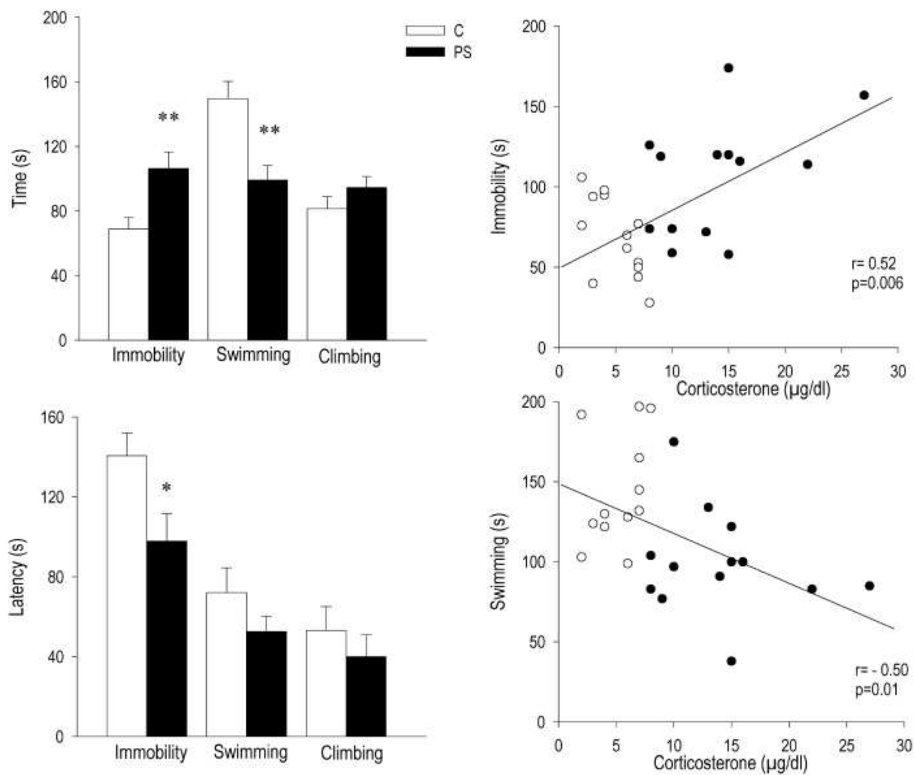

Fig. 2. Left: effects of prenatal stress in the forced swim test. Immobility, swimming and climbing shown by control (C) and PS male rats at 4 months of age. * $P<0.05$ and ** $P<0.01$ vs. controls. Results are expressed as mean±S.E.M. Right: Pearson's correlation analysis between corticosterone levels (60 min after stress) and duration of immobility and swimming in the forced swimming test.

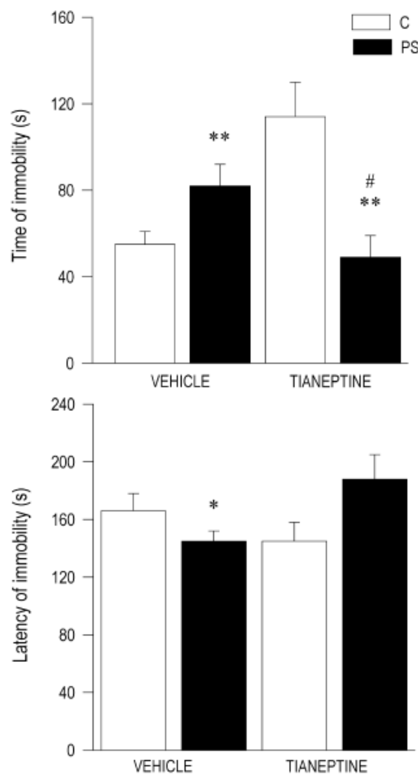

Fig. 3. Influence of chronic tianeptine treatment on immobility and latency in the forced swimming test. C and PS rats were injected for 21 days either with vehicle, either with 10 mg/kg tianeptine. * $P<0.05$ and ** $P<0.01$ PS vs. controls. # $P<0.05$ PS–VEH vs. PS–TIA. Results are expressed as mean±S.E.M.

decreased time spent in immobility in PS rats ($t=2.34$, $P<0.05$) whereas latency was significantly increased ($t=2.74$, $P<0.05$). In the control group, antidepressant treatment increased immobility ($t=3.30$, $P<0.01$) and had no effect on latency.

When considering swimming and climbing activities (see Table 1), ANOVA revealed a significant group by treatment interaction for duration of swimming ($F_{1,24}=15.27$, $P<0.001$). Namely, PS–VEH showed a trend for reduced levels of swimming ($t=2.02$, $P=0.06$) when compared with the corresponding control group. Chronic treatment with tianeptine increased levels of swimming in PS rats ($t=3.38$, $P<0.05$ vs. PS–VEH and $t=3.59$, $P<0.05$ vs. C–TIA) whereas it decreased duration of swimming in controls ($t=2.27$, $P<0.05$ vs. C–VEH). Climbing behavior was not affected by antidepressant treatment.

In the present study, PS male rats previously characterized by their prolonged corticosterone response to an acute stress, displayed high levels of immobility behavior in the forced swim test. Our findings confirm and extend previous data obtained in female rats in other PS models [1,12]. Behavioral performance in the forced swim test at 4 months and corticosterone response at 3 months of age were correlated. Moreover, there is already evidence for the involvement of corticosterone secretion in the behavioral performance in the forced swim test [4,33]. In this view, the finding of such a correlation reinforces our previous data indicating a prominent role of HPA axis in behavioral alterations produced by PS [13,42]. In addition to glucocorticoids, other factors could be involved in the effect of PS on immobility. For instance, the increased amount of REM sleep shown by PS rats [13,36] could be also taken into account, given that REM sleep deprivation, a non-pharmacological intervention known to exert antidepressant effects, induces anti-immobility effect in the forced swim test [3].

Since antidepressant must be administered for at least 2 weeks before clinical benefits are seen in human beings, PS rats were administered tianeptine chronically. Chronic antidepressant treatment significantly reduced their immobility behavior thus indicating predictive validity for this model. The specific increase of swimming behavior after tianeptine treatment is also consistent with what observed with classic antidepressants [21]. Given that tianeptine has shown to attenuate HPA axis activation in stressed animals [11], its restoring action on the hyperactivity of the HPA axis of PS rats has to be hypothesized. Suppression of behavioral deficits by tianeptine has already been reported in other studies [6,8], and its ability to reduce immobility in the forced swim test supports the clinical aspects of its antidepressant action [43].

The increased immobility shown by control treated by tianeptine was not shown in previous reported studies [21], although they assessed effects of an acute treatment of this antidepressant. Our results in control treated animals, may reflect the prediction of an additional anxiolytic activity which has already been published concerning the product [2]. Indeed, anxiolytics and tranquilizers increase the

Table 1
Effects of chronic tianeptine treatment on swimming and climbing in the forced swimming test

	C–VEH	PS–VEH	C–TIA	PS–TIA
Swimming (s)	151.7±13.7	114.3±13.4	108.1±13.4*	168.4±10.1$^{\$\#}$
Climbing (s)	93.3±9.6	103.4±11.2	77.0±8.2	82.6±13.2

Antidepressant treatment decreased duration of swimming behavior in C rats whereas it increased in PS rats. No differences were observed for climbing behavior between the groups. * $P<0.05$ vs. C–VEH; $ $P<0.05$ vs. PS–VEH; # $P<0.05$ vs. C–TIA.

immobility in certain animal models of depression [40]. In this regard, chronic treatment could be an additional variable.

Finally, it should be noted that tianeptine has shown to prevent stress-induced deficits in neuroplasticity [9,28,31,45]. In stress-related neuropsychiatric disorders such as recurrent depressive illness, there is evidence of structural changes in the hippocampus, a brain region extensively studied with regard to stress and antidepressant action [38] and a recent hypothesis links the reversal or prevention of these structural changes to the antidepressant therapy [14,29]. Little is known about effects of exposure to stress during a critical period such as prenatal life, but there is recent evidence of a lifespan reduction of neurogenesis in the hippocampal region [25]. Since tianeptine increases neurogenesis in adult animals [9], its antidepressant effect in PS rats could be related to its regulatory properties in synaptic plasticity. This aspect could be considered for future research.

Although the face validity and predictive validity of the PS rat model requires certainly further validation, the present data show that it could represent an interesting animal model for the testing of antidepressants' efficacy. Moreover, the efficacy of pharmacotherapeutic intervention in psychiatric disorders has often been addressed using non-stressed control animals, whereas, clinically, antidepressants affect neither the mood nor the behavior of non-depressed individuals. Therefore, data obtained in control rats have a limited relevance for understanding both the neurobiological abnormalities observed in depression and the beneficial effects of antidepressant drugs. Our results indicate that the use of early stressed rather than control animals might provide a more useful model in evaluating the mechanism of their action.

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Chronic treatment with imipramine reverses immobility behaviour, hippocampal corticosteroid receptors and cortical 5-HT_{1A} receptor mRNA in prenatally stressed rats

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Abstract

Prenatal stress in the rat induces enhanced reactivity of the hypothalamus–pituitary–adrenal (HPA) axis, disturbances in a variety of circadian rhythms and increased anxiety-like behaviour. Such abnormalities parallel those found in human depressed patients. Prenatally stressed (PS) rats could represent, therefore, an interesting animal model for the evaluation of the efficacy of pharmacotherapeutic intervention in psychiatric disorders that has often been addressed using control animals. In the present study, PS and non-stressed rats were chronically treated with the tricyclic antidepressant imipramine (10 mg/kg i.p. for 21 days) and assessed in the forced swim test. Glucocorticoid receptor binding sites in the hippocampus were measured and 5-HT_{1A} receptor mRNA levels in the frontal cortex were also assessed. PS rats were characterised by increased immobility in the forced swim test, reduced hippocampal corticosteroid receptor binding and increased levels of cortical 5-HT_{1A} mRNA. All these parameters were significantly reversed by chronic imipramine treatment. Conversely, no significant effects were observed for non-stressed rats. All these effects are consistent with the expected pharmacotherapy of depression-like abnormalities in PS rats. These results further indicate that PS rats are a relevant animal model of depression.
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Keywords: Prenatal stress; Depression; Animal model; Forced swim test; Tricyclic antidepressant

1. Introduction

Clinically, the effects of antidepressant drugs do not appear earlier than two or three weeks after the onset of treatment (Quitkin et al., 1996). Nevertheless, only a limited number of studies have employed chronic administration of antidepressants during a clinically relevant time in animals (Reul et al., 1993; Yau et al., 2002a,b). Also, the efficacy of pharmacotherapeutic intervention in psychiatric disorders has often been addressed using control animals, whereas, clinically, antidepressants affect neither the mood nor the behaviour of non-depressed individuals (Gelfin et al., 1998; Wilson et al., 2002; Bonne et al., 1999). Therefore, data obtained in control rats have a limited relevance for understanding both the neurobiological abnormalities observed in depression and the beneficial effects of antidepressant drugs.

Many of the neurobiological abnormalities found in prenatally stressed (PS) rats parallel those found in human depressed patients. For instance, a long-lasting impairment of HPA axis feedback inhibition has been reported in PS rats (Maccari et al., 1995; Koehl et al., 1997, 1999; Vallee et al., 1997; Morley-Fletcher et al., 2003), associated with a life-span impairment of hippocampal neurogenesis (Lemaire et al., 2000). Alterations

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in sleep–wakefulness parameters with increased amounts of paradoxical sleep are also observed in these animals (Dugovic et al., 1999), as well as disturbances in a variety of circadian rhythms such as corticosterone secretion and locomotor activity (Koehl et al., 1997, 1999; Van Reeth et al., 1998). Furthermore, there is evidence of dysfunctions in the serotonergic system at the cortical level (Peters, 1988, 1990), and in other neurotransmitters (Takahashi et al., 1992; Day et al., 1998). Finally, PS rats show a general impairment at the behavioural level including reduced sexual behaviour (Ward, 1983; Rhees et al., 1999) and increased reactivity and anxiety-like behaviour when faced with novelty (Vallee et al., 1997; Weinstock, 2001). In this model, the alterations reported are stable throughout life-span, since they can be observed at early (Henry et al., 1994; Lemaire et al., 2000) as well as later stages of development (Vallee et al., 1999). This aspect makes a dramatic difference with respect to the transitory disturbances observed in other animal models of depression (Yadid et al., 2000; Willner and Mitchell, 2002).

The aim of this study was therefore, to evaluate the effects of a chronic treatment (three weeks) with the tricyclic antidepressant imipramine on PS rats. In order to assess antidepressant efficacy on behaviour, animals were subjected to the forced swim test at the end of antidepressant treatment. Imipramine effectiveness on HPA axis and on the serotonergic system was evaluated in the same animals by measuring, respectively, MR and GR corticosteroid receptor binding in the hippocampus and mRNA encoding 5-HT_{1A} receptors in the prefrontal cortex, a brain region in which those receptors are highly expressed (Lanfumey and Hamon, 2000).

2. Materials and methods

2.1. Animals

Sprague–Dawley nulliparous female rats weighing approximately 250 g were purchased from a commercial breeder (Iffa Credo, France). Animals were kept at constant temperature (22 ± 2 °C), with a 12 h light/dark cycle (lights-on at 08:00 h). Water and food were available ad libitum. For a week after arrival, females were group-housed (four per cage) to co-ordinate their oestrus cycle. They were then placed with a sexually experienced male for a whole oestrous cycle, after which they were housed individually in Plexiglas cages ($50 \times 35 \times 25$ cm). Pregnant females were then randomly assigned to the PS or control group ($n = 12$ in each group).

2.2. Stress procedure

Prenatal stress was conducted as previously described (Maccari et al., 1995; Barbazanges et al., 1996): from day 14 of pregnancy until delivery, pregnant female rats were subjected daily to three stress sessions starting at 09:00, 12:00 and 17:00 h, during which they were placed in plastic transparent cylinders (diameter = 7 cm; length = 19 cm) and exposed to bright light for 45 min. Control pregnant females were left undisturbed in their home cages. Male and female offspring were weaned 21 days after birth, and only male offspring from litters containing 10–14 pups with a comparable number of males and females were used for the experiments. After weaning, male rats from each experimental group were housed in groups of four and maintained under the same social conditions throughout the study. All experiments followed the rules of the European Communities Council Directive 86/609/EEC.

2.3. Antidepressant drug administration

Imipramine hydrochloride (Sigma) was dissolved in saline (0.9%) and injected intraperitoneally (i.p.) at the dose of 10 mg/kg in a volume of 2 ml. Injections were performed once daily for 21 days two hours before lights-off (18:00 h). Controls received injections of vehicle in the same volume (saline, 2 ml/kg). A maximum of two male pups were taken for each experimental condition (vehicle or imipramine) from each litter to remove any litter effect. All the animals were used both for the forced swim test and for neurochemistry assessement. Animals were four months old at the onset of antidepressant treatment.

2.4. Forced swimming test

An adapted version of the forced swim test (Porsolt et al., 1978) was used. A cylindrical container (height = 59 cm; diameter = 25 cm) was filled with 25 °C water up to a level of 36 cm. In the first session (day 1, Pretest), rats were placed in water for a 15 min assessment. Then, they were removed from the water and allowed to dry in a heated room before being returned to their home cages. Twenty-four hours later (day 2, Test), rats were put back into the cylinder for 5 min and latency and duration of immobility behaviour (floating in the water with only movements necessary to keep the head above water) were measured by an observer blind to the rat treatment (Observer v2.0 Noldus, Wageningen, The Netherlands). Since pharmacologically diverse antidepressant drugs affect different patterns of active behaviour in the forced swim test (Lucki, 1997), climbing (active movements

with forepaws usually directed towards the walls) and swimming (active swimming movements around cylinder) behaviour was also scored.

2.5. Neurochemistry

One week after the end of antidepressant treatment, animals were killed by decapitation, brains were quickly removed, cerebral cortex and hippocampus dissected out, frozen on dry ice and stored at –80 °C until neurochemical analysis.

2.5.1. Hippocampal corticosteroid receptor binding

Total corticosteroid (MR + GR) receptors were estimated using a single saturating ligand concentration (for a previous use of this procedure, see Catalani et al., 2002). In order to eliminate endogenous corticosterone, an exchange assay was used as previously described (Casolini et al., 1993, 1997) with some modifications. Aliquots of cytosol (140 μl) were incubated with tritiated corticosterone at a concentration of 40 nM (specific activity 76.5 Ci/mmol; New England Nuclear, Italy). Non-specific binding was determined in the presence of a 500-fold excess of unlabeled corticosterone. Since previous work showed that the affinity of corticosteroid receptors is not affected by exposure to prenatal stress (Henry et al., 1994; Maccari et al., 1995; Barbazanges et al., 1996; Koehl et al., 1997, 1999), in the present study, we only measured the density of corticosteroid receptors.

2.5.2. Quantitative determination of $5\text{-}HT_{1A}$ receptor mRNA in the cortex

The method used to measure mRNAs was based on a competitive reverse transcriptase–polymerase chain reaction (RT-PCR) assay (Siebert and Larrick, 1992), in which mRNAs of the analysed gene are reverse-transcribed and amplified in the presence of a homologous deleted internal standard mRNA using an RT-PCR Access System Kit (Promega, Madison, WI, USA). Quantitative determination of $5\text{-}HT_{1A}$ receptor mRNA in the frontal cortex was performed as described by Le Poul et al. (2000). Reverse transcription (45 min at 48 °C) proceeded with 0.5 μg of total tissue RNA in the presence of standard deleted RNA at increasing dilutions (10^{-6} to 3×10^{-8}). The sequences of upstream and downstream oligonucleotide primers (Albert et al., 1990) were 5′-CTCTACGGGCGCATCTTCAGA-3′ (nucleotides 762–782), and 5′-CCCAGAGTCTTCACCGTCTTC-3′ (nucleotides 1165–1145, respectively). PCR amplification was performed with 1–2 units of Tfl DNA polymerase, 1 mM $MgSO_4$ and 1 pg/μl of each primer for 30 cycles (1 min at 95 °C, 1 min at 58 °C and 1 min at 72 °C). After electrophoretic separation in 2% agarose gel stained with 4% ethidium bromide, both standard and tissue RT-PCR products were quantified with a gel analyzer software (NIH 1.6). RNA levels are expressed as attomoles (amol) of synthetic standard RNA per microgram of tissue total RNA.

2.6. Statistical analysis

Data were analyzed using parametric analysis of variance (ANOVA), with group (Control vs PS) and treatment (vehicle vs imipramine) as between-subject variables, followed by Student's *t*-test. Significance was set at $P < 0.05$.

3. Results

3.1. Effects of imipramine on behaviour in the forced swim test (Fig. 1)

ANOVA conducted on both the Pretest and the Test day indicated no effect of group nor of day, whereas a marked effect of antidepressant treatment was found ($F_{1,40} = 6.40$, $P < 0.05$). Separate analysis on Test day revealed on the other hand, a main effect of group ($F_{1,40} = 4.59$, $P < 0.05$) and of treatment ($F_{1,40} = 8.05$, $P < 0.01$) although the interaction was not significant. PS saline-treated rats spent more time being immobile than saline-treated control animals (*t*-test: $t = -2.56$, df = 20; $P < 0.05$) and chronic imipramine significantly reversed their immobility time (*t*-test: $t = 2.79$, df = 20; $P < 0.05$). No effects of imipramine were observed in control animals.

No differences between saline-treated PS and saline-treated control groups were observed for climbing or

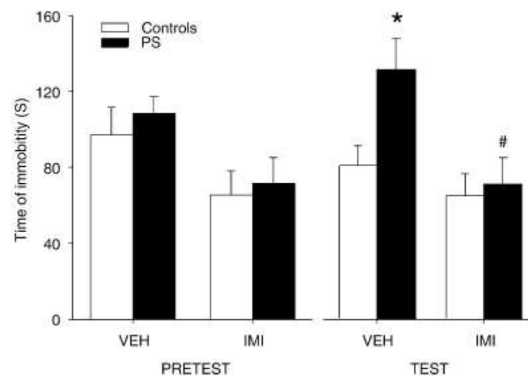

Fig. 1. Effect of chronic imipramine treatment (10 mg/kg i.p. daily, 21 days) on time spent in immobility (mean ± SEM) in the forced swimming test for control and PS adult rats on the pretest and test day ($n = 10$–12 each treatment group). On test day, prenatal stress increased immobility time whereas imipramine treatment reduced it down to the value of control rats. Imipramine had no effect in the latter animals. * $P < 0.05$ vehicle-treated PS animals vs vehicle-treated control group; # $P < 0.05$ imipramine-treated PS group vs vehicle-treated PS group.

for swimming activities on Test day. Conversely, a main effect of treatment was found for climbing behaviour ($F_{1,40} = 10.08$, $P < 0.01$). Separate analysis indicated that antidepressant treatment increased climbing in PS rats (82.9 ± 15.1 s for vehicle vs 129.88 ± 16.2 s for imi-treated PS rats, $t = 2.59$, df = 20; $P < 0.05$) whereas it had no effect in the control group (95.74 ± 20 for vehicle vs 128.29 ± 17.73 s for imi-treated control rats).

3.2. *Effects of imipramine on hippocampal corticosteroid receptor density (Fig. 2)*

In the hippocampus, ANOVA revealed a significant group by treatment interaction ($F_{1,26} = 4.20$, $P < 0.05$). Specifically, reduced levels of total (MR + GR) corticosteroid receptor binding were measured for PS saline-treated animals (*t*-test: $t = 2.08$, df = 10; $P < 0.05$) which were significantly increased following antidepressant treatment (*t*-test: $t = -2.5$, df = 10; $P < 0.05$). No significant effects of the antidepressant were observed for control groups.

3.3. *Effects of imipramine on cortical 5-HT$_{1A}$ receptor mRNA levels (Fig. 3)*

ANOVA revealed a main effect of group ($F_{1,18} = 4.48$, $P < 0.05$) and of treatment ($F_{1,18} = 7.07$, $P < 0.05$), although their interaction was almost significant

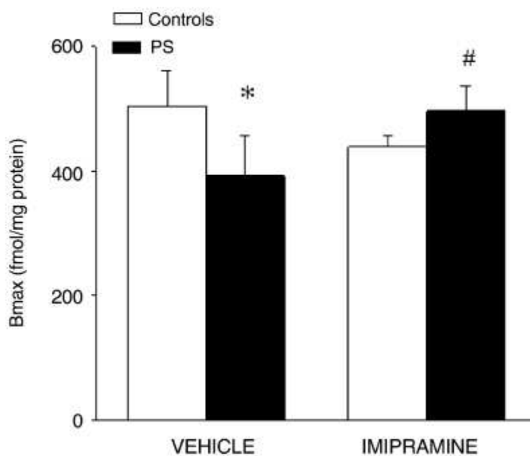

Fig. 2. Effect of chronic imipramine treatment (10 mg/kg i.p. daily, 21 days) on binding capacities (mean ± SEM: Bmax, fmol/mg/prot.) of total corticosteroid (MR + GR) hippocampal receptors in control and PS adult rats ($n = 6$ for each treatment group). Prenatal stress reduced corticosteroid receptors density whereas imipramine treatment increased it up to the value of control rats. Imipramine had no effect in the latter animals. * $P < 0.05$ vehicle-treated PS animals vs vehicle-treated control group; # $P < 0.05$ imipramine-treated PS group vs vehicle-treated PS group.

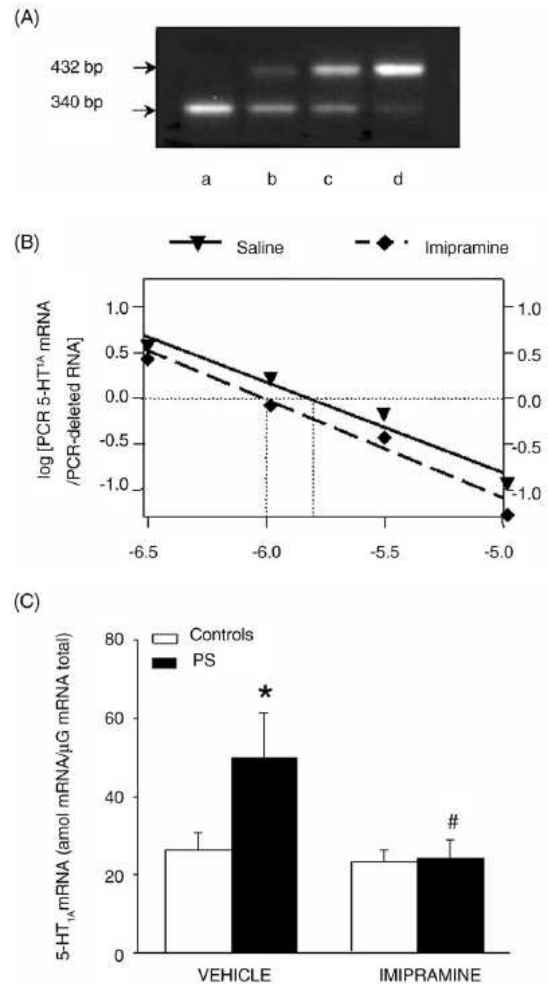

Fig. 3. Quantitation of 5-HT$_{1A}$ receptor mRNA by competitive RT-PCR in the frontal cortex. (A) Example of electrophoretic separation of RT-PCR products in 2% agarose gel stained with ethidium bromide of RT-PCR products. a, b, c, d represent the dilutions of synthetic deleted mRNA (initial concentration: 0.72 µg/µl; a: 10^{-5}; b: 3×10^{-5}; c: 10^{-6}; d: 3×10^{-7}). (B) Plot for the quantification of PCR products: the logarithmic ratio of the amounts (OD measurements) of the specific mRNA (432 bp) over those of the synthetic deleted RNA (340 bp) is plotted as a function of the logarithm of each serial dilution of the synthetic deleted RNA. The line intersection with the *x*-axis gives the equivalent dilution of the synthetic deleted RNA, thus the equivalent amount of specific 5-HT$_{1A}$ mRNA in the rat. (C) Effect of chronic imipramine treatment (10 mg/kg i.p. daily, 21 days) in control and PS adult rats ($n = 5$ for each group). Values are expressed as attomole-mRNA per microgram of total RNA (mean ± SEM). Prenatal stress 5-HT$_{1A}$ mRNA expression increased 5-HT$_{1A}$ mRNA expression whereas imipramine treatment reduced it down to the value of control rats. Imipramine had no effect in the latter animals. * $P < 0.05$ vehicle-treated PS animals vs vehicle-treated control group; # $P < 0.05$ imipramine-treated PS group vs vehicle-treated PS group.

($F_{1,18} = 3.92$, $P = 0.06$). 5-HT_{1A} receptors mRNA levels in the frontal cortex were significantly higher (+89%) in PS saline-treated rats than in saline-treated controls (t-test: $t = -2.4$, df = 10; $P < 0.05$). A marked reduction (−51% as compared to the saline-treated PS group) to levels equal to controls was observed after chronic imipramine treatment in the PS group (t-test: $t = 2.51$, df = 9; $P < 0.05$). In contrast, no significant effects of imipramine were observed in the control group.

4. Discussion

The aim of this study was to investigate the predictive validity of the PS rat as an animal model of depression by treating prenatally stressed animals chronically for three weeks with the tricyclic antidepressant imipramine and, measuring antidepressant efficacy on some parameters of behaviour, HPA axis activity and serotonergic system. Our results indicate that PS rats showed reduced immobility in the forced swim test and significant modifications of the cortical 5-HT_{1A} system as well as hippocampal corticosteroids in response to imipramine treatment. Interestingly, imipramine had no effect on control animals.

PS rats showed more immobility behaviour than controls in the forced swim test. Chronic imipramine significantly reduced immobility behaviour in PS rats whereas it had no effect in controls. Our results extend previous work on PS rats (Alonso et al., 1991, 1999; Drago et al., 1999; Frye and Wawrzycki, 2003) and are also in accordance with a recent study (Morley-Fletcher et al., 2003) showing reduced immobility following chronic tianeptine treatment in prenatally stressed males.

The forced swim test is a behavioural paradigm that is used as a screening test for antidepressant activity in rodents (Porsolt et al., 1978). Although this paradigm has one of the highest degrees of pharmacological predictive validity in terms of identifying antidepressants, a concern exists about the interpretation of immobility during forced swimming as reflecting a failure of the animal to cope as a successful mean of passive behavioural adaptation aimed at conserving energy in an inescapable situation (West, 1990). There are several evidences of a direct involvement of elevated levels of corticosterone in this behavioural response (Mitchell and Meaney, 1991; Korte et al., 1996; Morley-Fletcher et al., 2003). In this regard, we think that the immobility behaviour observed in PS rats in this study and a previous one (Morley-Fletcher et al., 2003) could be due to the increased corticosterone response after a stressful exposure such as the forced swim test.

In this study, we were interested to use this test mainly for its efficacy in screening antidepressant activity. Also, the forced swimming test not only detects antidepressant drugs with a common core behaviour (immobility) but also patterns of active behaviour in this test, such as climbing and swimming, revealing multiple components of antidepressant responses that are responsive to specific drug classes (Lucki, 1997). Drugs acting on serotonin and noradrenaline such as imipramine increase climbing behaviour and we show a specific increase in climbing behaviour following imipramine treatment in PS rats. This supports the action of antidepressant on the serotonergic system as well as the noradrenergic systems, both known to be impaired in those animals (Peters, 1988; Takahashi et al., 1992).

In this regard, we show for the first time that prenatal stress induces an upregulation of cortical 5-HT_{1A} mRNA. Dysregulation of the serotonergic system as well as of the HPA axis are implicated in the pathophysiology of depression and anxiety (Meltzer et al., 1987). Reciprocal influences do exist between the serotonin system and the HPA axis (de Kloet et al., 1986; Meijer and de Kloet, 1994). Herein, prenatal stressed animals showed a reduction in the binding capacity of hippocampal corticosteroid receptors. These results confirm and extend previous data showing a downregulation of corticosteroid receptors (Maccari et al., 1995; Barbazanges et al., 1996). Following imipramine treatment, corticosteroid receptor density increased, whereas the expression of cortical 5-HT_{1A} receptors at the mRNA level was downregulated by this treatment. Such changes are consistent with the well-reported efficacy of antidepressants to inhibit some changes evoked by glucocorticoids as well as hyperactivity of HPA axis often observed in depression (Seckl and Fink, 1992; Reul et al., 1993; Holsboer and Barden, 1996). Previous studies have shown that the normal responsiveness of corticosteroid receptors can be restored by chronic treatment with antidepressant drugs, notably those acting through the serotonin system (Semont et al., 2000). It is also suggested that 5-HT_{1A} receptors participate in the mechanism of action of this type of antidepressants (Srinivas et al., 2001).

In the present study, we reported a downregulation of cortical 5-HT_{1A} receptors only in PS rats and not in control rats. It has to be considered that PS animals were killed a week after cessation of imipramine treatment. This protocol was chosen in order to evidence long-lasting effects of the antidepressant. In this regard, the persistent difference in the response to antidepressant observed in prenatal stress animals compared to the control group after one week of cessation common enhances the higher sensitivity to pharmacological treatment in this animal model.

In accordance with our results, works conducted on a genetic animal model of depression such as the Flinder Sensitive Line (FSL, Overstreet et al., 1996),

indicate a higher density of postsynaptic 5-HT_{1A} receptors in the frontal cortex, together with greater immobility in the forced swim test (Gonzalez et al., 1998). Moreover, FSL rats display enhanced response to chronic antidepressant treatment in comparison to control rats both in the forced swim test (Overstreet et al., 1995), and at the level of the serotonergic system (Zangen et al., 1997).

Overall, the findings of an enhanced response to antidepressant treatment in PS rats in comparison to control rats underlie the importance of using appropriate animal models. There is evidence that in human patients significant mood-elevating and other psychological effects of antidepressants appear to be induced only when symptomatic targets exist (Wilson et al., 2002; Bonne et al., 1999). Frequently, rodent models have good predictive validity for antidepressants but they are often shown to be sensitive to acute administration of these compounds whereas common symptoms of depression are only ameliorated after chronic drug treatment. In the present study, the parallel normalisation of some of the behavioural and neurochemical abnormalities in PS rats after a chronic imipramine treatment provides evidence of the validity of this model for the study of antidepressants' action.

Our study was conducted on male subjects. In this regard, there is still very few experimental evidence of the effects of prenatal stress on females on behavioural or neurochemical parameters linked to depression (Alonso et al., 1991; Schmitz et al., 2002; Frye and Wawrzycki, 2003) although in humans depressive disorders affect more female than male subjects. This issue should be considered for future studies.

In conclusion, the present study indicates that the PS rat model has sufficient construct, face, and predictive validity to become an interesting "pathological" model for research on pharmacotherapeutic approaches of depression-like disturbances.

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Prenatal stress alters Fos protein expression in hippocampus and locus coeruleus stress-related brain structures

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Paraventricular nucleus

Summary Prenatal stress (PS) durably influences responses of rats from birth throughout life by inducing deficits of the hypothalamo-pituitary-adrenal (HPA) axis feedback. The neuronal mechanisms sustaining such alterations are still unknown. The purpose of the present study was to determine whether in PS and control rats, the exposure to a mild stressor differentially induces Fos protein in hippocampus and locus coeruleus, brain areas involved in the feedback control of the HPA axis. Moreover, Fos protein expression was also evaluated in the hypothalamic paraventricular nucleus (PVN) that reflect the magnitude of the hormonal response to stress. Basal plasma corticosterone levels were not different between the groups, while, PS rats exhibited higher number of Fos-immunoreactive neurons than controls, in the hippocampus and locus coeruleus in basal condition. A higher basal expression of a marker of GABAergic synapses, the vGAT, was also observed in the hypothalamus of PS rats. Fifteen minutes after the end of the exposure to the open arm of the elevated plus-maze (mild stress) a similar increased plasma corticosterone levels was observed in both groups in parallel with an increased number of Fos-immunoreactive neurons in the PVN. Return to basal plasma corticosterone values was delayed only in the PS rats. On the contrary, after stress, no changes in Fos-immunoreactivity were observed in the hippocampus and locus coeruleus of PS rats compared to basal

Abbreviations AP-1, transcription factor activator; AVP, arginin-vasopressin; CA3, amon horn 3; C, control; CRH, corticotropin-releasing hormone; DG, dentate gyrus; GR, glucocorticoid receptor; HPA axis, hypothalamo-pituitary-adrenal axis; MR, mineralocorticoid receptor; PaMP, PVN, medial parvocellular part; PaLM, PVN, lateral magnocellular part; PS, prenatal stress; PVN, paraventricular nucleus; vGAT, GABA vesicular transporter; vGlut1, vesicular glutamate transporter 1.

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condition. After stress, only PS rats presented an elevation of the number of activated catecholaminergic neurons in the locus coeruleus. In conclusion, these results suggest for the first time that PS alters the neuronal activation of hippocampus and locus coeruleus implicated in the feedback mechanism of the HPA axis. These data give anatomical substrates to sustain the HPA axis hyperactivity classically described in PS rats after stress exposure.
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1. Introduction

In rats, development of the central nervous system can be durably altered by deleterious perinatal environment, predisposing the organism to long-term behavioural abnormalities (for review, Chapillon et al., 2002; Maccari et al., 2003; Weinstock, 2005). Adult PS rats display an increased 'anxiety' (Vallée et al., 1997) and depressive-like behaviour (Morley-Fletcher et al., 2003, 2004). The HPA axis activity was associated with these behavioural alterations (Vallée et al., 1997, 1999). In fact, adult PS rats show increased stress-induced plasma concentrations of adrenocorticotrophic hormone (ACTH) (Takahashi and Kalin, 1991; McCormick et al., 1995), prolonged stress-induced corticosterone secretion and decreased binding capacity of hippocampal corticosteroid receptors (Weinstock et al., 1992; Maccari et al., 1995) suggesting a reduced feedback mechanisms of HPA axis in PS rats. An increased basal corticosterone levels was observed in the evening before the light-off (Koehl et al., 1999). Efferent limbic fibres as well as ascendant brainstem inputs are known to modulate the HPA axis activity (for review Herman and Cullinan, 1997) and PS rats show reduced noradrenaline content and increased noradrenaline turnover in the hippocampus and neocortex (Takahashi et al., 1992; Muneoka et al., 1997). Moreover, PS rats exhibit alterations of the monoaminergic system and cholinergic neurones (Peters, 1986; Poland et al., 1995; Day et al., 1999; Morley-Fletcher et al., 2004). These data strengthens evidence of the existence of deficient HPA axis feedback mechanisms in PS rats thought an altered neurotransmission. The HPA axis activity is under the control of interacting brain structures that generate adapted responses to stress, as hippocampus and locus coeruleus. Hippocampal neurons exert a tonic inhibitory control of corticosterone release, act directly on the hypothalamic paraventricular nucleus (PVN) neurons (Sapolsky et al., 1984; Cullinan et al., 1993) and/or indirectly via neurons located close to the PVN that regulate the corticotrophin releasing factor (CRH) and arginin-vasopressin (AVP) release (Herman et al., 2002). Moreover, the locus coeruleus, a brainstem catecholaminergic structure that project to both hippocampus and PVN, is also directly or indirectly involved in the regulation of the HPA axis (Ziegler et al., 1999; Daftary et al., 2000). For example, a lesion of noradrenergic ascending pathways reduces corticosterone secretion in rats exposed to novelty and increases the number of hippocampal mineralocorticoid receptors (Maccari et al., 1992). In the PVN, neurosecretory neurons (CRH and AVP) integrate the inhibitory and excitatory signals coming from hippocampus and locus coeruleus, and target ACTH release in order to mediate the stress response. Furthermore, literature data suggest that a substantial proportion of PVN excitation and inhibition is gated, respectively, by glutamatergic and GABAergic neurons located in the peri-PVN region, as well as by local intrahypothalamic neurons (Boudaba et al., 1996; Bowers et al., 1998; Herman et al., 2002, 2004).

Pace et al. (2005) using a cellular marker of activation, the expression of Fos protein, has recently demonstrated, that a novel experience increased the number of Fos-immunoreactive neurons in stress-related structures like hippocampus, cortex and PVN. In PS rats, the neuronal circuit sustaining HPA axis alterations have not been fully understood. In this context, we examined whether PS induced differential expression of Fos protein in the PVN and in two structures principally involved in the feedback mechanisms of the HPA axis, the hippocampus and locus coeruleus, in both basal condition and after exposure to a mild stressor. We determined also whether PS affected catecholaminergic activated neurons located in the locus coeruleus. Finally, we evaluated the hypothalamic content in GABA and glutamate vesicular transporters that are directly correlated to GABA and glutamate concentrations (McIntire et al., 1997; Takamori et al., 2000) and reflects their involvement in the control of PVN neurons activity.

2. Methods

All experiments were conducted in accordance with the principles of laboratory animal care (European Communities Council Directive of 1986, 86/609/EEC).

2.1. Housing conditions

Adult virgin Sprague–Dawley female rats ($n=10$, Charles River, L'Arbresle, France) weighting 240 g were housed in groups of 5 per cage, for 2 weeks before mating, in order to coordinate their oestrus cycle. They were then housed overnight separately with a sexually experienced male (400 g). The following morning, a vaginal smear was performed on each female in order to determinate their date of fecundation. Every negative female was then placed back in the common housing cage for the day and given back to the male the following night until sperm were seen in the vaginal smear. Pregnant rats were then randomly assigned to prenatal stress (PS) or control (C) groups, individually housed in plastic cages, allowed ad libitum access to food and water, and maintained on a 12 h light/dark cycle (light on at 0700 h am) with constant temperature and humidity.

2.2. Prenatal stress paradigm

The PS procedure was performed every day from day 11 of pregnancy until delivery. As previously described (Maccari et al., 1995; Morley-Fletcher et al., 2003), pregnant females were restrained for 45 min three times a day during the light phase, in a transparent plastic cylinder (7 cm in diameter and 19 cm long) exposed to a bright light. Control females were left undisturbed in their home cages. Biological mothers raised the offspring until weaning, 21 days after birth. Only litters of 8–13 pups with similar sex ratio were kept for the study (Chapman and Stern, 1979) to standardize postnatal conditions and adequate milk supply. A maximum of two male pups was used from each litter to prevent from any 'litter effects'. Male rats from each experimental condition were housed in groups of five until they reached 2 months of age. They were then housed in groups of two until the experiments.

2.3. Experimental procedures

The mild stressor consisted of an exposure of adult PS and C rats to the open arm of the elevated plus maze for 5 min (Landgraf et al., 1999). Two weeks before the beginning of the experiment, rats had been manipulated every day in order to minimize any effect of handling.

Plasma corticosterone assay. Adult PS ($n=27$) and C ($n=26$) rats (4-month-old) were exposed or not to the open arm (open arm condition). They were subdivided in four groups corresponding to different times of blood samplings before and after the exposure to the mild stress of 5 min: (1) basal condition (PS rats, $n=6$; C rats, $n=6$); (2) 15 min after stress (T15; PS rats, $n=7$; C rats, $n=6$); (3) 60 min (T60; PS rats, $n=7$; C rats, $n=7$) and (4) 120 min after stress (T120; PS rats, $n=7$; C rats, $n=7$). The T120 group was used to perform the immunocytochemical study. In addition, 6 PS rats and 6 C rats were left undisturbed (basal condition). For each animal, blood samples were collected by the tail in tubes containing 5 ml EDTA (0.1% w/v). All experiments were performed between 0900 and 1200 h to avoid circadian variations of plasma corticosterone concentrations. Plasma corticosterone levels were measured with a radioimmunoassay kit (Kit ImmunChem TM, ICN Pharmaceuticals, Orsay, France) using a highly specific corticosterone antiserum. The minimum level of detection was 0.2 mg/100 ml and the intra- and interassay coefficients of variation were, respectively, 5 and 9%.

2.4. Immunohistochemical procedure

We used rats from basal condition (PS rats, $n=6$; C rats, $n=6$) and T120 group (PS rats, $n=6$; C rats, $n=6$). Since Fos protein expression reaches a maximum between 90 and 120 min after the stress (Krukoff, 1999), only the T120 after stress group was used to perform the immunocytochemical study related to the exposure to the mild stressor. After blood samplings, rats were immediately anaesthetized with sodium pentobarbital (60 mg/kg) and perfused with 200 ml saline (NaCl, 0.9%, w/v), followed by a cold solution of phosphate buffer (PB, 0.1 M, pH 7.4) containing paraformaldehyde (400 ml; 4%). Brain was removed, postfixed for two hours at 4 °C in the same fixative, then immersed overnight at 4 °C in PB containing 20% sucrose. Frontal sections (50 μm) of the brain and medulla were cut on a cryotome, rinsed in PB and processed for the immunocytochemical detection of Fos protein. Sections of each group were incubated for 48 h in PB containing 0.2%Triton X-100 and the primary Fos rabbit antiserum (Santa Cruz, USA; 1:10,000, sc-52). Then, the biotinylated donkey anti-rabbit serum (Jackson Immunoresearch, Immunotech, Marseille, France; 1:500) in PB containing 0.1%Triton X-100 was applied for 90 min. Finally, sections were revealed with the 3-3′diaminobenzidine glucose oxidase protocol (Shu et al., 1988), as previously detailed by Viltart et al. (2003).

In a second step, for catecholaminergic staining, the catecholamine synthetic enzyme tyrosine hydroxylase (TH) (Chemicon Int., Temecula, USA) was immunocytochemically detected on the same sections. The primary TH sheep antiserum was used

at the dilution of 1:2000 for 24 h at room temperature. The peroxidase activity was visualised using only 3-3′diaminobenzidine. The specificity of the immunostaining was assessed by omission of the primary or secondary antibody from the protocol. After processing, tissue sections were mounted onto gelatine-alum-chrome coated slides, dehydrated, cleared in toluene and coverslipped with Eukitt (Poly Labo, Strasbourg, France). Brain sections belonging to C and PS groups were treated in parallel to avoid differences due to the technical procedure.

2.5. Morphological analysis

Neurons immunoreactive for Fos protein (Fos-IR) exhibit a dark-brown nucleus, TH-immunoreactive (TH-IR) neurons had a brown cytoplasm and double labelled (Fos-IR/TH-IR) neurons show a brown cytoplasm containing a dark-brown nucleus. Fos immunoreactive nuclei were manually counted under a light microscope (Leica, Germany) by an independent experimenter. The sections levels were standardised according to the atlas of Paxinos and Watson (1997). The PVN was counted in the midhypothalamus level from −1.8 to −2.12 mm posterior to bregma. The dorsal hippocampus (CA1, CA2, CA3, DG) was counted from level −2.13 to −3.80 mm. Finally, all Fos-IR nuclei, TH-IR and Fos-IR/TH-IR neurons were counted in the locus coeruleus from level −9.3 to level −10.30 mm. A first observation was done at ×10 objective to identify the structure. Then, neurones were counted at the ×20 and/or ×40 objective to check the Fos labelling. For all brain regions considered, the total number of Fos-IR neurons was obtained by counting them bilaterally on each slice. For some rats, a slight difference exists between the number of collected slices. Thus, to obtain homogeneous values between rats, the total number of Fos-IR neurons counted for one region was divided by the total number of sections used.

2.6. Western blotting

Ten adult rats (C rats $n=5$ and PS rats $n=5$) were used. The hypothalamic area surrounding and including the PVN (from level −1.3 to −2.30 mm posterior to Bregma, according to the atlas of Paxinos and Watson, 1997) was rapidly dissected on dry ice, immediately frozen in liquid nitrogen and stored at −80 °C until use. Hypothalamus were homogenised at 4 °C in the buffer (320 mM sucrose, 5 mM Hepes-NaOH pH7.4 and 0.1 mM EDTA) containing a cocktail of protease inhibitors (0.2 mM phenylmethylsulfonyl fluoride, 1 mg/ml aprotinin, and 0.5 mg/ml leupeptin). Protein concentrations within homogenates were measured by BCA assay (Pierce, Germany) and adjusted to 2 mg/ml in Laemmli buffer (Laemmli, 1970) in order to load 40 mg/sample. Proteins were separated onto 12% polyacrylamide gel electrophoresis containing SDS (SDS-PAGE), and then transferred onto nitrocellulose membranes. In order to identify the proteins of interest, molecular size markers (110–21.4 kDa range, BioRad) were used in parallel. For the immunoblotting, membranes were blocked for 30 min at room temperature with 5% non-fat dried milk, 5% normal goat serum and 0.1% Tween 20 in Tris buffered saline, then incubated for 1 h at room temperature with one of the following primary antibodies: polyclonal vesicular glutamate transporter 1 (anti-vGlut1, 1:10,000 in blocking buffer, Synaptic System, Germany) or GABA vesicular transporter (polyclonal anti-vGAT, 1:2000 in blocking buffer, Chemicon, Germany). After, 5 min washes in blocking buffer, membranes were then incubated for 1 h at room temperature with an anti-rabbit HRP-conjugated antibody (1:5000 in blocking buffer, Bio-Rad, Germany). Finally, immunoreactivity was visualised with enhanced chemoluminescence (Amersham Biosciences, Germany) and was quantified by densitometry (Molecular Analyst Software; Bio-Rad, France). After each vGlut1 or vGAT immunodetection, tubulin was revealed on the same membrane with a monoclonal anti-tubulin primary antibody (1:20,000 in blocking buffer, Sigma, Germany) and an anti-mouse HRP-conjugated secondary antibody (1:5000 in blocking buffer, Biorad). Results were expressed as a ratio of optic densities (OD): OD vGlut1/OD tubulin or OD vGAT/OD tubulin.

2.7. Statistical analysis

Data are expressed as mean±SEM. The Shapiron-Wilk test was first used to evaluate the normality of the population distribution. Since the populations studied were not normal ($P<0.05$), values were analysed with the non-parametric statistical Mann-Whitney test (for within-group differences). Significance was set at $P<0.05$.

3. Results

3.1. Plasma corticosterone levels after exposure to the open arm

In basal condition, no significant difference was found between C and PS rats. Exposure to the open

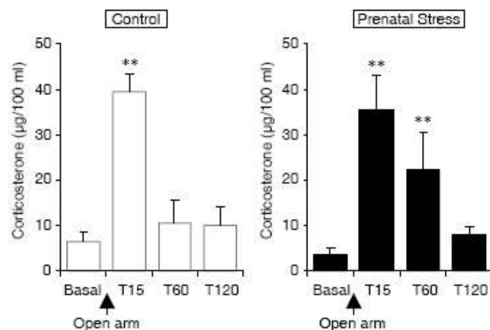

Figure 1 Mean plasma corticosterone level (±SEM) for control (C) and prenatal stressed (PS) rats, 15 min (T15; PS rats, $n=7$; C rats, $n=6$), 60 min (T60; PS rats, $n=7$; C rats, $n=7$), 120 min (T120; PS rats, $n=7$; C rats, $n=7$) after the end of the open arm exposure or just after removing the rats from their home cage for the basal group (basal, PS rats, $n=6$; C rats, $n=6$). Data were compared using a Mann-Whitney U-test: **, $P<0.01$, basal vs open arm.

arm induced a significant increase in the plasma corticosterone levels in both groups 15 min after the end of the exposure ($U=0$, $P<0.01$ for the C rats; $U=1$, $P<0.01$ for the PS rats; Fig. 1). This increase was followed by a delayed return to basal values in the PS rats: at T60 corticosterone level reached the baseline in C rats while in PS rats it remained elevated ($U=4$, $P<0.01$, T60 vs basal values), declining to baseline only at T120 min after stress (Fig. 1).

3.2. Expression of Fos protein in the PVN

Within the PVN, Fos-IR neurons were mostly found in the medial parvocellular region (PaMP) in basal and after stress conditions (Fig. 2A). In basal condition, no significant difference was noted between C and PS rats (Fig. 2B). Both in C and PS rats, exposure to the open arm significantly increased the number of Fos-IR neurons (respectively, $U=0$, $P<0.005$, $U=2.5$, $P<0.01$; Fig. 2B). Moreover, this stressor activated significantly more neurons in C than in PS rats ($U=2$, $P<0.01$; Fig. 2B). Considering the dorsal part of the PaMP, both C and PS rats showed a similar increase of the Fos-IR neurons number after exposure to the open arm ($U=5.5$, $P<0.05$ for C rats, $U=2$, $P<0.01$ for PS rats; Fig. 2C). In the ventral part of the PaMP, increased number of Fos-IR neurons was observed only in C rats after stress ($U=0$, $P<0.005$; Fig. 2D). Furthermore, the number of activated neurons after the open arm exposure was higher in C rats compared to PS rats ($U=3$, $P<0.05$; Fig. 2D).

3.3. Western blot analysis in the hypothalamus

Since vGlut-1 and vGAT are responsible for uptake and storage of, respectively, glutamate and GABA by synaptic vesicles in the central nervous system, we measured their respective expressions in the hypothalamus by western blot. No significant changes were observed for vGlut1 in the PVN and the surrounding hypothalamic area between C and PS rats. However, PS rats displayed a higher expression of vGAT in the same region ($U=0$, $P<0.01$; Fig. 3).

3.4. Expression of Fos protein in the hippocampus

Within the hippocampus, Fos-IR neurons were located in the pyramidal layer of the Ammon horns CA1, CA2, and CA3 and in the granular layer of the dentate gyrus in basal and after stress conditions (Fig. 4A). In basal condition, considering the whole hippocampus, PS rats exhibited a higher number of Fos-IR neurons than C rats ($U=0$, $P<0.005$; Fig. 4B). The exposure to the open arm had no effect on the activation of PS hippocampal neurons. On the contrary, the C rats showed a significant increase in the number of Fos-IR neurons after such a stressor ($U=0$, $P<0.005$, Fig. 4B). More specifically, in basal condition, both in CA3 and dentate gyrus, PS rats exhibited a higher number of Fos-IR neurons than C rats ($U=5$, $P<0.05$ in CA3, $U=2$, $P<0.01$ in dentate gyrus; Fig. 4C and D). Exposure to the open arm induced an increase in the number of Fos-IR neurons only in the C rats ($U=1$, $P<0.01$ in CA3, $U=0$, $P<0.005$ in dentate gyrus; Fig. 4C and D). After this stress, the number of activated neurons tented to be higher in the CA3 of C rats compared to PS rats ($U=7$, $P=0.07$; Fig. 4C).

3.5. Expression of Fos protein in the locus coeruleus catecholaminergic neurons

Within the locus coeruleus, Fos-IR neurons were scattered throughout the nucleus in basal and after stress conditions (Fig. 5A). In basal condition, PS had more Fos-IR neurons compared to control ($U=0$, $P<0.005$; Fig. 5B). Exposure to the open arm did not modify the number of Fos-IR in the locus coeruleus of PS rats, whereas a significant increase was noted in C rats ($U=0$, $P<0.005$; Fig. 5B). Catecholaminergic neurons were globally found

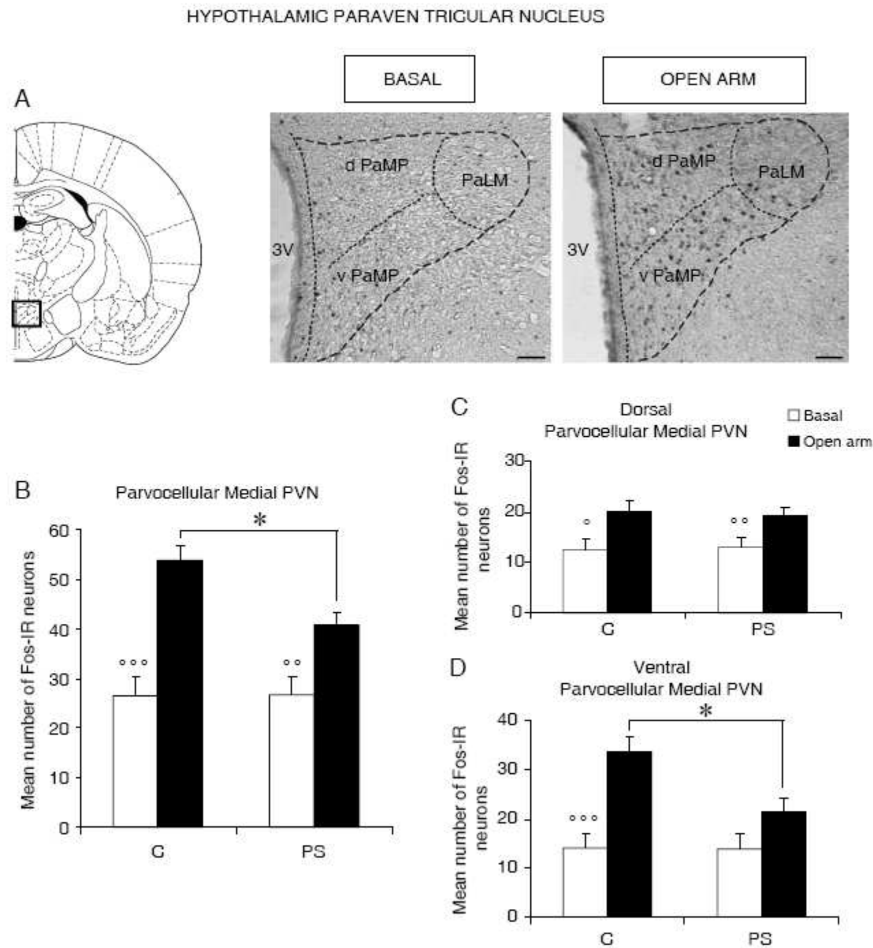

Figure 2 (A) Bright-field photomicrographs showing the distribution of Fos-immunoreactive (Fos-IR) neurons in the hypothalamic paraventricular nucleus (PVN) in control (C) rats in basal condition and after exposure to the open arm. Fos-IR neurons were mostly found in the dorsal and ventral regions of the medial parvocellular part of the PVN (respectively, d PaMP and v PaMP) and in the lateral magnocellular part of the paraventricular hypothalamic nucleus (PaLM). (B) Mean number of Fos-IR neurons in basal condition and after exposure to the open arm for C and prenatal stressed (PS) rats in the parvocellular part of the PVN and in detail for its dorsal (C) and ventral (D) subdivision. Bar: 100 μm. 3V: third ventricle; d PaMP and v PaMP: dorsal and ventral PVN, medial parvocellular part; PaLM: PVN, lateral magnocellular part; PVN: paraventricular nucleus of the hypothalamus. Open bars: basal condition; full bars: stress condition. o, $P<0.05$, oo, $P<0.01$, ooo, $P<0.005$, basal vs open arm; * $P<0.01$, control vs prenatal stress.

along the locus coeruleus, with a higher number in the dorsal than in the ventral region (data not shown). The number of TH-IR neurons was similar between the four groups of rats (Fig. 5C). Following exposure to the open arm, the percentage of the double-labelled neurons towards the TH-IR neurons population (Fos-IR/TH-IR) was higher in PS rats than in C rats ($U=4$, $P<0.05$, C vs PS rats; Fig. 5D). Moreover, only PS rats tended to present an increase in the percentage of double-labelled neurons after stress compared to basal condition ($U=6$, $P=0.06$, basal vs open arm; Fig. 5D).

4. Discussion

The present work provided evidence for the first time that PS has an impact on neuronal activation of

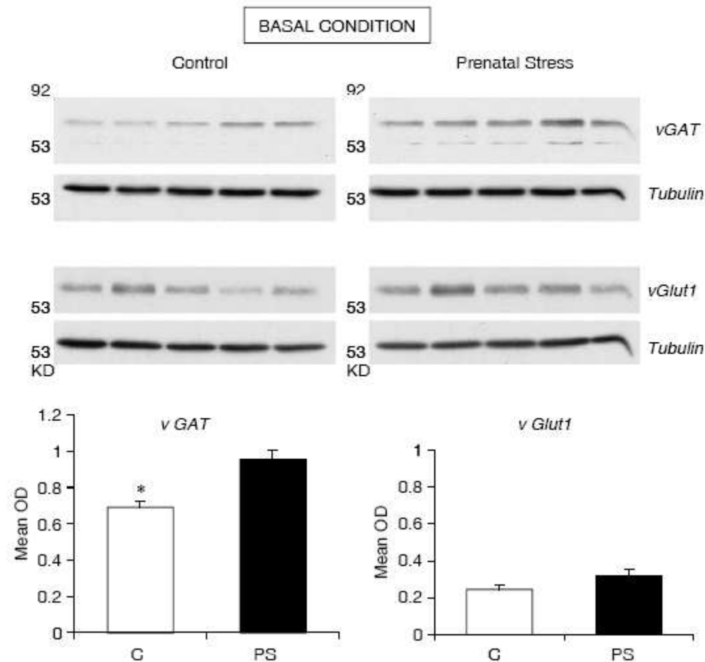

Figure 3 Western immunoblot of vesicular transporters for GABA (vGAT) and type-1 glutamate (vGlut1) in the hypothalamic area surrounding the hypothalamic paraventricular nucleus of control (C) and prenatally stressed (PS) rats. Tubulin was detected after vGlut1 or vGAT on the same membranes. Results are expressed as a ratio of optic densities (OD): OD vglut1/OD tubulin or OD vGAT/OD tubulin. PS rats displayed a higher expression of vGAT in the same region and no significant changes were observed for vGlut1 in the PVN and the surrounding hypothalamic area between control and PS rats. *, $P<0.05$.

the hippocampus and locus coeruleus, stress-related brain structures, implicated in the feedback mechanisms of the HPA axis. Our results give neuroanatomical basis to explain the neuroendocrine deficit of HPA axis feedback reported in PS rats by previous studies (Maccari et al., 1995; Barbazanges et al., 1996; Vallée et al., 1997; Dugovic et al., 1999). Moreover, the alteration of the plasma corticosterone levels found in our study reinforces these previous data underlying the high sensitivity of PS rats to stressors whatever their strength or duration. In fact, the mild stress, open arm exposure used here, was shorter (5 compared to 30 min) and was less stressful than the restraint stress used in the previous published experiments.

In basal condition, both PS and C rats exhibited similar plasma levels of corticosterone associated with a similar level of the Fos protein immunoreactivity in the parvocellular PVN. Furthermore, the hypothalamic area surrounding and including the PVN showed a significant increase of the hypothalamic vGAT and no change in the density of vGlut-1 in PS rats, suggesting a higher GABAergic inhibition in this area, even if an increased vGAT not always means a more release of GABA. Interestingly, PS rats presented a higher number of Fos-IR neurons in the hippocampus and locus coeruleus in basal condition. Given that we found an increased vGAT in the PVN and an increased hippocampal Fos immunoreactivity in PS rats, we would have expected a decreased Fos protein in the PVN of PS rats. We can explain the surprising absence of change in the PVN Fos immunoreactivity considering the increased locus coeruleus neuronal activation in PS rats. Locus coeruleus neurons provide stimulatory noradrenergic input to the PVN (Plotsky, 1987; Daftary et al., 2000). Indeed, this locus coeruleus excitatory effect at the PVN level (Saphier, 1989; Han et al., 2002) could be contrasted by the local vGAT inhibitor effect, in order to maintain in PS rats a basal activation of the CRH PVN neurons similar to C rats. Furthermore, an increased noradrenergic signal from the locus coeruleus to hippocampus reduces hippocampal

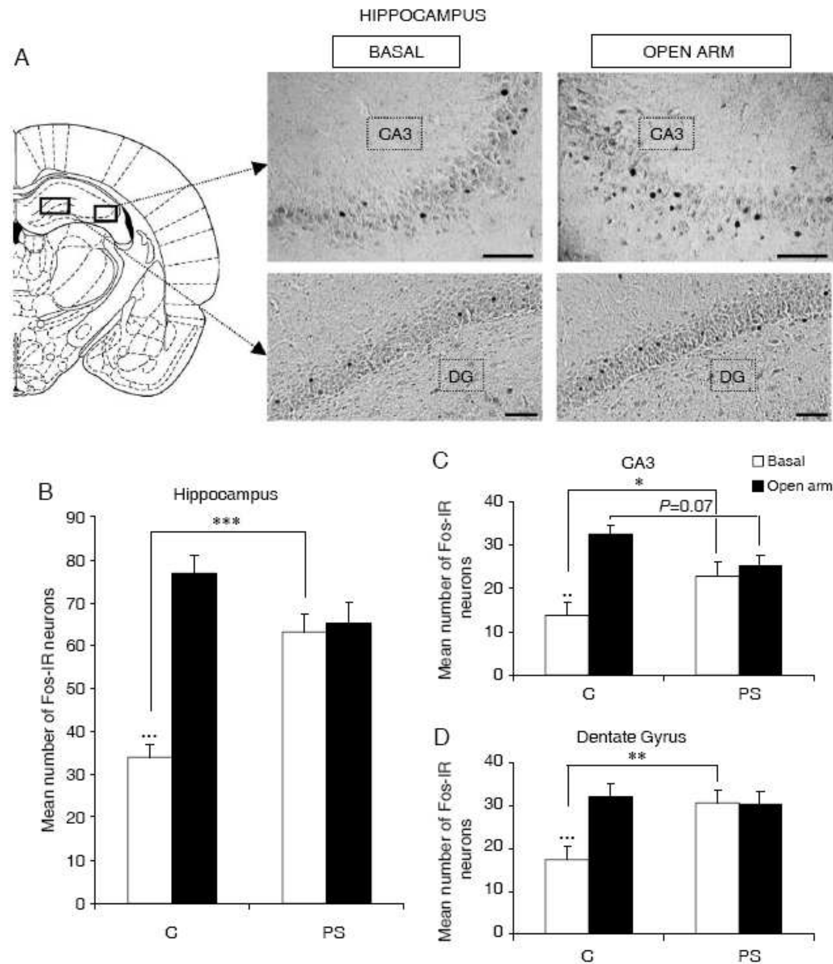

Figure 4 (A) Bright-field photomicrographs showing the distribution of Fos-immunoreactive (Fos-IR) neurons in the hippocampus in control rats in basal condition and after exposure to the open arm. Within the hippocampus, Fos-IR neurons were located in the pyramidal layer of the Ammon horns CA1, CA2, and CA3 and in the granular layer of the dentate gyrus (DG). Only CA3 and DG were illustrated. (B) Mean number of Fos-IR neurons in basal condition and after exposure to the open arm for control (C) and prenatal stressed rats (PS) in the hippocampus and in detail for CA3 (C) and DG (D). Bar: 100 μm. CA3: Ammon horn 3; DG: dentate gyrus. Open bars: basal condition; full bars: stress condition. oo, $P<0.01$, ooo, $P<0.005$, basal vs open arm; * $P<0.05$, ** $P<0.01$, *** $P<0.005$, C vs PS.

glucocorticoid and mineralocorticoid receptors (GR/MR) (Maccari et al., 1992; Kabbaj et al., 1995). Adult PS rats present a deficit in the hippocampal GR/MR at rest (Maccari et al., 1995). Thus, we can suggest that, in basal condition, the higher neuronal activity in the hippocampus of PS rats could be a physiological response to compensate the decreased hippocampal MR/GR in order to maintain in PS rats basal plasma corticosterone levels similar to the C rats.

After the exposure to open arm, plasma corticosterone secretion was similar in both PS and control groups 15 min after stress. Interestingly, in the whole parvocellular PVN, the exposure to open arm increased the number of Fos-IR neurons in both PS and control rats, even if the reactivity in the PS was lower than in C rats. The parvocellular PVN neurons counted included CRH neurons driving the HPA axis in the medial dorsal part of the PVN and the autonomic related neurons in the medial

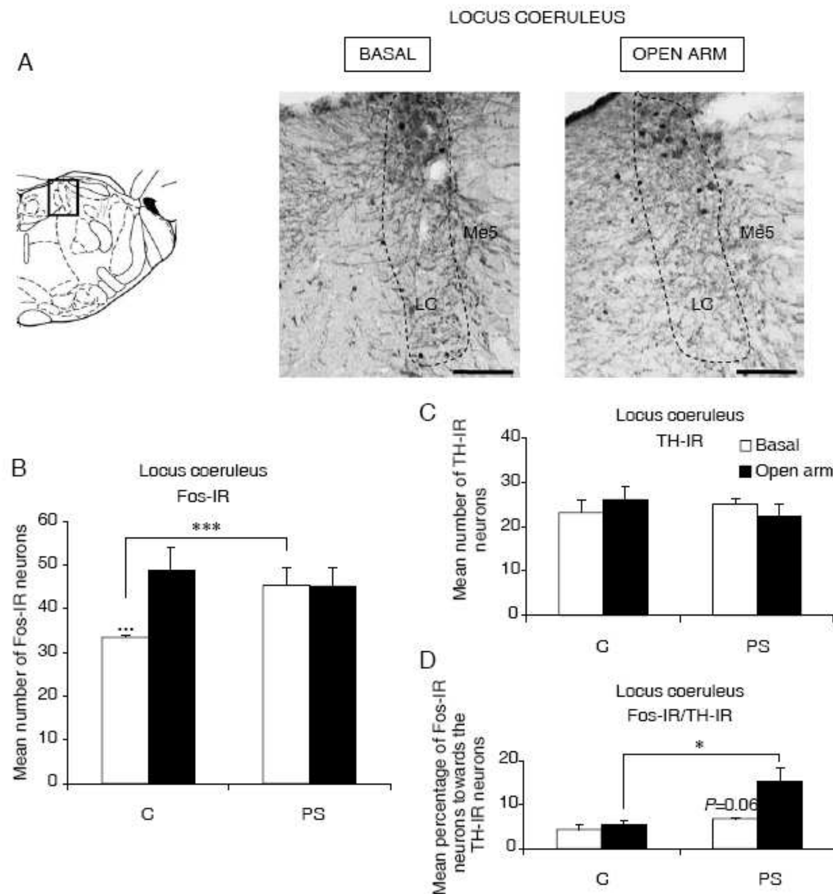

Figure 5 (A) Bright-field photomicrographs showing the distribution of Fos-immunoreactive (Fos-IR) neurons in the locus coeruleus in control rats in basal condition and after exposure to the open arm. Within the locus coeruleus, Fos-IR neurons were scattered throughout the nucleus. (B) Mean number of Fos-IR neurons in basal condition and after exposure to the open arm for control (C) and prenatal stressed rats (PS) in the locus coeruleus. (C) Mean number of TH-immunoreactive (TH-IR) neurons in basal condition and after exposure to the open arm. (D) Mean percentage of double labelled neurons (Fos-IR/TH-IR) towards the total population of catecholaminergic neurons in the locus coeruleus for C and PS rats in basal condition and after exposure to the open arm. Bar: 100 μm. LC: locus coeruleus; Me5: mesencephalic trigeminal nucleus. Open bars: basal condition; full bars: stress condition. o, $P<0.05$, oo, $P<0.01$, ooo, $P<0.005$, basal vs open arm; * $P<0.05$, ** $P<0.01$, *** $P<0.005$, C vs PS.

ventral part of the PVN. Both PS and C rats showed a similar neuronal activation in the dorsal of the medial parvocellular region (PaMP), while in the ventral part of PaMP Fos protein was more increased after stress in C compared to PS rats. These data underline that, consistently with the corticosterone levels at T15, activation of the HPA axis mediated by CRH neurons of medial dorsal part of the PVN, did not differ between PS and C rats. In PS rats, the reduced parvocellular PVN neuronal activation is due to a reduction of neuronal activity only in the ventral part of the parvocellular PVN. Nevertheless, we can suggest that the lesser neuronal reactivity observed in the ventral PaMP of PS rats may be explained by an increased catecholaminergic activity in the locus coeruleus which acts on autonomic neurons (Han et al., 2002).

After exposure to the open arm, PS and C rats differed in the time necessary to return to basal values, given that it took 60 min after stress for C and 120 min for PS rats. Since this stressor produced a similar increase in the HPA activation,

the difference between PS and C rats was related to the feedback mechanisms of the HPA axis. In fact, PS rats showed similar number of Fos-IR neurons in the hippocampus (mainly in CA3 and dentate gyrus) and locus coeruleus in basal condition and 120 min after stress, while it increased from baseline in C rats. This suggests no reactivity to stress of these brain structures in PS rats, structures involved in the feedback mechanisms. Thus, the absence of neuronal reactivity in the PS rats hippocampus after stress could explain this deficit in the HPA axis feedback mechanisms leading to a delay to return to basal corticosterone values. We also showed an increased basal levels of Fos in the hippocampus and LC of PS rats meaning a chronic and 'ceiling' activation of these neurons in the rest condition that could be explain at least in part the absence of hippocampus and LC reactivity. Furthermore, the absence of neuronal reactivity in the PS rats hippocampus can be in relation with the reduced number of hippocampal GR/MR in PS rats at rest (Maccari et al., 1995) and due to: (1) the increased percentage of the catecholaminergic neurons activated in the locus coeruleus in PS rats after stress, that in turn can act on hippocampal GR/MR number (Maccari et al., 1992; Kabbaj et al., 1995; Lai et al., 2003); (2) the already increased hippocampal neuronal activation in basal condition in PS rats (perhaps to compensate the decreased sensibility to corticosterone), which could prevent supplementary hippocampal activation increase after stress. This absence of hippocampal reactivity can contribute to the PS rats allostatic overload (McEwens and Wingfield, 2003): the unproductive try to restore homeostasis in a good time, as for a prolonged HPA response, resulting in the incapacity to cope efficiently with the challenge and finally in a disease state.

Finally, another possible mechanism explaining the absence of neuronal reactivity in the hippocampus and locus coeruleus of PS rats in response to stress could be the inhibition of the transcription factor activator protein-1 (AP-1). The AP-1 regulates the transcription of late genes when activated by the heterodimer c-Fos/c-Jun (as a result of the immediate early genes transcription, c-fos and c-jun) (Herdegen and Leah, 1998). The prolonged corticosterone secretion after stress observed in PS rats (Maccari et al., 1995) could repress the AP-1 given that glucocorticoids regulate AP-1 (Diamond et al., 1990; Yang-Yen et al., 1990; Unlap and Jope, 1994), and consequently the heterodimer c-Fos/c-Jun activity, resulting in the absence of increase in the number of Fos-IR neurons in the hippocampus and locus coeruleus after stress.

In conclusion, our work raises new anatomical arguments suggesting that PS can have long-term neuronal effects within hippocampus and locus coeruleus brain structures involved in the feedback mechanisms of the HPA axis. The PS may affect the organisation of pathways and/or neuronal circuitry responsible to maintain a basal tonic regulation of the HPA axis similar to controls. These neuronal 'reorganisation' could affect the PS rats reactivity to stress leading to alterations in behaviour related to HPA axis response to stress.

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Insulin-like growth factor 1 reduces age-related disorders induced by prenatal stress in female rats

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Abstract

Stress during the prenatal period can induce permanent abnormalities in adult life such as increased anxiety-like behavior and hyperactivity of hypothalamo-pituitary–adrenal (HPA) axis system. The present study was designed to investigate whether prenatal stress could induce spatial learning impairment in aged female rats. Furthermore, since it has been recently reported that insulin-like growth factor 1 (IGF-1) attenuates spatial learning deficits in aged rats and promotes neurogenesis in the hippocampus, we assessed the impact of a chronic infusion of IGF-1 on age-related disorders. Our results show that females stressed during prenatal life exhibit learning impairments in the water maze task. Chronic IGF-1 treatment restores their spatial abilities, reduces their HPA axis dysfunction and increases plasma estradiol levels. Parallel to these effects, chronic IGF-1 up-regulates neural proliferation in the dentate gyrus of the hippocampus. These findings support the hypothesis of an early programming of the vulnerability to some neurological diseases during senescence and reinforce the potential therapeutic interest of IGF-1 during brain aging.

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1. Introduction

Stressful events occurring during early life could increase vulnerability to the effects of stress later in life [19]. In rats, chronic stress during pregnancy exerts profound long-term influences on the offspring [33,54]. Prenatal stress induces an increase of the hypothalamo-pituitary–adrenal (HPA) axis activation in adult animals that is associated with a reduction in the number of hippocampal corticosteroid receptors [25,34]. This can be evidenced by a more prolonged elevation of plasma corticosterone after exposure to stress [34,51,55,56]. These HPA dysfunctions have been reported in young and aged animals, therefore suggesting a permanent effect of early stress [51]. In the brain, the main target of adrenal steroids is the hippocampal formation, which is involved in spatial memory processes [13]. Hippocampal neurons show remarkable plasticity, involving long-term potentiation, dendritic remodeling and neurogenesis, as well as a strong vulnerability to stressful experiences and to aging processes [20,38]. A recent study has provided evidence of a decrease of hippocampal neurogenesis after prenatal stress [28] and it was previously reported that prenatal stress increased age-related learning impairments [51]. Thus, HPA axis alterations by prenatal stress may be involved in the spatial memory impairments observed during aging, in agreement with the hypothesis of a "feed-forward" cascade whereby prolonged exposure to glucocorticoids damages the hippocampus and leads to cognitive deficits [45,46].

Extensive research suggests that exercise could have benefits for health and cognitive function particularly in aged individuals [9,11]. IGF-1 appears to play a major role in the effects of exercise on brain [6]. It regulates neurotrophic

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response after injury [7], brain vasculature [47], brain glucose consumption [8], increases mRNA of brain-derived neurotrophic factor [6] and stimulates hippocampal neurogenesis [2,50]. Interestingly, aging is associated with reductions in the plasma and brain levels of IGF-1 [49].

Although considerable evidences show a link between stress, HPA axis dysfunction, memory disorders and aging, only one study has addressed the effect of prenatal stress on cognition in aged male rats [51] and nothing is known on the consequences of prenatal stress in aged females. Furthermore, females are known to be more vulnerable to stress [24] and exhibit an hyperactivity of the HPA axis function in comparison to male animals [57]. The aim of the present study was then to characterize the cognitive effect of prenatal stress in aged female rats and to determine whether IGF-1 could correct age-associated disorders. Therefore, we evaluated the spatial learning abilities of 24 month-old females that had been exposed to prenatal stress and we tested the effect of chronic infusions of IGF-1 on spatial performances, HPA axis function, estradiol levels and cell proliferation in the dentate gyrus.

2. Materials and methods

2.1. Animals and prenatal stress procedure

Sprague Dawley female rats were maintained on a 12:12 h dark:light cycle (lights on from 8 a.m. to 8 p.m.), with free access to food and water. Manipulation of the animals was performed following the principles of laboratory animal care published by the French Ethical Committee and the rules of the European Union Normative (86/609/EEC). Special care was taken to minimize animal suffering and to set the number of animals to the minimum required. During the last week of pregnancy, from day 14 until parturition, pregnant females were individually placed in plastic transparent cylinders (7 cm diameter, 19 cm long) and exposed to bright light for 45 min [25,34]. Animals were submitted to such three daily stress sessions (9 a.m., 12 p.m. and 5 p.m.), whereas control pregnant females were left undisturbed in their home cages. After weaning (21 days old), the offspring were housed in groups of four and left undisturbed. A maximum of two females by litter were used to avoid any "litter effect". Animals with signs of respiratory distress or tumors were excluded. Two groups of 24 month-old animals were constituted: old control; old prenatally stressed. Moreover, a separate set of 2 month-old Sprague Dawley females (Charles River, France) was assigned to the young group.

2.2. Surgery

Animals were anesthetized using ketamine (50 mg/kg, i.p.) and xylazine (5 mg/kg, i.p.). A 28-gauge steel cannula (Alzet-2004® brain infusion kit) was implanted into the right lateral ventricle (from bregma, anteroposterior: 0.8 mm, lateral: 1.5 mm; coordinates based on Paxinos and Watson [40]) and connected to an Alzet® osmotic minipump that was placed subcutaneously in the neck/shoulder region. Animals subjected to prenatal stress received either vehicle (NaCl) or recombinant human IGF-1 (GroPep, Australia), delivered at a rate of 50 ng per 0.25 μl/h for 21 days (Fig. 1). The remaining old rats as well as a group of 2-month-old young females served as controls and were infused with NaCl.

2.3. Water maze

The water maze task has been validated as a valuable index of spatial learning in aged rodents [14,30]. Apparatus consisted of a plastic tank, 2 m in diameter and 0.6 m in height. The tank was filled with water (22 ± 2 °C) to a depth of 35 cm [51,52]. The platform (10 cm diameter) was 2 cm above the surface of the water during the pretraining and 3 cm below the surface of the water during spatial learning. The pool, walls and platform were all colored black and indirect lighting was used in the room, enabling the platform to be hidden from sight. Extra-maze visual cues around the room remained in a fixed position throughout the experiment.

The timeline is illustrated in Fig. 1. Before spatial learning assessment, three sessions (consisting of three daily trials) of pretraining with a visible platform were conducted in order to train the rats to swim and climb onto the platform. This procedure allows to reduce the non-cognitive components of this task (stress reactivity, motor performances) and to control any difference between experimental groups in visual or motor abilities. Four days later, preoperative spatial learning performances were evaluated using a submerged platform (positioned in a different site from the pretraining). Three sessions were conducted, each consisting of three trials with

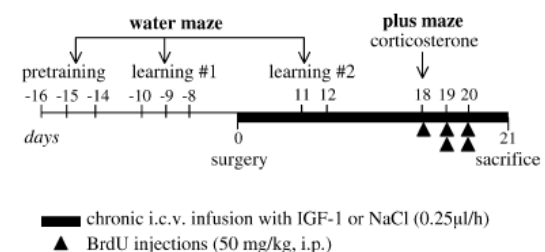

Fig. 1. Experimental design. The 24 month-old (control and subjected to prenatal stress) and 2 month-old young female rats were tested in the water maze. First, 16 days before surgery, animals received three sessions of pretraining with a visible platform, then they were evaluated for three sessions of spatial learning with a hidden platform (#1). One week later, an Alzet minipump was implanted for a chronic treatment with IGF-1 or NaCl. After 11 days of infusion, they were tested in water maze for a novel learning for two sessions (#2). After 18 days of treatment, rats were exposed 5 min in the elevated plus maze to assess anxiety levels and corticosterone secretion after stress. The same day in the afternoon, animals were injected with BrdU, then, the two consecutive days, they were treated with BrdU twice per day. Animals were killed for BrdU immunochemistry after 21 days of infusion.

distinct start locations. If the rat did not find the platform in 90 s, it was guided to the platform. One week later, animals were implanted with an osmotic minipump and infused in the lateral cerebral ventricle (i.c.v.) for 11 days with saline or IGF-1. Animals were then tested in the water maze for 2 days. The procedure used was similar to the preoperative learning procedure, except that the hidden platform was localized at a different site. Latency to reach the platform was recorded by an automated system (Viewpoint, Lyon, France).

2.4. Behavior in the elevated plus maze test and corticosterone response

After 18 days of i.c.v. infusion of IGF-1 or vehicle, animals were exposed to the elevated plus maze to study anxiety-like behavior [41] (Fig. 1). The elevated plus maze was made of white wood and was 60 cm above the floor. It had four arms radiating outward from a central square (15 cm × 15 cm). Two were open (50 cm × 15 cm) and two were closed with sidewalls (40 cm high). Each rat was placed on the central platform facing a closed arm, and allowed to freely explore the maze for 5 min. Exploration was recorded through an automated tracking system (Viewpoint, Lyon, France). The behavioral parameters scored were the number of entries into the closed and open arms and the time spent there. An entry was counted when the four paws were placed in the respective arm. Percentages of exploration of open arms (time and visits, ratio open/total arms) were calculated as an index of anxiety-like behavior.

Several studies have demonstrated an increase of corticosterone secretion after exposure to the elevated plus maze suggesting that this manipulation could be considered as stressful for the rat [27,44]. To determine plasma corticosterone after the plus maze exposure, blood samples (200 μl) were taken by cutting the tail (<2 min after removal from the plus-maze). Blood samples were then put into heparinized tubes, placed on ice, centrifuged and stored at −20 °C. The experiment was conducted between 10 a.m. and 1 p.m. to avoid the increase of plasma corticosterone induced by circadian rhythm.

2.5. 5-Bromo-2′-deoxyuridine injections and sacrifice

In the afternoon (5 p.m.) of the day 18 after the initiation of the i.c.v. infusion of NaCl or IGF-1, rats were injected with 5-bromo-2′-deoxyuridine (BrdU, 50 mg/kg, i.p.). Then, they received injections of BrdU twice per day (9 a.m., 5 p.m.) for 2 days and were killed between 15 and 20 h after the last injection of BrdU (Fig. 1).

Animals were deeply anaesthetized with pentobarbital and the cyclic status was determined by microscopic observation of vaginal smears. Adrenal glands were removed and weighed. Blood samples (500 μl) for plasma estrogen levels were taken by intracardiac punctures. Animals were then perfused intracardially with saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Brains were removed and immersed in the same fixative for 4 h at 4 °C and then rinsed in phosphate buffer.

2.6. Immunohistochemistry

For each brain, coronal sections (50 μm thick) of the entire dentate gyrus of the right cerebral hemisphere were obtained with a Leica Vibratome (Heidelberg, Germany). Endogenous peroxidase activity was quenched by incubating brain sections, for 30 min at room temperature, in 0.1 M phosphate buffer, with 10% methanol and 3% hydrogen peroxide. To ensure detection of BrdU-labeled nuclei, DNA was denatured by incubating the sections for 15 min at 37 °C, in 2 N HCl. After this step, sections were rinsed twice in 0.1 M borate buffer (pH 8.5), followed by a rinse in 0.1 M phosphate buffer, and then incubated for 1 h at room temperature, with 0.1 M phosphate buffer, 0.1 M, with 0.3% Triton X-100 and 0.3% bovine serum albumin. This buffer solution was used in the following washes and incubations. Sections were then incubated overnight, at 4 °C, with mouse anti-BrdU antibody (1:5000; Hybridoma Bank, Iowa City, IA). After rinsing in buffer, sections were incubated for 90 min at room temperature in biotinylated goat anti-mouse IgG (1:1000; Pierce, Rockford, IL), rinsed and transferred to the peroxidase avidin biotin complex (1:250; Pierce) for 45 min at room temperature. Peroxidase was detected using diaminobenzidine as chromogen.

2.7. Morphometric analysis of BrdU immunoreactive cells

The morphometric analysis of BrdU labeled cells was performed on coded sections. For each animal, BrdU positive cells were counted on every sixth section (300 μm apart) throughout the rostral-septal half of the dentate gyrus (from the rostral extreme of the hippocampus, at −1.80 mm from bregma, to the caudal end, at −6.80 mm from bregma). The same areas and number of sections were studied for all the animals and all the experimental groups. Sixteen sections were analyzed from each animal. All BrdU positive cells were counted with a 100× microscope objective. Cell counts were restricted to the granular cell layer (GCL) and the subgranular zone (SGZ) of the dentate gyrus. The SGZ was defined as a two-nucleus-wide band below the apparent border between GCL and the hilus. The total number of BrdU-labeled cells was estimated as previously described [4,23]. Briefly, BrdU-immunoreactive nuclei that came into focus while focusing down through the thickness of the section were counted, according to the optical disector principle [10], whereas BrdU-immunoreactive nuclei located in the uppermost focal plane were ignored. We considered as BrdU positive nuclei those completely filled with DAB product or fluorescent marker or showing patches of variable intensity. The number of BrdU-immunoreactive nuclei counted in the GCL/SGZ was multiplied by 6 (because every sixth section was used) to estimate

the total number of BrdU-immunoreactive cells in the hippocampus.

2.8. Corticosterone and estrogen assays

Plasma corticosterone and estrogen levels were measured with radioimmunoassay kits (corticosterone kit: ICN, Biomedical, Orsay, France; estrogen kit: Diagnostic Products Corporation, Los Angeles, CA). The minimum levels of detection were 0.2 μg/dl for corticosterone and 1.4 pg/ml for estradiol. Intra-assay and inter-assay coefficients of variation were, respectively, 7 and 8%, for estradiol and 4 and 8%, for corticosterone.

2.9. Statistics

Initial spatial learning performances were assessed by two-way analysis of variance (ANOVA) using group as between factor (three levels) and sessions as within factor (three levels). Learning performances after surgery, endocrine data and cell proliferation in the dentate gyrus were analyzed with one way ANOVA. When significant, ANOVAs were followed by planned comparisons with contrast analysis for specific comparisons. Comparison of the percentages of memory-impaired rats in each group, before and after treatment, was assessed with t-test for percentages comparison. Correlations were calculated using Pearson's test. Comparisons of the estrous cycle among groups were analyzed with a non-parametric test (χ^2). Differences were considered significant at $P = 0.05$.

3. Results

3.1. Prenatal stress affects spatial learning in old females

Visual and sensorimotor capacities were assessed after 3 days of pretraining. The mean latency covered to find the visible platform was similar across groups (young: 15 s ± 2.4; old C: 15 s ± 1.3; old PS: 18 s ± 4; $F(2,37) = 0.59$, NS). As depicted in Fig. 2, latencies to locate hidden platform decreased across the testing sessions showing that all groups learned (session effect, $F(2,74) = 24.68$, $P < 0.0001$). Spatial learning capacities differed between groups throughout the 3 days of learning (group effect, $F(2,37) = 7.26$, $P < 0.002$). Planned comparisons by contrast analysis revealed that old rats subjected to prenatal stress exhibited an increased latency to find the submerged platform in comparison to old controls or young rats (PS versus C, $F(1,37) = 7.86$, $P < 0.01$; PS versus young, $F(1,37) = 12.25$, $P < 0.001$). In contrast, old C and young rats showed similar performances ($F(1,37) = 1.46$, NS).

Correlative analysis conducted on old animals (limited to rats treated with vehicle) revealed a significant positive correlation between spatial learning impairment and the weight of adrenal glands. Indeed animals that exhibited poor performances, i.e. the ones with higher latencies (mean over the 3 days of testing) to find the hidden platform, had higher adrenal gland weights ($r = 0.64$, $P < 0.01$). In contrast, no significant correlation was observed between the ability to reach the visible platform and the weight of adrenal glands ($r = 0.30$, NS).

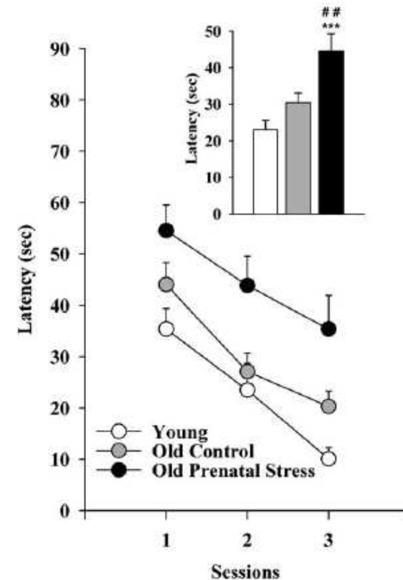

Fig. 2. Spatial learning performances in the water maze task. Latency (seconds) to find the hidden platform, over three sessions of testing, in young ($n = 8$) and old ($n = 16$ per group) control or prenatally stressed female rats. The mean over the 3 days of testing is presented in the inset. Means ± SEM are given. Old prenatal stress versus old control: $^{\#\#}P < 0.01$; old prenatal stress versus young: $^{***}P < 0.001$.

3.2. IGF-1 improves spatial performances in old females subjected to prenatal stress

Given that only the old females that were subjected to prenatal stress exhibited memory impairments compared to young animals, the assessment of the influence of chronic infusions of IGF-1 on spatial learning was limited to this group. The differences in spatial learning abilities across the groups, described above (Fig. 2), were also observed by comparing the spatial learning performances before chronic i.c.v. infusion (group effect, $F(3,26) = 4.09$, $P < 0.05$). Before IGF-1 treatment, PS-IGF-1 and PS-NaCl groups exhibited an impaired spatial memory compared to C-NaCl animals (latency, $F(1,26) = 5.36$, $P < 0.03$) and compared to young females (latency, $F(1,26) = 9.47$, $P < 0.01$). After 11 days of i.c.v. treatment, a new spatial learning test was conducted. There was no significant decrease of the latency to reach the hidden platform throughout the two sessions of the second learning (session effect, $F(1,26) = 3.23$, NS).

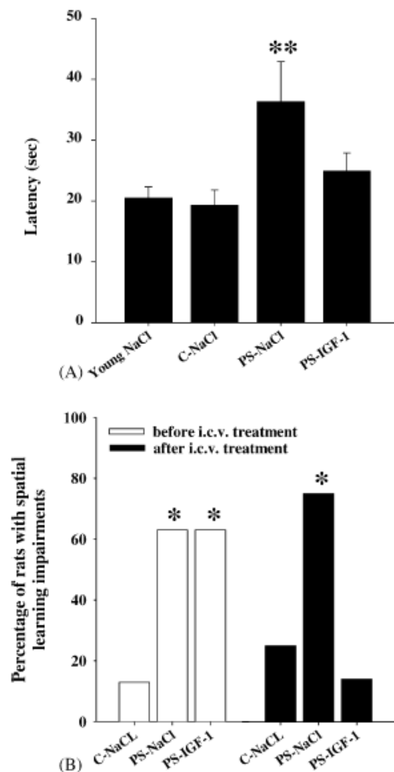

Fig. 3. Effect of IGF-1 on spatial learning performances in old prenatally stressed females. (A) Latency (seconds) to reach the hidden platform of the water maze over the two sessions of testing in young females, old control rats infused with saline (C-NaCl), old rats subjected to prenatal stress infused with saline (PS-NaCl) and old rats subjected to prenatal stress infused with IGF-1 (PS-IGF-1). Chronic infusion with IGF-1 attenuates the learning abilities deficits of the PS old females. Means ± SEM are given, $n = 7–8$ per group. $^{**}P < 0.01$, PS-NaCl versus C-NaCl or Young. (B) Percentage of old females with learning impairments before and after chronic i.c.v. treatment with NaCl or IGF-1 ($n = 7–8$ animals per group). Rats were defined as poor performers if their mean latency over the two sessions of learning was higher than 1 S.D. from the mean of young animals (before i.c.v. treatment: 38 s; after i.c.v. treatment: 25 s). Prenatal stress increases the percentage of old animals showing learning impairments. This effect is reduced by chronic treatment with IGF-1. $^{*}P < 0.05$ compared with C-NaCl and PS-IGF-1 after treatment.

However, as shown in Fig. 3A, the global performances differed across groups (group effect, $F(3,26) = 3.74$, $P < 0.05$). Planned comparisons indicated that learning performances were impaired in old PS-NaCl females as shown by the increased latency to reach the hidden platform in comparison with C-NaCl ($F(1,26) = 8.32$, $P < 0.01$). Subsequent analysis with young animals showed that the latency to reach the platform was similar across the groups, except for the old PS-NaCl females (young versus PS-NaCl, $F(1,26) = 7.42$, $P < 0.01$). Moreover, old PS females treated with IGF-1 exhibited an improvement of their spatial learning performances that just miss significance (PS-IGF-1 versus PS-NaCl, latency, $F(1,26) = 3.87$, $P = 0.059$). The percentage of old animals with learning impairments was determined based on the mean latency of young rats during the first two sessions, before and after chronic i.c.v. treatment (Fig. 3B). Before i.c.v. treatment, the percentage of rats showing learning impairments was 13% in the control group and 63% in animals exposed to prenatal stress ($P < 0.05$). After chronic treatment with IGF-1, this percentage was markedly decreased and reached the value of control females (after i.c.v. treatment, PS-NaCl versus PS-IGF-1 and C-NaCl, $P < 0.05$; PS-IGF-1 before treatment versus PS-IGF-1 after treatment, $P < 0.05$).

3.3. IGF-1 effects on anxiety, HPA axis function and estrogen levels in old females subjected to prenatal stress

In old rats, prenatal stress did not affect the percentages of time spent in the open arms (C-NaCl = 1.84 ± 0.91%, PS-NaCl = 6.78 ± 2.85%, PS-IGF-1 = 3.96 ± 1.23%) or the number of visits (C-NaCl = 22.78 ± 7.43%, PS-NaCl = 28.12 ± 7.83%, PS-IGF-1 = 34.60 ± 7.03) whatever the treatment (time spent, $F(2,19) = 2.12$, NS; visits, $F(2,19) = 0.69$, NS). Young animals spent more time than old animals in the open arms (young = 17.41 ± 5.05%, old = 3.96 ± 1.00%; $F(1,28) = 15.76$, $P < 0.001$) whereas no significant difference was observed for the number of visits (young = 34.47 ± 6.13%, old = 28.53 ± 4.23%; $F(1,28) = 0.55$, NS).

After a plus maze exposure, plasma corticosterone levels differed across the groups ($F(3,24) = 3.95$, $P < 0.05$). Prenatal stress increased the corticosterone response (PS-NaCl versus C-NaCl, $F(1,24) = 11.52$, $P < 0.01$), whereas IGF-1 treatment appeared to attenuate this effect (PS-IGF-1 versus C-NaCl, $F(1,24) = 2.36$, NS) (Fig. 4A). The increase in corticosterone secretion induced by the exposure to the plus-maze test was not affected by aging (young = 73.94 ± 8.17 μg/dl, old = 69.52 ± 4.38 μg/dl, age effect, $F(1,26) = 0.24$, NS). A significant group effect was also observed for the weight of adrenal glands ($F(3,26) = 11.10$, $P < 0.001$). PS-NaCl exhibited higher adrenal gland weights than C-NaCl ($F(1,26) = 4.19$, $P < 0.05$). Infusion with IGF-1 reversed this effect (PS-IGF-1 versus PS-NaCl, $F(1,26) = 4.45$, $P < 0.05$) as demonstrated by the similar adrenal weights observed in PS-IGF-1 and C-NaCl groups ($F(1,26) = 0.01$, NS) (Fig. 4B). The weight of adrenals was decreased in old animals compared to young (young = 116.57 ± 4.54 mg/kg, old = 81.78 ± 3.62 mg/kg; age effect, $F(1,28) = 24.15$, $P < 0.001$). In old females, cyclic status was similar across groups (they mainly exhibited diestrus or estrus type of smear, $\chi^2 = 0.78$, d.f. = 2, NS). However, plasma estrogen levels differed between groups in aged animals ($F(2,14) = 6.79$, $P < 0.01$) with higher levels in PS-IGF-1 females compared to groups infused with NaCl (PS-IGF-1 versus C-NaCl, $F(1,14) = 8.53$, $P < 0.01$; PS-IGF-1 versus PS-NaCl, $F(1,14) = 10.63$, $P < 0.01$) (Fig. 4C).

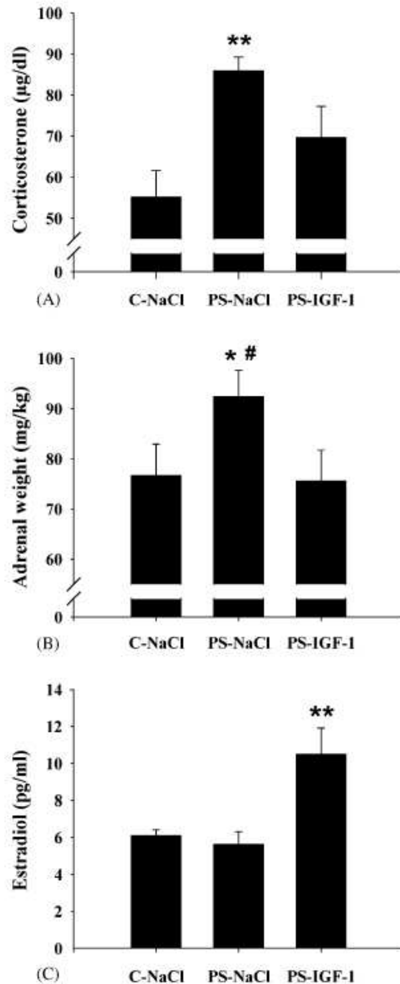

Fig. 4. Effect of IGF-1 on endocrine parameters in old prenatally stressed females. (A) Plasma corticosterone secretion (means ± SEM, $n = 6–8$ per group) in response to 5 min of elevated plus maze exposure in old C-NaCl, PS-NaCl and PS-IGF-1 animals. Prenatal stress increased corticosterone response after plus-maze ($^{**}P < 0.01$, PS-NaCl versus C-NaCl), whereas after IGF-1 treatment PS group did not differ from C group. (B) Adrenal weight (mg/kg) of old C-NaCl, PS-NaCl and PS-IGF-1 (means ± SEM, $n = 7–8$ per group). Adrenal weight was increased by prenatal stress ($^{*}P < 0.05$, PS-NaCl versus C-NaCl), IGF-1 treatment reversed this effect ($^{\#}P < 0.05$, PS-NaCl versus PS-IGF-1). (C) Plasma estradiol levels (means ± SEM, $n = 5–7$ per group) in old C-NaCl, PS-NaCl and PS-IGF-1. Chronic IGF-1 treatment increased estradiol levels in prenatally stressed rats ($^{**}P < 0.01$, PS-IGF-1 versus C-NaCl or PS-NaCl).

3.4. IGF-1 increases BrdU labeling in the dentate gyrus of old females subjected to prenatal stress

The number of BrdU-immunoreactive cells in the granular and subgranular layers of the dentate gyrus was drastically affected by aging (young, 6502 ± 2908; old, 483 ± 124; age effect, $F(1,18) = 93.10$, $P < 0.001$). However, in old animals, the number of BrdU-positive cells differed between groups ($F(2,12) = 8.83$, $P < 0.01$) and was increased after 21 days of IGF-1 infusion in PS old females (PS-IGF-1 versus C-NaCl, $F(1,12) = 14.88$, $P < 0.01$; PS-IGF-1 versus PS-NaCl, $F(1,12) = 11.38$, $P < 0.01$) (Fig. 5). The number of BrdU-positive cells in the dentate gyrus of PS rats treated with IGF-1 showed a 2.5–3 time fold increment compared to C-NaCl or PS-NaCl groups.

The number of BrdU-labeled cells in the dentate gyrus and the weight of adrenal glands were negatively correlated in the

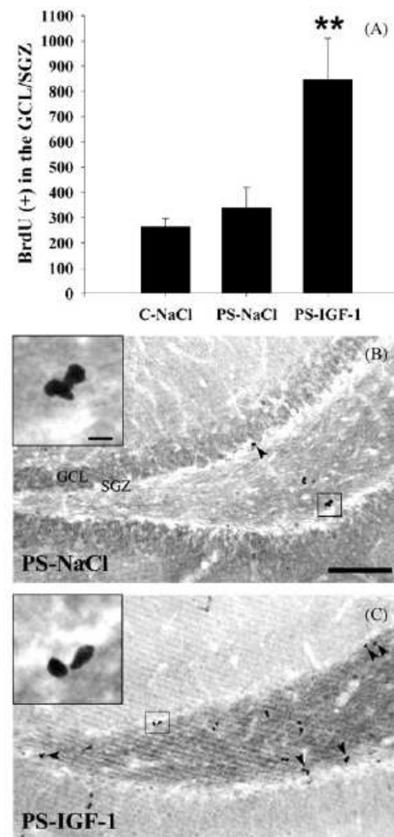

Fig. 5. Effect of IGF-1 on the number of BrdU-labeled cells, in the granular and subgranular layers of the dentate gyrus, in old prenatally stressed females. (A) Quantification of BrdU-positive cells in the granular cell layer and subgranular zone (GCL/SGZ) of the dentate gyrus in old C-NaCl, PS-NaCl and PS-IGF-1 rats (means ± SEM, $n = 5$ per group). Chronic IGF-1 infusion increased the number of BrdU-positive cells of the PS group. $^{**}P < 0.01$, in comparison with C-NaCl and PS-NaCl. Representative photomicrographs of BrdU-labeled cells in subgranular zone of the dentate gyrus from old females subjected to prenatal stress chronically infused with (B) NaCl or (C) IGF-1. Scale bar for (B) and (C): 100 µm. Insets represent a high magnification of a cluster of newborn cells showing BrdU immunoreactive cells. Scale bar for insets: 10 µm.

prenatally stressed animals (Pearson's correlation; $r = -0.73$, $P < 0.05$). The number of BrdU cell was also negatively correlated with corticosterone levels after stress in this experimental group (Pearson's correlation; $r = -0.89$, $P < 0.001$). No significant correlations were shown in the other experimental groups.

4. Discussion

Our results show that females stressed during prenatal life exhibit spatial learning impairments with aging. Chronic IGF-1 treatment restores their spatial abilities, reduces their HPA axis dysfunction and increases plasma estradiol levels. Parallel to these effects, chronic IGF-1 up-regulates neural proliferation in the dentate gyrus of the hippocampus. Although mechanisms underlying the effects of IGF-1 remain to be fully elucidated, our results suggest an "anti-aging" activity of this neurotrophin.

Among the factors involved in the vulnerability to aging-associated cognitive disorders, stress and glucocorticoids have been proposed to play a major role [38,39]. Here we demonstrate for the first time that spatial learning is impaired in 24 month-old females exposed to stress during the prenatal period. These results confirm and extend a previous work showing an impairment of spatial memory in a spontaneous recognition task in old male rats subjected to prenatal stress [51]. Our results demonstrate that chronic infusion of IGF-1 suppresses spatial learning deficits in cognitively-impaired old animals. Indeed, aged females subjected to prenatal stress, that exhibited a marked impairment in their learning abilities before treatment, reached the performances of old control and young females after i.c.v. infusion of IGF-1 for 11 days. This result reinforces previous findings showing an improvement in spatial reference memory and in object recognition in old male rats after chronic treatment with IGF-1 [36,49]. Water maze is a stressful task, especially with water at 22 °C [32]. We have used water at 22 °C based on previous studies conducted in aged animals in a similar paradigm [51,52]. This stressful situation allows maintaining high levels of motivation in aged animals and prevents behavioral strategies such as floating behavior often observed in these animals at higher temperatures. The lack of effect of IGF-1 on anxiety, observed in the elevated plus-maze, suggests that the enhancement of learning performance by IGF-1 in rats subjected to prenatal stress was not related to a secondary effect of IGF-1 such as a decrease of fearfulness in the water maze.

Old females subjected to prenatal stress exhibited higher corticosterone after plus-maze exposure and had increased adrenal gland weights according to previous reports in males [28,53]. Age-related changes of the HPA axis have been shown to be involved in cognitive aging. We observed a significant increase of adrenal function between memory impaired and memory non-impaired aged rats, but surprisingly not between old and young animals. The use of very young females (2 months old) may explain our results. In this view, adrenal hypertrophy with aging is only observed when old animals are compared to adult rats (6 months old) whose growth is essentially completed [58]. When compared to younger animals (28 days or 3 months old), old rats show lower adrenal gland weight [28]. Although extensive evidence indicates that prenatal stress induces HPA dysfunction in young rats [25,37,55], we show here for the first time that the effects of prenatal stress on the HPA axis may persist until senescence in females. The elevated plasma corticosterone levels indicate that hormonal response to a stressor is more pronounced in rats subjected to prenatal stress. A chronic activation of the adrenal glands in aged animals, resulting in heightened release of corticosterone, has been shown to be associated with hypertrophy of the adrenal glands due to the hyperstimulation by the pituitary [43]. Considering both control and prenatally stressed females, our results give evidence of a link between spatial learning and HPA axis dysfunction in aged animals. Indeed, old females with higher learning deficits in the water maze had higher adrenal gland weights. This observation strongly supports previous findings in animals and humans of a correlation between the hyperactivity of the HPA axis and the development of cognitive impairments with aging [22,26,31,42,59].

We observed that chronic i.c.v. infusion of IGF-1 attenuated HPA axis activity and increased plasma estradiol levels. The mechanisms underlying this effect remain to be elucidated. It could be hypothesized that central IGF-1 is involved in the feed back processes of HPA axis activity through a decrease of CRH and/or ACTH release or through a modulation of hippocampal glucocorticoid receptors and/or a modulation of neurotransmitters regulating this axis. Moreover, since the ovaries of old rats are capable of near normal function under appropriate gonadotropic stimulation [21], IGF-1 action could be mediated by a modulation of the hypothalamo-pituitary function and/or ovarian function, resulting in an increase in estradiol synthesis [12]. It is also possible that the increase in circulating estradiol was related to changes in the clearance of estradiol. The inhibition of HPA axis hyperactivity and/or the stimulation of estradiol secretion may be involved in the processes by which IGF-1 restored cognitive function in aged females. Indeed, chronic exposure to high levels of glucocorticoids are damaging for the hippocampus, inducing atrophy, synaptic loss and a decrease of cell proliferation [38]. In contrast, estrogen replacement improves spatial reference memory in aged female rodents [15,35] and stimulates cell proliferation in the hippocampus [3]. Furthermore estrogen and IGF-1 have been proposed to interact in the regulation of brain plasticity [5,17].

Our data indicate that chronic i.c.v. infusion of IGF-1 increases cell proliferation in the dentate gyrus of old female rats exposed to chronic stress during fetal life. This finding corroborates recent studies conducted in male rats showing that IGF-1 promotes hippocampal neurogenesis in young adults [1,50] and in old animals [29]. Recent data showing a correlation between spatial learning performances in the water maze and cell proliferation in the hippocampus [28]

suggest that an increase of cell proliferation induced by IGF-1 may be important in its behavioral effect. Interestingly, we report here that the enhancement of cell proliferation induced by IGF-1 treatment was associated with an improvement of HPA axis function in the same animals. IGF-1 and adrenal steroids have been suggested to work antagonistically to regulate hippocampal neurogenesis. Exposure to stressful experiences, including early stress events, reduces cell proliferation [18,28,48]. However, although prenatal stress resulted in an increased weight of adrenal glands in old females, no differences were observed in hippocampal cell proliferation between prenatal-stressed and control old females. This observation is apparently in contrast with the recent finding showing a reduced neurogenesis in old male rats exposed in utero to the same chronic stress [28]. A gender difference could be taken into account to explain this discrepancy, since higher levels of estrogen and transcortin occurring in females throughout life could protect hippocampus. In this view, it has been shown that in comparison with male rats, females exposed to chronic restraint stress exhibited higher corticosterone levels but lower hippocampal atrophy [16].

In conclusion, our results demonstrate for the first time, that chronic IGF-1 treatment in aged females stressed during fetal life reduces spatial learning impairment in the water maze. Although additional work is necessary to unravel the mechanisms underlying the beneficial effect of IGF-1, it could be hypothesized that hormonal changes (reduced corticosterone and increased estradiol levels) induced by IGF-1 treatment may help to restore brain plasticity and/or neuroprotection of the hippocampus. These results support the interest of studying the effects of IGF-1 in age-related disorders and reinforce the hypothesis that early adverse events may have a profound impact on future adaptive abilities of an organism throughout his life-time, from adulthood to senescence.

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Estradiol, insulin-like growth factor-I and brain aging

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Tau

Summary
The decrease in some hormones with aging, such as insulin-like growth factor-I (IGF-I) and estradiol, may have a negative impact on brain function. Estradiol and IGF-I may antagonize the damaging effects of adrenal steroids and other causes of brain deterioration. The signaling of estradiol and IGF-I interact to promote neuroprotection. Estrogen receptor α, in an estrogen-dependent process, can physically interact with IGF-I receptor and with the downstream signaling molecules of the phosphotidylinositol 3-kinase (PI3K)/Akt/glycogen synthase kinase 3 (GSK3) pathway. Estradiol and IGF-I have a synergistic effect on the activation of Akt, which in turn decreases the activity of GSK3. This may be one of the mechanisms used by estradiol to promote neuronal survival, since the inhibition of GSK3 is associated to the activation of surviving signaling pathways in neurons. Furthermore, estradiol may control Tau phosphorylation by modulating the interactions of estrogen receptor α with GSK3 and β-catenin, another molecule involved in the regulation of neuronal survival and the reorganization of the cytoskeleton. All these actions may be involved in the neuroprotective effects of the hormone. Possible aging-associated changes in the expression or activity of these signaling molecules may affect estradiol neuroprotective effects. Therefore, it is important to determine whether aging affects the signaling of estradiol and IGF-I in the brain.
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1. Introduction

The aging process affects all tissues and organs, including the brain. Individual variations in decline of cognitive skills, development of affective disorders and neurodegenerative diseases with aging suggest that brain deterioration is not only the result of age per se and probably represents a

Abbreviations: ERE, estrogen response elements; ERKs, extracellular-regulated kinases; GSK3, glycogen synthase kinase 3; HPA, hypothalamo–pituitary–adrenal; IGF-I, insulin-like growth factor-I; IRS-1, insulin receptor substrate-1; MAPK, mitogen-activated protein kinases; PI3K, phosphotidylinositol 3-kinase

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failure to adapt to age-associated homeostatic changes (Mattson and Magnus, 2006). Hormones are involved somewhat in the aging process since the levels of many of them change in plasma with aging. Several hormones such as growth hormone, IGF-I, dehydroepiandrosterone and sex hormones decrease with aging in mammals (Lamberts et al., 1997). In humans, their change is associated in time with the progression of neurodegenerative disorders, increased depressive symptoms, and other psychological disturbances (Resnick and Maki, 2001; Azcoitia et al., 2003; Sonntag et al., 2005). This suggests that the modification in hormone levels with aging may have a negative impact on brain function. Alternatively, since the brain is an important center for endocrine control, brain aging may be involved in the hormonal changes. These hormonal changes may represent a positive adaptive response to the aging process, since accumulating evidence suggests that animal lifespan may be subject to endocrine regulation (Holzenberger et al., 2004).

2. Influence of IGF-I and estradiol on age-related disorders

Even if the hormonal changes are a general positive adaptation to aging, they may have a negative impact on the brain. The decrease in the levels of neuroprotective hormones in aged people may result in a reduced protection against the environmental and genetic factors that promote neurodegeneration. However, not all hormones exert neuroprotective effects. Considerable evidences show a link between stress, hypothalamo–pituitary–adrenal (HPA) axis dysfunction, memory disorders and aging (Sapolsky et al., 1986; McEwen, 2002a). The hippocampus is one of the brain areas the most vulnerable to stress and to aging processes. Stress and stress hormones alter hippocampal neurogenesis (Mirescu and Gould, 2006) and may lead to hippocampal damage. Not only stress in adulthood may increase brain damage and brain aging. Even stress during the brain development may result in permanent brain abnormalities in adult life. Prenatal stress increases anxiety-like behavior and induces dysfunction of the negative feedback of the HPA axis (Maccari et al., 2003). With aging, rats subjected to prenatal stress exhibit hyperactivity of the HPA axis associated to spatial learning impairments (Vallée et al., 1999; Darnaudéry et al., 2006).

Some hormones, such as estradiol and IGF-I, may antagonize the damaging effects of adrenal steroids. IGF-I attenuates spatial learning deficits in aged rats and promotes neurogenesis in the hippocampus (Darnaudéry et al., 2006). Chronic IGF-I infusion in the brain restores the spatial learning abilities of aged rats that were stressed during prenatal life (Fig. 1A). IGF-I also up-regulates neurogenesis in the hippocampus of these animals and reduces their HPA axis dysfunction (Fig. 1B and C). Interestingly, IGF-I increases estradiol levels in the plasma

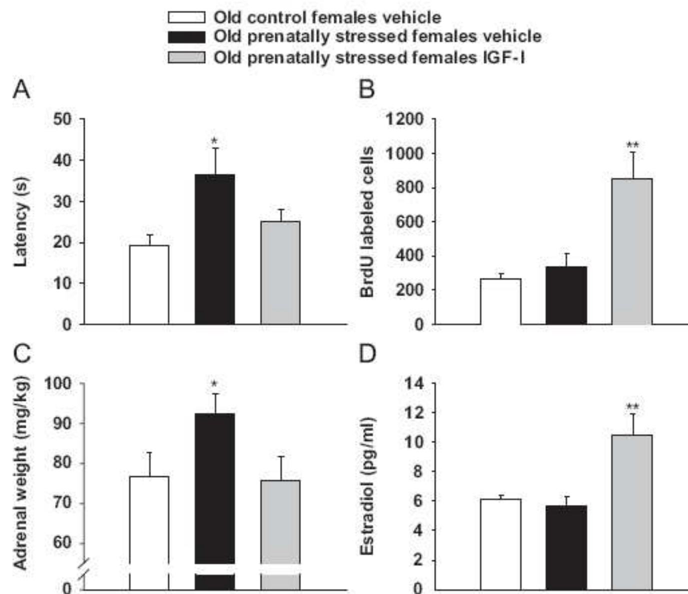

Figure 1 Impact of chronic intracerebroventricular IGF-I treatment in old female rats subjected to prenatal stress. (A) IGF-I attenuates aged-related impairments of prenatally stressed females in the water maze test, * $p<0.05$ versus old control females treated with vehicle (NaCl). (B) IGF-I increases cell proliferation in the hippocampus of prenatally stressed female animals, ** $p<0.05$ versus old prenatally stressed females treated with vehicle. (C) Adrenal gland hypertrophy observed in prenatally stressed animals is attenuated after IGF-I administration, * $p<0.05$ versus old prenatally stressed females treated with vehicle. (D) Plasma level of estradiol is enhanced after IGF-I treatment in prenatally stressed rats, ** $p<0.05$ versus old prenatally stressed females treated with vehicle.

of aged rats that were submitted to prenatal stress (Fig. 1D). Estradiol, in turn, stimulates neurogenesis in the hippocampus of young and old rats and prevents hippocampal damage induced by excitotoxic injuries (Garcia-Segura et al., 2001). In addition, the signaling of estradiol and IGF-I interacts to promote neuroprotection (Mendez et al., 2005). Therefore, different hormones affected by the aging process may act in cooperation or antagonistically. In consequence, low levels of protective hormones, such as IGF-I and estradiol, in old individuals may increase the risk of neural damage induced by stress hormones or by previous stressful experiences. To understand how these hormones affect the aging process in the brain and to develop adequate protocols for possible hormone therapies to prevent brain deterioration, we need to know their neuroprotective mechanisms and how these are modulated in the aged brain.

Estradiol regulates gene expression, neuronal survival, neuronal and glial differentiation and synaptic transmission (McEwen, 2002b). It is also well established that estradiol has neuroprotective and reparative properties and prevents cognitive and neuronal loss in several experimental animal models of neurodegeneration (Garcia-Segura et al., 2001). However, there has not been an unambiguous translation of the data from animal models to human studies. The results from hormone therapy (estrogens and progestins) or estrogen-only therapy on neurological and cognitive function in women are controversial (Resnick and Maki, 2001; Saunders-Pullman, 2003; Wise, 2003; Brinton, 2004) and emphasize the need for a better understanding on the effects of estradiol in the brain.

3. Interactions between estradiol and IGF-I signaling pathways

As in other tissues, the effects of estradiol in the nervous system may be exerted by the activation of classical nuclear estrogen receptors α and β (Fig. 2). In addition, estradiol may elicit rapid actions in the nervous system by the interaction with the cytoplasmic and membrane compartments of neurons and glial cells (Kelly et al., 2002; McEwen, 2002b; Beyer et al., 2003; Toran-Allerand, 2004). Estradiol may also act in the nervous system through non-specific receptors, such as neurotransmitter ion channels (Fig. 2) and through non-receptor-mediated mechanisms, for example, as an anti-oxidant (Behl, 2002). The actions of estradiol

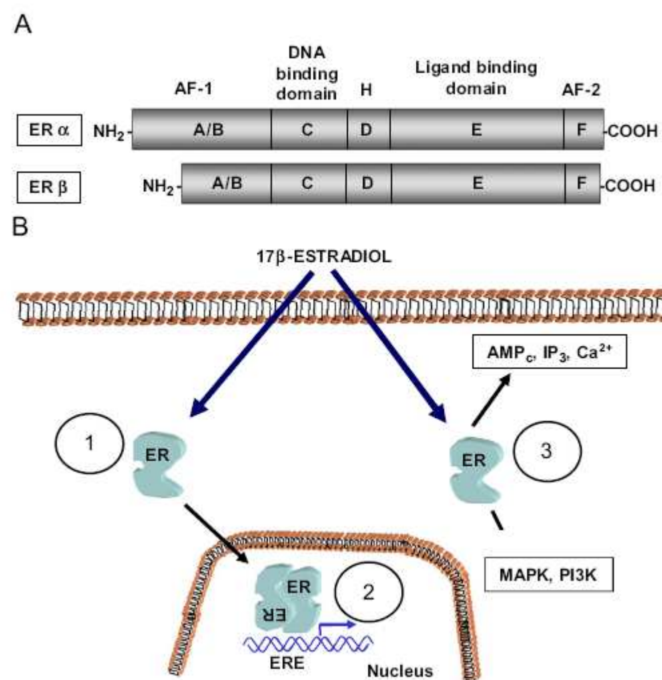

Figure 2 Estradiol acts on the brain by multiple mechanisms that may be affected by aging. The classical mechanism of action is via nuclear estrogen receptors. (A) Schematic representation of nuclear estrogen receptors, showing the different protein domains. (B) Estradiol binds to the ligand-binding domain and activates the receptors (1). Then, the receptors homo- or heterodimerize and bind, via their DNA-binding domain, to estrogen response elements (ERE) in the promoters of target genes to regulate transcription (2). In addition, estradiol may elicit rapid actions by the interaction with cytoplasmic and membrane signaling (3). Estradiol may interact with membrane receptors, ion channels or with uncharacterized cytoplasmic molecules and modify the intracellular levels of calcium, ATP and the activity of kinases, affecting signaling pathways such as the MAPK pathway or the PI3K/Akt pathway. These membrane and cytoplasmic effects allow the interaction of estradiol with the signaling of growth factors, such as IGF-I.

at the membrane and the nuclear receptors are inextricably linked: the gene products generated by estradiol-dependent activation of nuclear receptors and transcription can be post-transcriptionally modified by cell signals activated by membrane estrogen receptors. Transcription itself can be augmented or reduced by co-activators and co-repressors previously modified through membrane-associated estrogen actions (Fig. 2). Finally, estradiol not only drives the transcription of genes whose promoters bind nuclear estrogen receptors, but also of genes that are transactivated by other transcription factors modified after membrane estradiol signaling.

We do not have an adequate knowledge yet on how aging regulates affects all these mechanisms of action of estradiol in the brain. Changes in the expression of membrane and nuclear estrogen receptors, in the availability of transcriptional cofactors or in the interaction of estrogen with the signaling of other factors may result in different effects of the hormone in young and aged brains. One of the factors that may regulate estradiol signaling is IGF-I, one of the hormones that decline in plasma with aging.

As in other organs, IGF-I signaling in the brain is modulated by IGF-binding proteins and IGF-I receptors. Within the brain there is abundant co-expression of nuclear estrogen receptors and IGF-I receptor in the same cells and both factors cooperate in neuroprotection in an excitotoxic *in vivo* animal model of hippocampal injury and in an experimental model of Parkinson disease (Mendez et al., 2005).

IGF-I can activate extracellular-regulated kinases (ERKs), members of the family of mitogen-activated protein kinases (MAPK), leading to the phosphorylation of estrogen receptors. In parallel, estrogen receptor α, in an estrogen-dependent process, can physically interact with IGF-I receptor and with the downstream proteins insulin receptor substrate-1 (IRS-1) and PI3K, enhancing IGF-I signaling in the brain (Mendez et al., 2005). The neuroprotective actions of estradiol may be in part mediated by the activation, through the IGF-I receptor-signaling cascade, of the anti-apoptotic kinase Akt. In addition, estradiol induces a transient activation of GSK3 in the adult female rat hippocampus, followed by a more sustained inhibition. This hormonal action may enhance neuronal survival, since the inhibition of GSK3 is associated to the activation of surviving signaling pathways in neurons. Estradiol also regulates the interaction of estrogen receptor α with GSK3 and β-catenin, another molecule involved in the regulation of neuronal survival and the reorganization of the cytoskeleton. Furthermore, estradiol controls the interaction of the microtubule-associated protein Tau with GSK3, β-catenin and elements of the PI3 kinase complex and reduces the hyperphosphorylation of Tau, one of the molecular markers of Alzheimer's disease. All these actions may be involved in the plastic and neuroprotective effects of the hormone (Fig. 3).

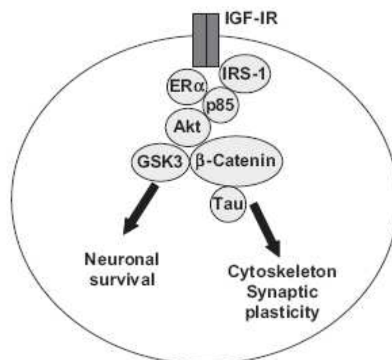

Figure 3 Postulated interactions of estrogen receptor α (ERα) with components of the IGF-I receptor (IGF-IR) signaling cascade based on immunoprecipitation experiments in the rat brain. Estradiol induces the association of ERα to a macromolecular complex including insulin receptor substrate-1 (IRS-1), the p85 subunit of the PI3K, the anti-apoptotic kinase Akt, the kinase GSK3, β-catenin and the microtubule-associated protein Tau. These interactions may mediate effects of estradiol on neuronal survival, cytoskeletal remodeling and synaptic plasticity.

4. Conclusion

The intricate association of estradiol and IGF-I signaling may be highly relevant for their effects in the aging brain. Aging-associated changes in IGF-I levels, IGF-binding proteins, IGF-I receptors and IGF-I receptor-associated signaling molecules may affect the neuroprotective effects of estradiol in the brain. Therefore, to predict the outcome of hormonal therapies we need first to determine how aging affects the signaling of estradiol and IGF-I in the brain.

Role of the funding source

University of Lille 1, USTL (France) contributed with editorial assistance, reviewed drafts of the manuscript and contributed to the decision to submit the manuscript for publication. The authors retained full editorial control and responsibilities throughout the preparation of the manuscripts.

Conflict of interest

None declared.

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STRESS PRECOCES ET SURCHARGE ALLOSTASIQUE CHEZ LA DESCENDANCE

Etat de stress post-traumatique

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Effects of a single footshock followed by situational reminders on HPA axis and behaviour in the aversive context in male and female rats

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CRH;
Memory;
Stress

Summary Gender is an important factor in the vulnerability to develop psychopathologies. At the biological level, stress-related pathologies such as depression or post-traumatic stress disorder (PTSD) are associated with profound disturbances of the hypothalamo-pituitary-adrenal (HPA) axis. The aim of the present study was to assess sex-differences in the long-term effect of an intense stressful procedure on HPA function and behaviour in the aversive context in rats. Female and male rats experienced an aversive procedure consisting in an electric footshock (2 mA, 10 s) in a dark chamber followed by 3 weekly situational reminders (SR, 2 min in the white chamber close to the footshock chamber). Our results indicate that 41 days after the end of the aversive procedure, female rats showed an increase of the corticosterone negative feedback in response to restraint stress, whereas such effect was not observed in males. Despite this change in the hormonal response, glucocorticoid receptors mRNA expression in the hippocampus was not affected in shocked females. In contrast, a significant increase of the mineralocorticoid receptors mRNA was observed in the CA2 of the hippocampus in shocked males. Finally, CRH mRNA levels in the paraventricular nucleus of the hypothalamus (PVN) were decreased in both female and male animals exposed to the aversive procedure. Behavioural observation revealed that shocked males and shocked females showed a high level of avoidance. However, the latency to visit the shock box was lower in females, which spent also more time in this area than males. In conclusion, our results suggest that gender might be a key factor impacting the direction of the effects induced by an intense stress. Interestingly, only females exhibited an

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increased negative feedback of the HPA axis response to stress, which could parallel endocrine changes of PTSD.
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1. Introduction

Epidemiological studies demonstrate that women are more vulnerable than men to stress-related psychopathologies (Carter-Snell and Hegadoren, 2003). For instance, after confrontation to a traumatic event, women are twice as likely as men to meet criteria for post-traumatic stress disorder (Stein et al., 2000). At the biological level, stress-related psychopathologies share profound alterations of the HPA axis (Ehlert et al., 2001) and, sex-differences in the HPA function have been proposed to contribute to the greater vulnerability to stress in women (Young, 1998). However, whereas sex-differences in the structure of limbic regions as well as differences in circulating sex steroids and transcortin levels are well established, sex-dimorphisms in stress-related HPA axis responses remain inconsistent (Kudielka and Kirschbaum, 2005).

In rats, sex-differences in HPA axis responses to stress are clearly described from many years. Indeed, as early as 1960s, several studies have pointed out that females exhibit higher levels of corticosterone than males in basal conditions and in response to an acute stress (Kitay, 1961). Female animals show a higher magnitude and duration of corticosterone response after both neurogenic (Kitay, 1961; Weinstock et al., 1998) and systemic stressors (Harbuz et al., 1999; Rivier, 1999). However, despite marked differences between males and females in corticosterone levels, sex-dimorphisms in the central pathways of the HPA axis are more contradictory. In this view, it has been reported an increase of the expression of CRH in the PVN of male rats after acute or chronic stress, but no significant variations are observed in female animals (Duncko et al., 2001; Viau et al., 2005). Similarly, corticosteroid receptors mRNA levels are more affected by an acute stress in males than in females (Karandrea et al., 2002). Finally, even if a single adverse event may have long-term consequences (van Dijken et al., 1993; Valles et al., 2002; Armario et al., 2004), most studies examining sex differences only focus on the immediate effects of stress.

We have recently reported in female rats that an intense stress (2 mA, 10 s footshock) followed by SRs could induce lasting behavioural disturbances associated with an hypocorticosteronism in response to the stress context (Louvart et al., 2005a). This model developed by Pynoos et al. (1996), has been proposed to be a good tool to study interindividual differences in behavioural response to an intense stress (Rasmusson and Charney, 1997). Therefore, the aim of the present study was to assess sex-differences in the HPA axis reactivity and behaviour in rats exposed to this traumatic procedure. More than 2 months after the footshock, male and female rats were compared for the corticosterone response to restraint stress, corticosteroid receptors and the CRH expression as well as for behaviour in the context of the aversive procedure.

2. Methods and materials

2.1. Animals

Three weeks before the experiment, three-months old Sprague-Dawley female and male rats (Harlan, France) were individually housed under controlled conditions of temperature (23$\pm$1 °C) and photoperiod (lights from 0800 to 2000 h), with food and water ad libitum. Manipulation of the animals was performed following the principles of laboratory animal care published by the French Ethical Committee and the rules of the European Union Normative (86/609/EEC).

2.2. Aversive procedure

Treatments were randomly assigned to animals in order to obtain four groups: no shock males ($n=14$), no shock females ($n=14$), shock males ($n=14$) and shock females ($n=14$). On day 0, animals were exposed to an aversive procedure. This procedure consisted in a single exposure to footshock on day 0 followed by three weekly exposures to SR, on days 7, 14 and 21 (Louvart et al., 2005a,b). After a 2-min adaptation period, a guillotine door was opened and a bright light was turned on in the first compartment. The door remained open until the animal has entered the second compartment, which remained dark. Then, the door was closed and the rat received an inescapable 2 mA footshock for 10 s in the dark compartment. The no shock groups received the same treatment, but with the shock mechanism inactivated. All groups were exposed to a reminder of the situation for 2 min, once per week for 3 weeks. This was accomplished by placing the animal in the light compartment with the door closed to prevent

any entry in the shock compartment. Exposure to SRs has been proved to induce behavioural sensitisation and, this type of SR was chosen to impede extinction, which might occur if the animal had entered the shock compartment without receiving electric footshock (Pynoos et al., 1996).

2.3. Restraint stress procedure and corticosterone radioimmunoassay

Forty one days after the last SR, each animal was exposed to restraint stress in a plastic tube (7 cm diameter, 19 cm long) under bright light for 30 min. Blood was collected from the tail vein in tubes containing EDTA immediately after the rat was placed in the restrainer (less than 2 min after removal from the home cage), 30, 60 and 120 min after the start of the restraint stress. To minimise the effects of circadian rhythms on corticosterone concentrations, blood was collected between 0830 and 1200 h. Aliquoted plasma samples were stored at −20 °C until assayed. Plasma corticosterone levels were determined using a radioimmunoassay kit (^{125}I corticosterone; ICN Biomedicals, France). The sensitivity of the assay was 0.1 μg/100 ml. The average intra-assay and inter-assay coefficients of variation were, respectively, 9 and 10%.

2.4. Tissue collection and in situ hybridisation

Forty-nine days after the last SR, animals were sacrificed. A vaginal smear was performed and the stage of the oestrous cycle was microscopically checked. Brains were quickly removed, frozen on dry ice and stored at −80 °C until sectioning. Coronal sections of the brain (12 μm) were cut in a cryostat at −20 °C, mounted onto twice gelatine-coated slides, dried on a slide warmer and kept at −80 °C. Sequential matched sections of the hypothalamus at the level of the PVN (Bregma −1.3 to −2.12 mm; Paxinos and Watson, 1998) and of the amygdala at the central nucleus level (CeA, Bregma −2.12 to −2.80 mm; Paxinos and Watson, 1998) were hybridised for CRH mRNA detection. Hippocampal sections (Bregma −3.60 to −4.30 mm; Paxinos and Watson, 1998) were hybridised for GR and MR mRNA detection. In situ hybridisations were performed as previously described (Leonhardt et al., 2002; Lesage et al., 2002). The CRH probe was a 770-bp *Bam*H1 fragment of the rat CRH gene (Thompson et al., 1987) subcloned into pGEM3 (supplied by Dr L. Bain, University of Michigan, Ann Arbor, USA) and linearised with *Hind*III (antisense probe). The MR and GR probes were, respectively, 513-and 674-bp fragments of rat cDNA clones encoding the 3′-regions of MR and GR mRNAs, subcloned, respectively, into pGEM4 and pGEM3 (supplied by Dr J.R. Seckl, University of Edinburgh, Edinburgh, UK) and linearised, respectively, with *Hind*III and *Ava*I (antisense probes). Riboprobes were labelled using [^{35}S] uridine triphosphate (Amersham Biosciences, France) and synthesised with Sp6/T7 transcription kit (Roche diagnostics, Germany). Controls included hybridisation with sense probes; no specific hybridisation signals were observed in these conditions.

2.5. Quantification of the hybridisation signal

For each treatment group, we respectively used 4-6 and 6-8 animals for CRH and MR or GR quantification. Sections from no shock and shock groups were processed in the same hybridisation and exposed together at room temperature to Biomax film (Kodak, France). The autoradiographic hybridisation signal was quantified using GS-700 densitometer coupled with a computer-assisted image analysis system using Multi-Analyst software (Bio-rad Laboratories, USA). The values for each rat were calculated from an average of measurements in two or three matched sections after substraining background. For MR and GR hybridisation, optical densities (OD) were measured in the whole hippocampus i.e. in CA1, CA2, CA3 and dentate gyrus (DG) areas. Similarly, CRH hybridisation signal was measured in the PVN and in the CeA. Results were expressed as OD per square millimetre.

2.6. Behaviour in the aversive context

Forty-nine days after the last SR, each rat was placed for 5 min in the light compartment (no shock box) of the footshock apparatus, its head facing the wall opposite to the opened door. The latency to visit the footshock compartment, the time spent in this area, the number of visit and the duration of each visit were assessed as well as the number of rearing per minute in the white compartment. A latency of 300 s was attributed when an animal never explored the dark compartment. Behaviour was recorded with a video camera for offline manual scoring with the help of Observer V5.0 software (Noldus, The Netherlands).

2.7. Statistical analysis

Statistics were performed using Statistica package (Version 6, Statsoft, France). Corticosterone

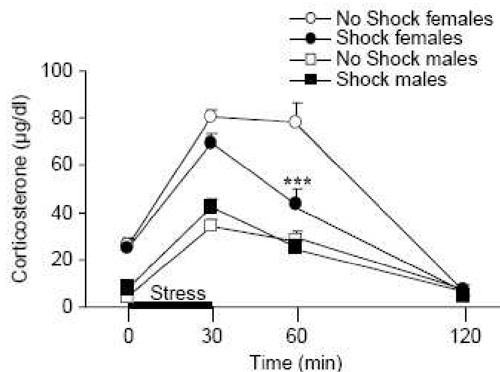

Figure 1 Corticosterone (mean ± SEM, µg/dl) response to a 30 min restraint stress in male and female rats, 41 days after the last SR. ***$P<0.001$ No shock females versus Shock females.

response to stress and hybridisation measures were assessed by analysis of variance (ANOVA) with sex (male, female) and treatment (no shock, shock) as between factors. Post-hoc Scheffe's test were used to assess significant differences between specific groups. Since, the majority of shocked males reached the cut off (i.e. 300 s) for the latency to enter the shock compartment or for the time spent in the shock compartment, behavioural data were analysed with non parametric analysis of variance Kruskall-Wallis, followed by Mann-Whitney tests when a significant group effect was observed. $P<0.05$ indicated a significant difference.

3. Results

3.1. Corticosterone response to restraint stress

Regardless of the treatment, female rats had higher levels of corticosterone than male rats (sex effect, $F_{(1,52)}=127.96$, $P<0.0001$, Fig. 1). Forty-one days after the last SR, the aversive procedure affected corticosterone response to restraint stress only in female rats (sex × treatment × time effect, $F_{(3,156)}=4.31$, $P<0.01$). 60 min after the restraint stress, shocked female exhibited blunted plasma levels of corticosterone in comparison to the no shock female group (post hoc Sheffe's test, $P<0.001$).

3.2. Central corticosteroid receptors and CRH mRNA expression

All but four female rats were in proestrous at the time of the sacrifice, the four resting were in metoestrous (three from the no shock and one from the shock groups). All animals were included in the present study, since data of female rats in metoestrous did not differ from the others, all animals were included in the present study.

The aversive procedure tended to differentially affect the distribution of MR mRNA in the four subfields of the hippocampus in male and female animals (treatment × sex × subfields effect, $F_{(3,72)}=2.53$, $P=0.06$). In female rats, MR mRNA were not affected by the aversive procedure (treatment effect, $F_{(1,12)}=0.03$, ns, Fig. 2a). In male rats, the aversive procedure increased MR mRNA in the CA2 area (treatment × subfields effect, $F_{(3,33)}=4.26$, $P<0.05$; post hoc Sheffe test in CA2 no shock versus shock, $P<0.05$, Fig. 2c).

GR mRNA were differently distributed in the four subfields of the hippocampus (subfields effect, $F_{(3,78)}=1167.3$, $P<0.001$, Fig. 2(b-d)). GR mRNA levels were neither affected by sex (sex effect, $F_{(1,26)}=1.23$, ns) nor by the aversive procedure (treatment effect, $F_{(1,25)}=0.001$, ns).

Male and female rats showed similar CRH mRNA levels in the PVN (sex effect, $F_{(1,18)}=0.09$, ns) and in the CeA (sex effect, $F_{(1,19)}=2.93$, ns). As shown in Fig. 3, the aversive procedure induced a decrease of CRH mRNA levels in the PVN (treatment effect, $F_{(1,18)}=15.89$, $P<0.001$). In contrast, CRH mRNA levels in the CeA were not affected by the aversive procedure (treatment effect, $F_{(1,19)}=2.84$, ns, data not shown).

3.3. Behaviour in the aversive context

As depicted in the Table 1, the exploration of the aversive context differed across groups (Kruskall-Wallis, exploration of the footshock compartment: latency to enter, $H_{(3,56)}=29.28$, $P<0.0001$; time spent, $H_{(3,56)}=30.23$, $P<0.0001$; number of visits, $H_{(3,56)}=30.32$, $P<0.0001$; duration of visits, $H_{(3,44)}=16.5$, $P<0.0001$). Groups comparison indicates that both shocked males and shocked females increased their latency to visit the shock compartment (Mann-Whitney, females: $U=28$, $P<0.001$, males: $U=15$, $P<0.0001$) and reduced the time spent in this box (females: $U=17.5$, $P<0.0001$, males: $U=17$, $P<0.0001$). Shocked males also showed a significant decrease of the number of visits to the footshock compartment ($U=16.5$, $P<0.0001$), whereas it was unchanged in shocked females ($U=94.5$, $P=0.87$). In fact, only 22% of the population explored the shock compartment in male animals, while the percentage reached 93% in females. However, in female rats, the aversive procedure induced a significant reduction of the duration of visit to the shock

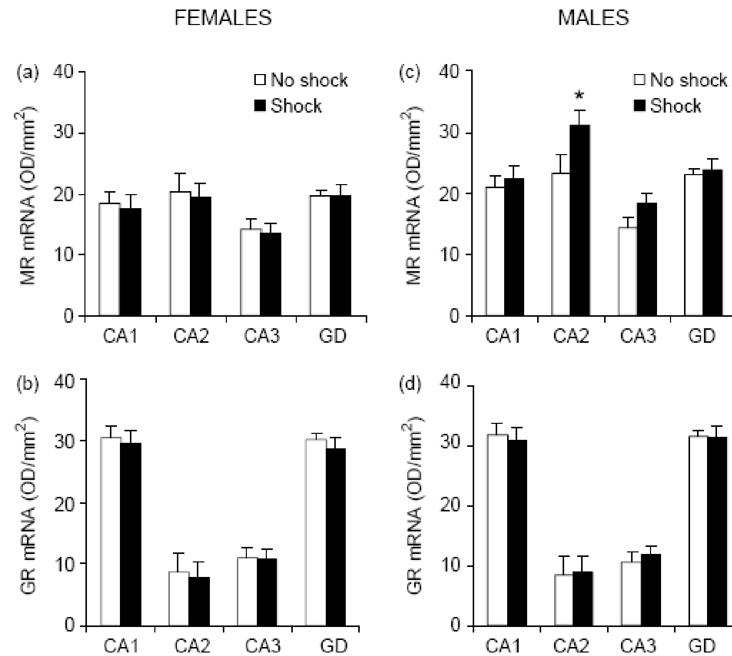

Figure 2 Corticosteroid receptors mRNA in the different areas of the hippocampus 49 days after the last SR. Left panel: MR (a) and GR (b) mRNA levels (mean±SEM, OD/mm^2) in female rats. Right panel: MR (c) and GR (d) mRNA levels (mean±SEM, OD/mm^2) in male rats. *$P<0.05$ No shock males versus Shock males.

compartment ($U=49.5$, $P<0.05$), whereas it remained stable in shocked males ($U=20.5$, $P=0.95$). The avoidance of the footshock compartment was higher in shocked males than in shocked females (latency to enter $U=29$, $P<0.001$, time spent, $U=45$, $P<0.05$, number of visit, $U=14.5$, $P<0.0001$), but shocked females exhibited greater activity in the light box than shocked males (rearing per minute, group effect: $H_{(3,56)}=9.23$, $P<0.05$, shocked females versus shocked males: $U=41.5$, $P<0.01$).

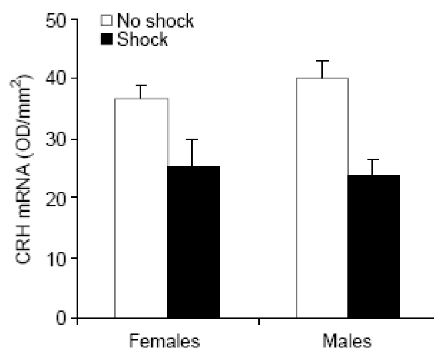

Figure 3 CRH mRNA levels (mean±SEM, OD/mm^2) 49 days after the last SR, in the PVN of male and female rats.

4. Discussion

Aim of this study was to evaluate sex differences in the long-term effects of an intense stressful procedure on HPA axis and on behaviour in the aversive context in male and female rats. Although central HPA axis alterations after a single brief footshock administration followed by repeated SRs were observed for both sexes, this paradigm induced long-lasting peripheral HPA axis changes only in females. Furthermore, male and female animals exhibited long-term retention of the aversive event, while they showed differential behavioural response in the aversive context.

We demonstrate in the present study, that a traumatic procedure does not alter the corticosterone response to stress in male rats, while such response is attenuated in females, 41 days post-shock. This work is the first demonstration of a sex-dependent HPA axis response to a heterotypic stress and, suggests that female rats are more sensitive to long-lasting changes in the corticosterone response to stress. An increase of the HPA axis feedback has been described using an homotypic stress of immobilisation in male rats (Marti et al., 2001; Dal Zotto et al., 2003) and, we have previously observed reduced corticosterone levels

Table 1 Behaviour in the footshock apparatus 49 days after the last SR in male and female rats.

	Females		Males	
	No Shock	Shock	No Shock	Shock
Footshock compartment				
Latency to enter (s)	13.6±3.5	79.1±24.5[a]	31.7±8.3	243.5±30.1[b,c]
Time spent (s)	260.4±9.9	157.1±21.2[a]	266.4±8.3	55.3±36.9[b,c]
Number of visit	2.6±0.5[d]	2.5±0.4	1.2±0.1	0.2±0.1[b,c]
Duration of visit (s)	157.4±26.8[d]	91.9±23.1[a]	244.9±15.4	263.7±13.7
Population which enter (%)	100	93	100	22
Light compartment				
Rearing per minute	2.9±0.8	2.8±0.6	1.1±0.5	1.2±0.5[c]

At least $P<0.05$.
[a] Shock females versus No shock females.
[b] Shock males versus No shock males.
[c] Shock females versus Shock males.
[d] No shock females versus No shock males.

in shocked females after the re-exposure to the footshock apparatus (Louvart et al., 2005a). In contrast, results on heterotypic stress are more conflicting in the literature (van Dijken et al., 1993; Marti et al., 2001). In our study, corticosterone levels were assessed in response to a restraint stress (first exposure to this stress), suggesting that the blunted corticosterone response observed in females does not reflect a habituation. Social environment strongly influences the HPA function and thus, we cannot exclude that our results are modulated by a differential vulnerability to social isolation in male and female animals (Weiss et al., 2004). Also, the aversive procedure has a different effect on social behaviour in male and female rodents. Indeed, Pynoos et al. (1996) mentioned a high level of aggressive behaviour in male mice exposed to the footshock procedure and, we have recently observed a decrease in social behaviour in shocked female rats (Louvart et al., 2005a).

We observed sex differences in MR expression following the aversive procedure, with an increase of mRNA levels in the hippocampal CA2 area in males and no change in females. Although the functional significance of the heterogeneous neuronal expression of MR in the hippocampal subfields still remains unknown (de Kloet, 2003), previous works suggest that specific manipulations selectively modulate MR mRNA levels in the CA2 of the hippocampus. Indeed, amygdala kindling (Clark et al., 1994) or chronic antidepressive treatment in aged rats (Yau et al., 2002) increase MR mRNA expression in the CA2 area. Our results confirm that MR levels exhibited a sexually dimorphic regulation upon stress and, suggest that differential effects may be observed after acute and chronic stress, as well as when effects are studied short or long-term after stress (Karandrea et al., 2002). GR expression was not affected by the intense stress procedure. GR are involved in the increased feedback of the HPA axis (de Kloet et al., 1998; Liberzon et al., 1999). Surprisingly, hippocampal GR mRNA levels were not affected in shocked females which exhibited change in their corticosterone response. However, we cannot exclude a change in the affinity or the number of glucocorticoid receptors in this group. Furthermore, the increase of the negative feedback of the HPA axis may be due to non-classical genomic effects of GR (Makara and Haller, 2001). CRH in the PVN is the central node of the HPA axis and CRH in the CeA is implicated in behavioural fear (Liang et al., 1992). In our study, we demonstrate that the aversive procedure induces a long-term decrease of CRH expression in the PVN without affecting the expression in the amygdala. Our results contrast with the heightened CRH expression often observed after different kinds of psychogenic stress (Albeck et al., 1997; Hsu et al., 1998; Givalois et al., 2000; Viau et al., 2005). However, CRH variations in the PVN strongly depend on the procedure used, since CRH increases after repeated exposure to footshock (Bruijnzeel et al., 2001) and decreases after repeated exposure to immobilisation stress (Marti et al., 2001) or water deprivation stress (Sebaai et al., 2002).

In the present work, behavioural observation reveals that 70 days after the footshock exposure, both male and female rats exhibit a marked increase of the latency to visit the footshock compartment and a decrease of the time spent in this area. These results extend our previous findings obtained in female rats, using the black and white box test, which indicate a high latency to visit the black compartment that recalled the shock

compartment (Louvart et al., 2005a). We demonstrate here, that both genders are affected by the traumatic procedure, but they exhibit a different behavioural response to the aversive situation. In comparison to male animals, which in most case never explore the footshock box, female rats return in the aversive compartment. However, when shocked males returned to the footshock compartment (22% of the population), they showed the same duration of visits as non-shocked males, suggesting an extinction of the conditioned response. The lower avoidance of the traumatic context in females could be explained by their high locomotor activity (rearing per minute). Indeed, our results extend the observation that sex differences in learning may be attributable to differences in motor performances (Shors et al., 2000). Memory consolidation is related to corticosterone levels elicited by training (Sandi et al., 1997; Cordero et al., 2002) and, it is well known that females release more corticosterone than males in response to stress. In this view, we have reported that corticosterone levels in response to a footshock are positively correlated to a long-term avoidance in female rats (Louvart et al., 2005a). Therefore, our results may reflect a conflict in shocked females between the aversion for the light environment and the avoidance of the dark box associated with the footshock. Interestingly, we have recently documented a similar behaviour when we applied the same footshock procedure in the female offspring from mothers submitted to chronic restraint stress during the last week of pregnancy as compared to the shocked offspring of undisturbed mothers (Louvart et al., 2005b).

Although the mechanisms underlying the differential avoidance between males and females (as well as between prenatally stressed and control animals) remain to be explored, we could hypothesise that hippocampal BDNF is involved in this effect. Indeed, BDNF expression seems sex-dependant (Matsuki et al., 2001) and, Rasmusson et al. (2002) have recently shown that a re-exposure to the context associated to the footshock induces a reduction of the hippocampal BDNF expression in male rats.

In conclusion, the present study confirms that a single intense stress could induce long-lasting behavioural and neuroendocrinological disturbances in rats. Interestingly, only female animals exhibit an enhanced corticosterone negative feedback and at the behavioural level, despite a good memory of the traumatic event, females return more frequently in the aversive context. One has to note that some individuals with PTSD fail to exhibit conditioned avoidance or strategic planning when confronted with situations that should be regarded as potentially dangerous. Furthermore, women are more vulnerable than men to develop PTSD and, the increased sensitivity of the HPA axis feedback is a well-documented feature of PTSD (for review: Yehuda, 2002). Taken together, our results reinforce the importance to study both sexes in animal models of stress-related disorders.

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Long-term behavioural alterations in female rats after a single intense footshock followed by situational reminders

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Summary Post-traumatic stress disorder (PTSD) affects a vulnerable sub-population of individuals exposed to a traumatic event. This psychopathology induces long-lasting hypothalamo-pituitary-adrenal (HPA) axis hypoactivity, hyperarousal and avoidance of trauma-like situation. PTSD also manifests a high co-morbidity with anxiety disorders. The aim of the present study was to characterise long-term biobehavioural alterations in female rats in an animal model of PTSD consisting in an intense footshock (2 mA, 10 s) followed by three weekly situational reminders. This procedure induced several long-term alterations: increased anxiety behaviour, reduced time spent in an 'aversive-like' context, altered social behaviour and blunted corticosterone response to stress. These results demonstrate that exposure to an intense footshock associated with repeated situational reminders elicited long-term disturbances which lasted more than 1 month after the footshock administration. Our findings suggest that this paradigm could provide a useful animal model of PTSD.
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1. Introduction

Individuals confronted to a stressful experience exceeding their adaptive capacities (a traumatic event) might develop post-traumatic stress disorder (PTSD). This psychopathology is characterised by three long-lasting symptomatic clusters: re-experiencing (flashbacks, intrusive recollections, recurrent nightmares), avoidance of associated stimuli and hyperarousal (American Psychiatric Association, 1994). Moreover, at the biological level, PTSD patients present with hypocortisolism (Yehuda, 2000). In regard to the complexity to conduct prospective studies of PTSD in humans,

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numerous animal models have been developed using different types of traumatic events (for review, see Foa et al., 1992; Yehuda and Antelman, 1993; Ramusson and Chainey 1997). However, in these animal models, alterations are observed rather early after the traumatic paradigm (Servatius et al., 1995; Liberzon et al., 1997; Cohen et al., 2000, 2003; Wang et al., 2000), whereas PTSD is characterised by a long-lasting symptomatology (American Psychiatric Association, 1994).

Long-lasting effects of a single stress are somewhat difficult to obtain in animals. In fact, data on the time-course of footshock consequences remain unclear (Maier, 2001). Moreover, behavioural tests are often conducted in the context associated with the aversive event (Servatius et al., 1994; Wang et al., 2000). In other words, behavioural effects likely reflect a conditioned process. Yet, it has been proposed that the temporal persistence of PTSD results from re-experiencing (Brewin and Holmes, 2003). However, in animals, the concept of re-experiencing is difficult to address. Exposure to contextual cues present during an intense stressful situation may induce re-experiencing of the aversive event (Wagner, 1981; Maier, 2001; Gisquet-Verrier et al., 2004). Indeed, Maier (2001) demonstrates that exposure to an environment previously associated to a footshock is capable of extending behavioural effects of the footshock. The effectiveness of this environment does not weaken with multiple exposures, suggesting that environmental cues are themselves stressors. In this view, Pynoos et al. have developed a paradigm in which male mice are exposed to a brief intense footshock (2 mA, 10 s) followed by situational reminders (SR) of this aversive event (Pynoos et al., 1996). Such a procedure is the only demonstration of an apparent sensitisation of a behavioural response similar to a symptom of PTSD (Rasmusson and Charney, 1997). The effects of the footshock decline with time. Contrariwise, multiple exposures to SR induce over time a progressive increase in the magnitude of the startle reflex (Pynoos et al., 1996). Moreover, SR could parallel situations in humans in which traumatised individuals are often exposed to reminders of the trauma but not to the traumatic event itself (Brunet, 1996).

However, effects of this aversive procedure on other behavioural alterations and on plasma corticosterone responsiveness remain unknown. In this context, the aim of our study was to determine whether a single intense footshock followed by SR would lead to long-term consequences on anxiety-like disturbances, on behaviour in an aversive-like context and on social interaction in female rats. Furthermore, we assessed effects of the aversive procedure on corticosterone stress response.

2. Materials and methods

2.1. Animals

Animals were maintained on a 12 h light/dark cycle (light on: 08:00–20:00 h), with free access to food and water. Manipulation of the animals was performed according to the guidelines of laboratory animal care published by the French Ethical Committee and the rules of the European Union Normative (86/609/EEC). At 5 months of age, Sprague Dawley female rats (Iffa Credo, France) experienced the aversive procedure ($n=24$) or were decapitated for basal plasma levels of corticosterone ($n=4$). Twenty-eight females were individually housed 3 weeks before the experiment. Animals were not handled before the aversive procedure.

2.2. Aversive procedure

Female rats were randomly assigned to two groups: no shock and shock ($n=12$ per group). Another group ($n=4$) was added to determine basal diurnal levels of corticosterone in the home cage. On day 0, females were exposed to an aversive procedure. This procedure, adapted from Pynoos et al. (1996), consisted in a single exposure to footshock on day 0 followed by three weekly exposures to SR, on days 7, 14 and 21 (Fig. 1). This procedure, which included both an intense electric footshock and SRs, was chosen since it has been previously shown to induce behavioural sensitisation (Pynoos et al., 1996). Each rat was placed in the black compartment of a two-sided box. After a 2-min adaptation period, a guillotine door was opened and a bright light was turned on in the first plastic compartment. The door remained open until the animal entered the second plastic compartment, which was kept dark. Then, the door was closed and the rat received an inescapable 2 mA shock for 10 s in the dark compartment. The no shock group received the same treatment but the shock mechanism was inactivated. The two groups were exposed to reminders of the situation for 2 min once a week for 3 weeks. This was achieved by placing the animal in the light compartment with the door closed to prevent from any entry in the shock compartment. This SR was chosen to impede extinction which might have occurred if the animal

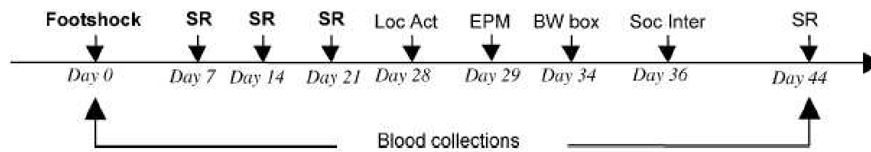

Figure 1 Experimental design. Briefly, rats were exposed to the aversive procedure followed by three weekly exposures to situational reminder (SR). Animals were then submitted to: elevated plus-maze (EPM), black and white box (BW box) and social interaction (Soc Inter). Blood samples were collected 5 min after exposure to the footshock or to a SR.

had entered the shock compartment without receiving a shock (Pynoos et al., 1996).

2.3. Behavioural tests

As depicted on Fig. 1, behavioural assessments were conducted after the aversive procedure. Behaviour was recorded with a video camera for offline scoring.

2.3.1. Elevated plus-maze

On day 29 post-footshock, behaviour was assessed for 5 min in the elevated plus-maze test. According to the description of Pellow et al. (1985), the wooden apparatus consisted of two open arms (50× 10 cm) alternating at right angles with two arms enclosed by 40 cm high walls. The four arms delimited a central area of 10 cm^2. The whole apparatus was placed 60 cm above the floor. The test began with the placement of the rat in the centre of the maze its head facing a closed arm. The time spent and visits in open and closed arms were recorded and a four-paws criterion was used for arm entries. Percentages of time spent and visits in open arms with respect to total time and visits in both open and closed arms were calculated.

2.3.2. Black and white box

The apparatus consisted of a wooden chamber subdivided into two compartments: a black compartment closed (30×32×40 cm high) and a white one open (45×32×40 cm high) (Costall et al., 1989). The compartments were connected by a small divider (10×15 cm high). On day 34, each rat was placed in the light compartment facing the wall opposite to the opening. Latency to enter the dark compartment and time spent in each compartment were assessed for 5 min.

2.3.3. Social interaction

Thirty six days after footshock administration, social behaviour was assessed during a 10-min session in a novel environment. The behaviour of each rat was recorded after introducing a pair of animals (same group, same treatment) in two opposite corners of an open box (45×32×40 cm high). Each session was manually scored with the help of Observer v2.0 software (Noldus, Wageningen, The Netherlands). Separate scores were obtained for each individual in a pair but, since the two values cannot be considered statistically independent, pair means were used for further analysis (File and Seth, 2003). Therefore, presented data refer to a total of six pairs of rats from each group. Three discrete social behaviours were selected: sniffing, allogrooming and crawling over the partner. Moreover, the number of social interactions and the time spent in social interaction were defined by the sum of sniffing, allogrooming and crawling over.

2.4. Corticosterone radioimmunoassay

To minimise the effects of circadian rhythm on corticosterone concentrations, blood was collected between 09:00 and 12:00 h (n=6-7 per group, except for basal levels: n=4 per group). For basal levels, animals were taken from their home cage and immediately decapitated. Five minutes after footshock on day 0 and 5 min after a reminder on day 44, blood was collected from the tail vein in tubes containing EDTA (Fig. 1). Aliquoted plasma samples were stored at −20 °C until assayed. Plasma corticosterone levels were determined using a radioimmunoassay kit (^{125}I corticosterone; ICN Biomedicals, France). The sensitivity of the assay was 0.1 μg/100 ml. The average intra-assay and inter-assay coefficients of variation were, respectively, 9 and 8%.

2.5. Statistical analysis

Statistics were performed using independent two-sided Student's t-test to compare the two groups (no shock, shock) or by analysis of variance (ANOVA) with treatment (no shock, shock) and time (day 0, day 44) as between factors. Pearson's coefficient was used to determine the correlation. Post hoc Scheffe's test was used to assess significant differences between specific groups. A significant difference was indicated by $P<0.05$.

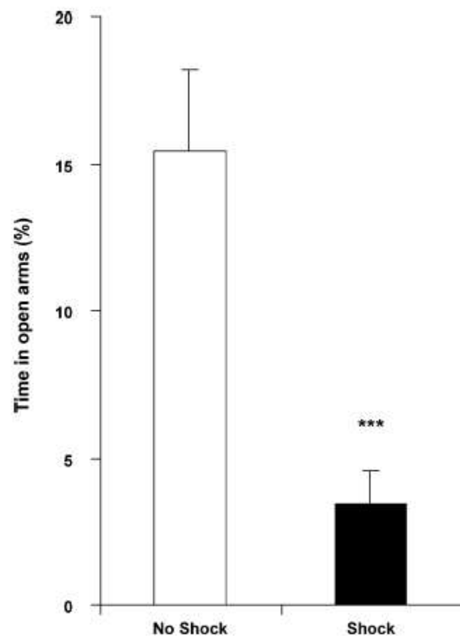

Figure 2 Elevated plus-maze. Percentage of time spent (mean $\pm$ SEM) in the open arms of the elevated plus-maze in shock and no shock groups. $^{***}P<0.001$ no shock group versus shock group. $n=12$ per group.

3. Results

3.1. Elevated plus-maze (8 days after the last SR)

Eight days after the end of the aversive procedure the percentage of time spent in the open arms was reduced (Student's t-test, $t_{(22)}=2.81$; $P<0.01$, Fig. 2) and the percentage of visits to those arms tended to be lower (no shock group: 21.8 ± 3.4; shock group: 12.1 ± 3.6; Student's t-test, $t_{(22)}=1.96$, $P=0.06$, data not shown). Moreover, the number of visits to the closed arms was reduced by previous footshock exposure (no shock group: 9.5 ± 0.5; shock group: 6.8 ± 0.8; $t_{(22)}=2.89$, $P<0.01$, data not shown).

3.2. Black and white box (13 days after the last SR)

The latency to escape from the white compartment was around 10 s and the time spent in this compartment was around 75 s in no shock animals. Animals shocked 34 days before in the black box of a footshock chamber exhibited an increase of the latency to escape from the white compartment ($t_{(22)}=3.58$, $P<0.001$, Fig. 3A) and of the time spent in this compartment ($t_{(22)}=3.33$, $P<0.01$, Fig. 3B). The latency to escape from the white compartment was positively correlated to plasma levels of corticosterone after footshock or exposure to the chamber on day 0 (Pearson's correlation, $r=0.58$, $P<0.05$).

3.3. Social interaction (15 days after the last SR)

As depicted on Fig. 4, 15 days after the end of the aversive procedure, social behaviour was reduced in shocked animals (number, $t_{(10)}=3.09$, $P<0.01$; time spent $t_{(10)}=2.30$, $P<0.05$). As shown on Table 1, sniffing (number: $t_{(10)}=2.81$, $P<0.05$;

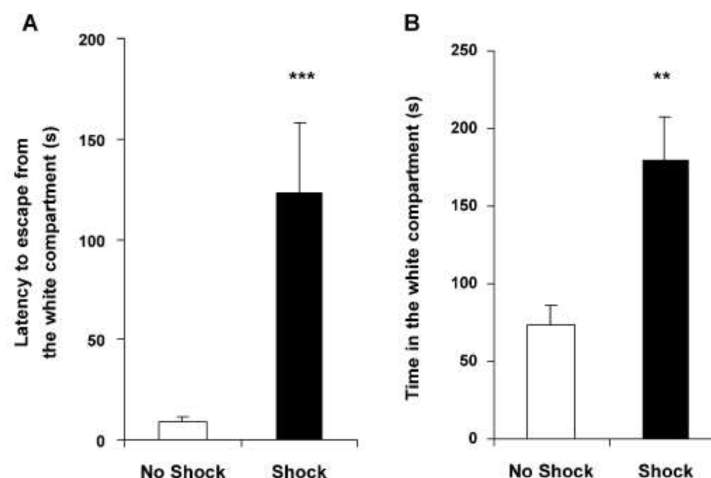

Figure 3 Black and white box. (A) Latency to escape and (B) time spent in the white compartment of the black and white box test (mean $\pm$ SEM) in shock and no shock groups. $^{***}P<0.001$; $^{**}P<0.01$. $n=12$ per group.

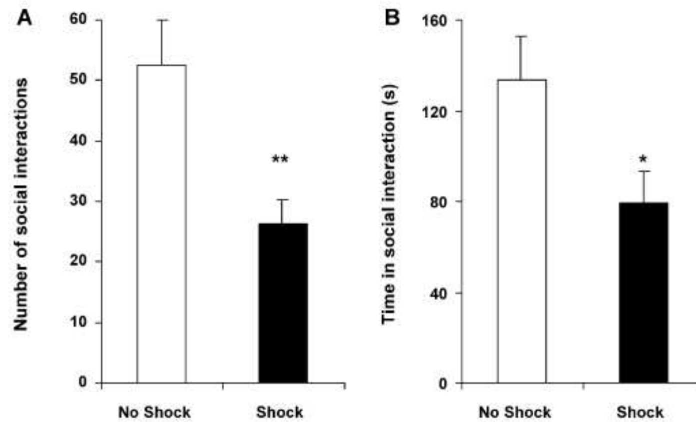

Figure 4 Social behaviour. (A) Number of social interactions and (B) time spent in social interaction (mean ± SEM) in shock and no shock groups. **$P<0.01$; *$P<0.05$. $n=6$ pairs of rats per group.

time: $t_{(10)}=1.85$, ns), allogrooming (number: $t_{(10)}=2.27$, $P<0.05$; time per period: $t_{(10)}=2.26$, $P<0.05$) and crawling over (number: $t_{(10)}=2.84$, $P<0.05$; time: $t_{(10)}=2.57$, $P<0.05$) were reduced in shocked animals. Moreover, the percentage of time spent in the open arms of the elevated plus-maze was positively correlated to the number of social interactions ($r=0.47$, $P<0.05$) and to the time spent in social interaction ($r=0.42$, $P<0.05$).

3.4. Plasma corticosterone levels after footshock administration and after situational reminder (23 days after the last SR)

As shown on Fig. 5, exposure to the chamber ($t_{(9)}=4.14$, $P<0.01$) and exposure to the foot-shock ($t_{(8)}=7.61$, $P<0.001$) on day 0 increased plasma corticosterone levels compared to basal levels. Plasma corticosterone levels were similar after exposure to the chamber or to the footshock ($t_{(11)}=1.75$, ns). Forty-four days later, plasma corticosterone levels were differently affected by the SR among groups (ANOVA, time × treatment effect, $F_{(1,11)}=8.26$, $P<0.05$). In the previously shocked rats, plasma corticosterone levels were reduced compared to footshock exposure on day 0 (post hoc Sheffe's test, $P<0.05$). In the no shock group, plasma corticosterone levels did not differ between the two exposures to the chamber (post hoc Scheffe's test, ns). Moreover, on day 44,

Table 1 Sniffing, allogrooming, and crawling over behaviours in a social interaction test in shock and no shock groups (mean ± SEM).

	No shock	Shock
Sniffing		
Number	44 ± 5.5	25 ± 3.9*
Time (s)	109.2 ± 11.2	76.5 ± 13.6
Allogrooming		
Number	1.5 ± 0.6	0.2 ± 0.2*
Mean time per period (s)	2.4 ± 0.7	0.5 ± 0.5*
Crawling over		
Number	7 ± 2	1.2 ± 0.4*
Time (s)	19.3 ± 6.6	2.2 ± 0.9*

*$P<0.05$. $n=6$ pairs of rats per group.

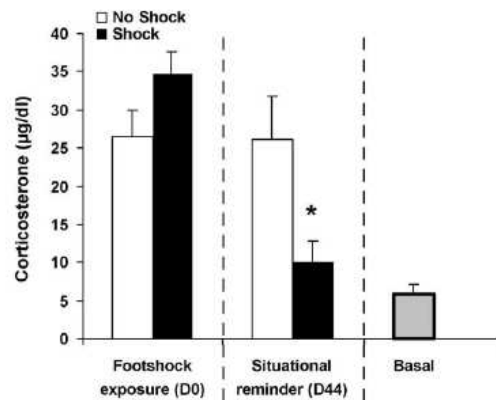

Figure 5 Plasma levels of corticosterone (µg/dl) (mean ± SEM): on day 0, 5 min after exposure to the shock chamber (no shock group), 5 min after the electric footshock (shock group); on day 44, after a SR and in basal situation. *$P<0.05$ electric footshock exposure versus SR. $n=4$-7 per group.

plasma corticosterone levels in the shocked group were similar to basal levels ($t_{(8)}=1.20$, ns).

4. Discussion

The present study was designed to evaluate long-term effects of an aversive procedure on behavioural and neuroendocrinological parameters in female rats. Our results demonstrate that a single brief footshock administration followed by repeated SRs induces behavioural and neuroendocrinological alterations which last more than 1 month after the aversive event and more than 20 days after the last SR occurred.

Previous studies have assessed long-lasting avoidance (from 2 h up to 2 weeks) of stressful situations such as the elevated plus-maze in shocked animals (Steenbergen et al., 1991; Grahn et al., 1995; Koba et al., 2001). Our results demonstrate for the first time that animals submitted to a footshock and SRs not only presented long-term higher anxiety (in the elevated plus-maze) but also altered social behaviour and a specific avoidance of stimuli associated with the aversive event (in the black and white box). In fact, these animals exhibited a locomotor hypoactivity, as shown by the decreased number of visits to the closed arms in the elevated plus-maze. The number of visits to the closed arms has been validated as a good index of anxiety-independent locomotor activity (Ramos et al., 1997; Salome et al., 2002). This result is in accordance with the studies of Van Dijken et al. (1992) and Van den Berg et al. (1998) showing an hypoactivity in male rats in the open field, respectively, 28 and 15 days after one or 5 exposures to electric footshock. However, these results contrast with those of Pynoos et al. (1996) describing that a footshock associated to SRs did not affect locomotor activity in an open field 3 and 6 weeks later in male mice. Further experiments assessing locomotor activity would be necessary to confirm rats hypoactivity in such conditions. It has to be noted that our data show a positive correlation between the percentage of time spent in the open arms of the elevated plus-maze and the intensity of social behaviour, which further reinforces the idea that social interaction can be considered as a good index of anxiety (File and Seth, 2003). To our knowledge, only short-term effects of an intense stress on social behaviour have been assessed in rats. For instance, a decreased social interaction has been described immediately or one hour after the exposure to footshock (Hajos-Korcsok et al., 2003) or to the odour of a predator (Zangrossi and File, 1992). In contrast, our study demonstrates a significant long-term anxiogenic effect of a single intense stress and SRs on social behaviour. In the black and white box test, rats generally spend more time in the black than in the white compartment (Sanchez, 1996; Ramos et al., 2002). Interestingly, in our study, 34 days after the footshock, rats still avoided the black box indicating that they exhibited a fear generalisation of context a behaviour which known to be facilitated when unconditional stimulus intensity is elevated (Laxmi et al., 2003). In this regard, the black and white box test represents an aversive-like context in view of its spatial configuration similar to the footshock chamber even if the two apparatus were constructed in different materials, had different dimensions and were located in different rooms. One has to keep in mind, however, that one limitation in applying such an animal model to the human pathology is the fact that only a vulnerable sub-population of individuals exposed to a traumatic event will develop PTSD. In our study although, most of females exposed to the aversive procedure developed behavioural alterations. Nevertheless, a large inter-individual variability in the black and white box test was observed for the latency to visit the black compartment, indicating that some animals returned in an environment similar to the shock context. Further studies should assess factors involved in the behavioural intra-group differences in response to the aversive procedure.

We found a blunted plasma corticosterone stress response in animals previously exposed to a 2 mA electric footshock which represents a traumatic procedure according to Cordero et al. (2002). This hypocortisism could result from a habituation or an unusual hyporesponsivity of the HPA axis. In no shock rats, the two exposures to the light chamber similarly increased corticosterone levels demonstrating that no habituation occurred between two exposures separated by a long delay. Thus, shock animals seem to display a hyporesponse of the HPA axis. This result is in agreement with data showing that a footshock administration increases basal (Fleshner et al., 1995) or stress levels (Johnson et al., 2002) of corticosterone only during few days after the stress exposure and reduces subsequent corticosterone response to stress (King et al., 2001). Moreover, we have recently found that shocked females exhibited a hypersensitive feedback of the HPA axis during a restraint stress response

62 days after footshock (unpublished data). Taken together, the decreased corticosterone level in the footshock chamber and the increased HPA axis feedback, respectively, 44 and 62 days later, could mimic HPA alterations generally reported in PTSD patients (Yehuda, 2000). However, it has to be noted that the corticosterone response was assessed 5 minutes after the exposure to the shock or to the SR and was thus not representative of the whole corticosterone responsiveness. It would be interesting to determine if the aversive procedure alters the peak value and the return to baseline levels of corticosterone. Nevertheless, the blunted corticosterone response we observed 5 minutes after the shock or a SR is a first report suggesting that the aversive procedure has immediate as well as long-lasting effects on the HPA axis. Interestingly, plasma corticosterone levels in response to the aversive event are predictive of the latency to escape from the white compartment 34 days later. This finding extends previous data demonstrating a positive correlation between the magnitude of corticosterone levels and the fear-related behavioural inhibition exhibited in the context associated with the footshock 24 h and 7 days later (Cordero et al., 1998). These data support the involvement of corticosterone in the long-term memory of aversive events. Studying HPA axis in female rats raises the problem of the oestrous cycle. Although several studies report that estrogens have a stimulatory role on the HPA axis (Atkinson and Waddell, 1997; Viau, 2002), discrepancies have emerged between reports (Guo et al., 1994; Rivier, 1999). This indicates that the influence of the stage of the cycle still remains unclear. Moreover, since oestrus cycle is involved in inter-individual differences in women, elimination of this variability in animal studies would be deleterious in modelling a human psychopathology. Finally, numerous epidemiological studies have demonstrated that gender is an important risk factor in the emergence of PTSD since women more frequently develop this pathology when they are submitted to an intense trauma (Stein et al., 2000; Gavranidou and Rosner, 2003). Our results demonstrate long-lasting behavioural disturbances in female rats, however, further studies should be conducted to determine if female rats exhibit more alterations than male animals after the aversive procedure.

In conclusion, a single stressor (10 s) followed by three SRs is a procedure able to produce long-term alterations lasting up to 1 month in female rats. Shocked animals exhibited anxiety, avoidance of stimuli related to the aversive event, altered social behaviour and hypocortisism in response to stress. These results extend the initial study of Pynoos et al. (1996) which demonstrated anxiety-like behaviour alterations and sensitised startle reflex in male mice after six SRs. The disturbances observed in our model mimic major key symptoms of PTSD in humans: hypocortisolism (Yehuda, 2000; McEwen, 2002), alterations of social behaviour (Yehuda, 1999), comorbidity with anxiety disorders (Brown et al., 2001; Golier et al., 2001) and specific avoidance of traumatic-like context (Brewin and Holmes, 2003). In this view, using such an aversive procedure could provide a useful model to study neurobiological disturbances associated with PTSD and to develop new therapeutic approaches for this disabling psychopathology.

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Research report

Prenatal stress affects behavioral reactivity to an intense stress in adult female rats

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Abstract

In rats, prenatal stress has been shown to influence behavioral and neuroendocrinological immediate responsivity to several kinds of mild stress in adulthood. Indeed, prenatal stress increases anxiety-like behavior, depressive-like disturbances and alters the hypothalamo-pituitary–adrenal (HPA) axis response to stress. However, long-term effects of an intense stress on behavior of prenatally stressed rats remain unknown. Moreover, most studies focus on male offspring. The aim of our study was to evaluate long-term behavioral effects of an aversive procedure consisting of an intense footshock (2 mA, 10 s) followed by three weekly situational reminders in prenatally stressed female rats. Prenatal stress was achieved by restraining the pregnant dams under bright light three times per day for 45 min during the last week of pregnancy. The aversive procedure induced long-term behavioral alterations in adult animals: an increase of immobility in the footshock chamber, hypoactivity in a novel environment and decreased avoidance of an "aversive-like" context. Interestingly, the procedure induced opposite effects in control and prenatally stressed females, suggesting bi-directional manifestation in some situations. In conclusion, prenatal stress affects behavioral response to an intense footshock associated with repeated situational reminders. These results suggest that early stress may interact with later ability to cope with intense stress in adulthood.

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Theme: Neural basis of behavior
Topic: Stress

Keywords: Post Traumatic Stress Disorder; Early stress; Reactivity to novelty; Black and white box; Immobility; Avoidance

1. Introduction

In humans, several studies underline the major role of stressful events during early life and adulthood in the vulnerability to develop psychopathologies such as depression [24] or Post Traumatic Stress Disorder (PTSD) [23]. Contrary to depression, apparition of PTSD is totally dependent of a confrontation to a traumatic event [3]. Nevertheless, both of these psychiatric syndromes target a vulnerable subpopulation and share a high rate of co-morbidity [28]. In this view, it is well known that women are more likely to develop depression [48] and PTSD [21].

In rats, prenatal stress, i.e., stress applied to pregnant females, alters reactivity to stress in adult offspring [32]. Indeed, gestational stress induced by three times weekly unpredictable noise and flashing light increases plasma noradrenaline levels immediately after exposure to a novel environment or to electric footshock in the offspring [47]. Maternal restraint stress during the last week of pregnancy induces long-term disturbances in the offspring. It increases acetylcholine release in the hippocampus after mild stress such as saline injection [11] and prolongs corticosterone secretion after exposure to novelty [31] or restraint stress [31,44]. Prenatally stressed rats also show a

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higher behavioral emotionality in several stressful situations such as the elevated plus maze [44], restraint stress [31], exposure to novelty [12,38] or forced swim [35]. In different paradigms, prenatally stressed animals are characterized by high levels of anxiety and depressive-like alterations such as increased paradoxical sleep [15] and increased immobility in the forced swim test [35]. These alterations have been observed using relatively mild stress and in a short window of time (minutes following the stress exposure). However, nothing is known on prenatally stressed rats' abilities to cope with an intense stressful event and on the temporal persistence of stress-induced disturbances in these rats. Moreover, although effects of prenatal stress on hypothalamo-pituitary–adrenal (HPA) function are more marked in females [33,46], most of prenatal stress studies focus on male rats. We have developed in rats a model of "traumatic-like" procedure adapted from Pynoos [39], consisting of a single inescapable intense footshock followed by situational reminders. The aim of the present work was to determine how prenatal stress may affect later behavioral long-term adaptation to an intense stress in adulthood. Prenatal stress was achieved by restraining the pregnant dams under bright light three times per day for 45 min during the last week of pregnancy. Five-month-old female offspring experienced a single intense stress (2 mA, 10 s footshock) followed by situational reminders (7, 14 and 21 days later) and were assessed for immobility in the footshock chamber, locomotor reactivity to novelty and avoidance in an "aversive-like" context.

2. Materials and methods

2.1. Animals and prenatal stress procedure

Animals were maintained on a 12 h light/dark cycle (light from 8 a.m. to 8 p.m.), with free access to food and water. Manipulation of the animals was performed following the principles of laboratory animal care published by the French Ethical Committee and the rules of the European Union Normative (86/609/EEC). Sprague–Dawley adult virgin female rats (Iffa Credo, France) weighing 250 g were group-housed (4/cage) for at least 7 days after arrival to coordinate their estrus cycle. Female were individually housed in the presence of a sexually experienced male rat (weighing 400 g) for a whole estrus cycle. Pregnant females were then randomly assigned to restraint stress or control groups (n=10 for each group). Stress group was submitted to three daily restraint stress sessions (9 a.m., 12 p.m. and 5 p.m.) in a transparent cylinder (7 cm diameter, 19 cm length) under bright light for 45 min during the last week of gestation, as previously described [29]. Control dams were left undisturbed throughout the pregnancy. Offspring were weaned 21 days after birth and left undisturbed until the aversive procedure at 5 months of age. Forty females (prenatally stressed n=20 and control n=20) were individually housed 3 weeks before the experiment.

2.2. Aversive procedure

Treatment were randomly assigned to 5-month-old female rats in order to obtain four groups: no shock-control, no shock-prenatal stress, shock-control and shock-prenatal stress (n=10 per group). On day 0, control and prenatally stressed females were exposed to an aversive procedure. The aversive procedure was adapted from Pynoos [39], consisting of a single exposure to footshock followed by three weekly exposures to situational reminder (day 7, day 14 and day 21). After a 2-min adaptation period, a guillotine door was opened and a bright light was turned on in the first compartment. The door remained open until the animal entered the second compartment, which remained dark. Then, the door was closed and the rat received an inescapable 2 mA shock for 10 s in the dark compartment. The no shock groups received the same treatment, but with the shock mechanism inactivated. The four groups were exposed to reminder of the situation for 2 min, once per week for 3 weeks. This was accomplished by placing the animal in the light compartment with the door closed to prevent entry in the shock compartment. This situational reminder was chosen to prevent extinction, which might occur if the animal entered in the shock compartment without shock.

Behavioral assessments were conducted between day 21 and day 44 after the electric footshock in the adult female rats. Behavior was recorded with a video camera for offline scoring except for motor activity in a novel environment, which was counted by photocell beams.

2.3. Immobility in the footshock chamber (21 and 44 days after footshock)

Frequency of immobility was assessed during 1 min, on day 21 and day 44 in the light compartment of the footshock chamber and was expressed as a percentage of no shock groups (control or prenatal stress) mean values.

2.4. Locomotor reactivity to novelty (28 days after footshock)

Twenty-eight days after footshock administration, animals were individually placed in plastic transparent boxes (30×18×18.5 cm high) located inside racks equipped with two sets of photoelectric cells. These cages were connected to a computer which recorded the number of light beam interruptions (Imetronic, Pessac, France). Spontaneous exploratory behavior (number of rearing) was evaluated during a 30-min session. Gradual decrease of the activity represented an uninterrupted habituation. The exponential function $Y(t)=Y_0e^{-kt}$ (Y=amount of activity

H. Louvart et al. / Brain Research 1031 (2005) 67–73 69

per 5-min period, k=individual rate of habituation, t=time of session) was used as a model of habituation course of exploratory activity in individual animals, and was determined by using the method of linear regression, as previously described [14]. The individual rate of habituation (k-value) expresses the rapidity of locomotor activity decline.

2.5. Black and white box test (34 days after footshock)

The black and white box test consisted in a wooden chamber with two compartments: a black closed compartment (30×32×40 cm high) and a white open compartment (45×32×40 cm high) [10]. The two compartments were connected by a small partition (10×15 cm high). This design mimicked the apparatus where animals experienced the aversive procedure, and for this reason, this test was chosen to evaluate avoidance of a "aversive-like" environment. On day 34, each rat was placed in the light compartment, facing the wall opposite to the opening and the latency to enter the dark compartment and the time spent in each compartment were assessed for 5 min.

2.6. Statistical analysis

Statistics were performed using two-way analysis of variance (ANOVA) with group (control, prenatal stress) and treatment (no shock, shock) as between factors. Subsequent analyses were used to assess significant differences between specific groups, using orthogonal contrast and post hoc Scheffe's test. Pearson's coefficient was used to determine correlation. A significant difference was indicated by P<0.05.

3. Results

3.1. Immobility in the footshock chamber (21 and 44 days after footshock)

Footshock administration differently affects immobility behavior when control and prenatally stressed rats were reexposed to the situational reminder 21 or 44 days later [group×treatment×time effect, $F(1,36)$=7.54, P<0.01]. As shown in Fig. 1, immobility differed across time between shock-control and shock-prenatal stress rats [group×time effect, $F(1,19)$=31.17, P<0.001]. At day 21, shock-control group has higher number of immobility periods than shock-prenatal stress group (post hoc Sheffe test, P<0.05). Between day 21 and day 44, the number of immobility periods was stable in shock-control rats (post hoc Sheffe test, ns), whereas it increased in shock-prenatal stress rats (post hoc Sheffe test, P<0.001). At day 44, shock-prenatal stress rats exhibited higher immobility than shock-control rats (post hoc Sheffe test, P<0.01).

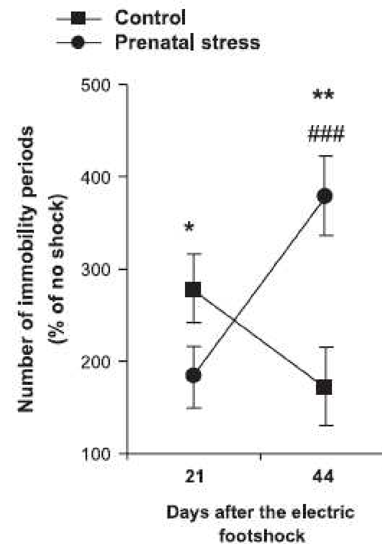

Fig. 1. Mean (±S.E.M.) number of immobility periods expressed as a percentage of no shock groups during 1 min of exposure to the electric chamber 21 and 44 days after electric footshock in control and prenatally stressed female rats. *P<0.05 shock-control group versus shock-prenatal stress group, **P<0.01 shock-control group versus shock-prenatal stress group, ###P<0.001 shock-prenatal stress group day 21 versus shock-prenatal stress group day 44.

3.2. Locomotor reactivity to novelty (28 days after footshock)

No shock-prenatal stress animals tend to exhibit an increase of the total number of rearing in a novel environment [group×treatment effect, $F(1,36)$=3.72, P=0.06, Fig. 2A]. Electric footshock (delivered 28 days before) does not affect total number of rearing in control rats, but decreases activity in prenatally stressed rats (P<0.05). The rate of habituation, as shown by the k-value, is differently affected by footshock among groups [group×treatment effect, $F(1,36)$=9.00, P<0.01, Fig. 2B]. Indeed, prenatally stressed rats which exhibit a poor habituation before electric shock are not affected by footshock (post hoc Scheffe test, no shock-prenatal stress versus shock-prenatal stress, ns). In contrast, in control rats, electric footshock decreases the habituation (post hoc Sheffe test, no shock-control versus shock-control, P<0.05).

3.3. Black and white box test (34 days after footshock)

As shown in Fig. 3, no shocked animals exhibited a weak latency to enter the black compartment (around 10 s) in the black and white box test. In contrast, animals shocked 34 days before in the black box of a footshock chamber exhibited an increase of the latency to visit the black compartment [treatment effect, $F(1,36)$=14.08, P<0.001]. However, this increase was lower in the shock-prenatal

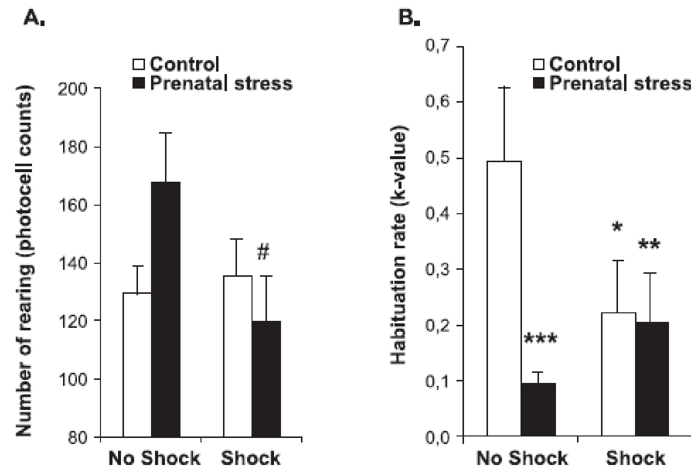

Fig. 2. (A) Mean (±S.E.M.) number of rearing during a 30-min session in a novel environment in control and prenatally stressed female rats, 28 days after footshock. #P<0.05 no shock-prenatal stress versus shock-prenatal stress. (B) Mean (±S.E.M.) habituation rates (k-value) during a 30-min session in a novel environment in control and prenatally stressed female rats. *P<0.05 no shock-control versus shock-control, **P<0.01 no shock-control versus shock-prenatal stress, ***P<0.001 no shock-control versus no shock-prenatal stress.

stress group than in shock-control rats (P<0.05). Time spent in the white compartment is increased in shocked animals [treatment effect, $F(1,36)=18.84$, P<0.001, data not shown]. Latency to enter the black compartment is positively correlated to the number of immobility periods during situational reminders at day 21 [Pearson's correlation r=0.53, P<0.001] and day 44 [r=0.70, P<0.001]. Thus, the more the animals were immobile during situational reminders, the more their latency to enter the black compartment was high.

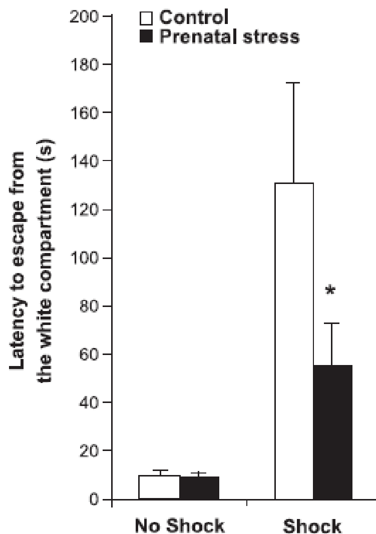

Fig. 3. Mean (±S.E.M.) latency (s) to enter the black compartment 34 days after electric footshock in control and prenatally stressed female rats. *P<0.05 shock-control versus shock-prenatal stress.

4. Discussion

In the present study, we demonstrate that prenatal stress affects long-term behavioral alterations resulting from an intense stress in adulthood. Indeed, shock-prenatal stress group exhibits an increase of immobility in the aversive context across time, a hypoactivity in a novel environment and a decreased avoidance of an "aversive-like" context.

Shock-prenatal stress rats do not get used to the context associated to the aversive event. Indeed, immobility increases between two situational reminders separated by an extensive delay (23 days), indicating a sensitization of this behavior and suggesting that repeated exposures to the stressor context reactivate memory in prenatally stressed rats. This result extends previous works describing an increase in unconditional [22,42] and conditioned [22] freezing behavior in male offspring of stressed mothers. In this context, prenatal stress could vulnerabilize animals to impaired extinction process and to time-dependent sensitization of immobility behavior after a an intense aversive event.

Biological substrates underlying this vulnerability are unknown. As mesoprefrontal dopamine neurons are involved in the normal extinction of fear responses [36], it could be hypothesized that altered dopaminergic function of prenatally stressed animals increases their vulnerability to behavioral sensitization to stress. Indeed, prenatal stress induces a higher dopamine turnover in prefrontal cortex [2,19] and higher dopaminergic D2 autoreceptors levels in the medial prefrontal cortex [4] and in the accumbens [25].

A deficit in the GABAergic transmission could also explain the alterations of fear conditioning reported here in prenatally stressed animals. Indeed, $GABA_A$ receptor antagonists increase fear behavior [17] and prenatal stress reduces the number of $GABA_A$ receptors in the hippocampus of adult female rats [20]. Interestingly, fear conditioning and time-dependent sensitization have often been suggested as potentate good animal models of PTSD [49]. Moreover, a recent study underlines that GABA low plasma levels in the aftermath of trauma exposure are predictive in the development of an acute PTSD [43].

Our results show that prenatal stress increases total rearing in a novel environment in female rats. This result is in accordance with previous studies showing an increase of locomotor reactivity to novelty in male prenatally stressed rats [12,37]. Twenty-eight days after footshock, prenatally stressed female rats exhibited a decreased exploration of a novel environment, whereas control rats are not affected. Although footshock induces a long-lasting hypoactivity in male rats [45], only transitory alterations have been observed in male mice in a paradigm similar to our aversive procedure [39]. However, it is well known that locomotor activity is strongly sex dependent [18]. In this regard, we suggest that prenatal stress vulnerabilizes female rats to persistent hypoactivity after footshock. Moreover, prenatal stress reduced locomotor habituation to novelty as shown by the lack of decrease in the number of rearing across time. A reduced habituation was also observed in control animals which have experienced an electric footshock. This result is consistent with hypervigilance described in a novel environment in shocked male mice [39]. At the neurochemical level, the cholinergic system is an important substrate of habituation [9]. In rats, cortical and hippocampal acetylcholine release is induced by stimuli that produce arousal (novelty or conditioned fear), whereas habituated animals exhibit low release of acetylcholine [1]. Acute intense stress induces long-lasting changes in cholinergic gene expression [27]. Since prenatal stress induces a hypersensitivity of the cholinergic hippocampal system [11], alterations of habituation in prenatally stressed shocked rats could be sustained by a hyperactivity of this system.

The black and white box test constitutes an “aversive-like” context, since animals shocked 34 days before in a black chamber, specifically avoided the black compartment. In this test, control rats typically exhibit a weak latency to enter the dark compartment and spend more time in the black than in the white compartment [40]. After footshock, the latency to enter the “aversive-like” context was weaker in prenatally stressed animals, suggesting cognitive alterations or fear-related disturbances in these animals. Indeed, prenatal stress has been shown to induce cognitive alterations [30] and specifically to reduce short-term retention in a passive avoidance task [13]. We show here that prenatal stress also impairs passive avoidance in a context resembling to the footshock chamber. However, in the footshock chamber, we demonstrated an increased immobility in prenatally stressed rats, suggesting that these animals have a long-term retention of the aversive event. Thus, despite a behavioral inhibition in an inescapable aversive situation, rats subjected to prenatal stress impulsively return to the hazardous context. This maladaptive behavior could result from a conflict in prenatal stressed rats between aversion for the light environment and conditioned fear of the footshock-associated context.

PTSD alters explicit memory and enhances emotional memory (intrusion symptomatic cluster) [16]. The neurobiological fundaments of this mnesic alterations dichotomy might be linked to peritraumatic excess of stress hormones. On one hand, a peritraumatic long-term excess of glucocorticoids could lead to the hippocampal atrophy [5,6]. On the other hand, a hypernoradrenergic drive at the amygdala level, which has been demonstrated to be implicated in long-term memory [26,34], might result in a higher acquisition of the traumatic experience [41]. Yet, in rats, prenatal stress prolongs corticosterone stress response [31] and increases plasma levels of noradrenaline after footshock [47]. This could explain why prenatally stressed rats exhibit both a weaker avoidance in an “aversive-like” context and a long-term memory of the aversive event.

Pre-traumatic depressive profile has been shown to be an important risk factors of PTSD [7,8]. Several studies demonstrate that prenatal stress could induce depressive-like alterations in rats (for a review, see Ref. [32]). In the present study, we demonstrate that prenatal stress modifies long-term alterations induced by an intense stressor followed by situational reminders in adult female rats. It could be hypothesized then that early adverse events may participate to interindividual variability in vulnerability to develop stress-related disorders in response to a traumatic event, thus contributing to the high comorbidity between depression and PTSD.

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STRESS PRECOCES ET SURCHARGE ALLOSTASIQUE CHEZ LA DESCENDANCE

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Short Communication

Impact of an intense stress on ethanol consumption in female rats characterized by their pre-stress preference: Modulation by prenatal stress

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ABSTRACT

We examined the influence of prenatal stress on alcohol preference in adult female rats exposed to an intense stress. To take into account interindividual variability, the study was conducted in animals categorized as low or high alcohol preferring. After footshock, control high-preferring rats strongly reduced their alcohol consumption; in contrast, alcohol consumption was not changed in high-preferring rats that were prenatally stressed.

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Alcohol has been hypothesized to serve as a coping mechanism against stress (Holahan et al., 2001). According to that view, stress-related psychopathologies, such as posttraumatic stress disorder, are highly co-morbid with alcohol abuse (Jacobsen et al., 2001). In animals, studies of the effects of stress on ethanol intake have produced conflicting results. Stress has been shown to induce an increase, a decrease or no effect on the alcohol intake (Nash and Maickel, 1985; Pohorecky, 1990; Sprague and Maickel, 1994; Van Erp and Miczek, 2001). This differential impact of stress on ethanol intake could result from marked individual differences in the spontaneous ethanol preference and/or in the response to stress. In rats, chronic stress during pregnancy exerts profound long-term influences on the offspring's ability to cope with stress in adulthood (Maccari et al., 2003). Prenatal stress induces an increase of anxiety-like behavior (Vallée et al., 1997) and depressive-like disturbances (Morley-Fletcher et al., 2003). It is also associated with a prolonged stress-induced corticosterone secretion (Maccari et al., 2003; Morley-Fletcher et al., 2003) and with an alteration of the dopaminergic function (Henry et al., 1995). Despite evidence for a higher vulnerability to psychostimulants in animals exposed to

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Abbreviations: CRH, corticotropin releasing hormone; CGRP, calcitonin gene-related peptide; GR, glucocorticoid receptor; HPA, hypothalamic-pituitary adrenal; MR, mineralocorticoid receptors; NPY, neuropeptide Y; SR, situational reminder

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prenatal stress (Déminière et al., 1992; Henry et al., 1995; Koehl et al., 2000; Morley-Fletcher et al., 2004), few studies have focused on the vulnerability to ethanol in this animal model (DeTurck and Pohorecky, 1987). We have recently reported that in adult female rats, prenatal stress affects the behavioral response to an intense stress consisting of an inescapable footshock followed by situational reminders (Louvart et al., 2005a).

The aim of the present study was to examine the influence of prenatal stress on alcohol preference in female rats exposed to this aversive procedure. To take into account the inter-individual variability in the spontaneous preference for ethanol, we studied the impact of the aversive procedure in animals previously categorized as low or high alcohol preferring, namely light or heavy drinkers. After 4 weeks of withdrawal, we measured in the same animals, brain levels of neuropeptides involved in the ethanol preference such as neuropeptide Y (NPY), calcitonin gene-related peptide (CGRP), neurotensin, corticotropin releasing hormone (CRH) and neurokinin A (Ehlers et al., 1998, 1999a,b; Slawecki et al., 2005). Analysis focused on the hippocampus and on the frontal cortex, two areas often associated with marked differences between high and low ethanol preferring animals and very sensitive to ethanol withdrawal (Ehlers et al., 1999a,b; Bison and Crews, 2003; Slawecki et al., 2005).

Sprague Dawley female rats (Harlan, France) were housed with sexually experienced males, and vaginal smears were inspected daily until the discovery of spermatozoids, after which, they were housed individually. Pregnant dams were then assigned to stress ($n=11$) or control groups ($n=12$). Prenatal stress procedure was performed as previously described (for review, see Maccari et al., 2003; Louvart et al., 2005a). Briefly, during the last week of pregnancy females were submitted to 3 daily restraint stress sessions lasting 45 min under bright light. The offspring were weaned 21 days after birth. The mean number of pups per litter was 12.9±0.7 (6.4±0.5 males and 6.5±0.4 females), no litter size or sex-ratio differences were observed between control and stressed dams. Only female offspring was used for the present study. As previously described (Déminière et al., 1992; Maccari et al., 1995; Vallée et al., 1997), a maximum of two rats per litter were used to prevent any possible "litter effect" (Chapman and Stern, 1979).

Animals were maintained on a 12-h light/dark cycle (light on at 8 a.m.), with free access to food and water. Manipulation of the animals was performed following the principles of laboratory animal care published by the French Ethical Committee and the rules of the European Union Normative (86/609/EEC). Prenatally stressed ($n=22$) and control ($n=24$) rats (2 months old) were singly housed 1 week before the onset of testing. Then, alcohol preference was assessed in a two bottles choice paradigm (water and ethanol 5%). The position of the bottles was alternated every week to control for side preference. Alcohol preference was assessed for 2 weeks in basal condition and then, for 4 weeks during the aversive procedure. Before the aversive procedure, the mean preference of the animals was evaluated and the population was separated into low (ethanol preference <40%; ethanol intake <2.5 g/kg/day; Control low preferring, $n=7$; Prenatal Stress low preferring, $n=7$) and high-preferring rats (ethanol preference >70%; ethanol intake >4.5 g/kg/day, Control high preferring, $n=6$; Prenatal Stress high preferring, $n=7$). The aversive procedure was conducted as previously described (Louvart et al., 2005a,b, 2006). It consisted of a single exposure to an inescapable footshock (2 mA, 10 s) followed by 3 weekly exposures to situational reminder (SR1, SR2, SR3). During the aversive procedure, the alcohol preference continued to be assessed in the two bottles choice paradigm. Animals were sacrificed after 4 weeks of withdrawal and frontal cortex and hippocampus were quickly dissected and frozen on dry ice. Brain levels of neuropeptides were assayed by radioimmunoassay as previously described (Mathé et al., 1990; Husum et al., 2002). Ethanol preference was analyzed by analysis of variance (ANOVA) with group (prenatal stress, control) and type (low preferring, high preferring) as between factors and time as within factor. Newman–Keuls post hoc and planned comparisons were used for specific comparisons. The normality of the distribution was assessed with the Shapiro–Wilk test. $P<0.05$ indicated a significant difference.

Basal preference (Fig. 1A) and basal intake (Fig. 1B) of ethanol 5% were not modified by prenatal stress (ANOVA, group effect, preference, $F(1,44)=0.61$, ns; intake, $F(1,44)=0.28$, ns) and were stable across time (time effect, preference, $F(1,44)=0.17$, ns; intake, $F(1,44)=0.01$, ns, data not shown). As shown in Fig. 1A, overall ethanol preference values in female rats were around 50%, indicating that animals did not show a preference for ethanol. The distribution of the basal preference followed a normal distribution in each population (control: $W=0.96$, $P=0.56$; prenatal stress: $W=0.93$, $P=0.17$). However, considering interindividual differences, the population could

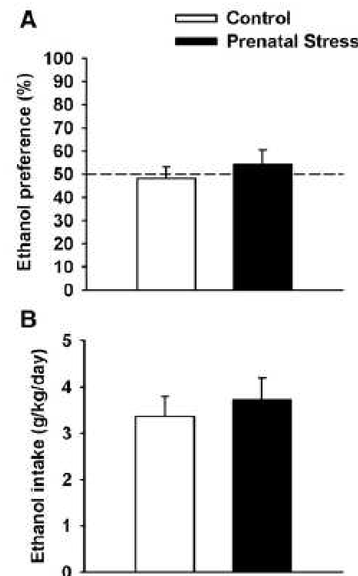

Fig. 1 – Basal condition. (A) The percentage of ethanol preference and (B) ethanol intake (g/kg/day) were similar between control and prenatal stress groups. Dotted line indicates a similar consumption of ethanol and water. Means±SEM are given.

BRAIN RESEARCH 1131 (2007) 181–186 183

be divided into low- and high-preferring rats that strongly differed with regard to their basal ethanol preference (type effect, $F(1,23)=204$, $P<0.0001$) and intake (type effect, $F(1,23)=110$, $P<0.0001$) (Figs. 2A, B). Taking into account the initial preference of the animals, the aversive procedure differently affected ethanol preference and ethanol intake across groups (group × type effect, preference $F(1,23)=6.68$, $P<0.05$; intake $F(1,23)=8.11$, $P<0.01$). In the high-preferring rats, after the footshock procedure, ethanol preference and ethanol intake were decreased in the control in comparison to the prenatally stressed animals (post hoc Newman–Keuls test, $P<0.05$). Furthermore, in comparison to the basal consumption, control high-preferring rats showed a progressive decrease throughout the aversive procedure of both preference (planned comparisons, basal versus shock, $P=0.056$, basal versus SR1, SR2, SR3, all $P<0.01$) and intake (basal versus shock, $P<0.01$; basal versus SR1, SR2, $P<0.001$; basal versus SR3, $P<0.01$). In fact, the mean preference over the 4 weeks of aversive procedure in control high-preferring rats did not significantly differ from 50% indicating that the footshock procedure abolished the ethanol preference. In contrast, in the prenatally stressed high-preferring rats, only a transient decrease of the ethanol preference and of the ethanol intake were observed during the first week of aversive procedure (planned comparisons for preference and intake, basal versus shock, $P<0.05$; basal versus SR1, SR2, SR3, *ns*).

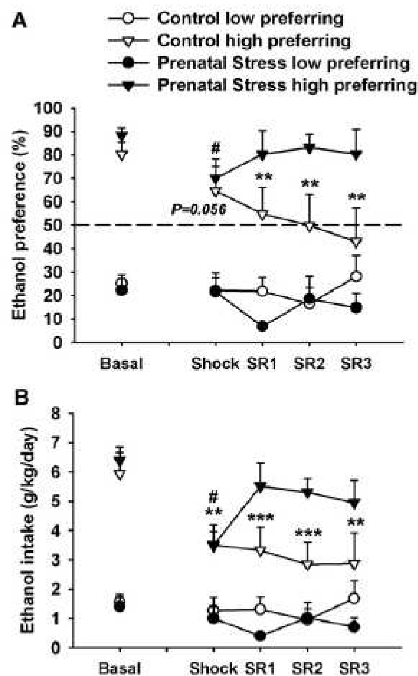

Fig. 2 – Aversive procedure (footshock followed by 3 situational reminders). (A) Percentage of ethanol preference and (B) ethanol intake (g/kg/day) in control and prenatal stress groups divided according to their pre-stress preference as low or high-preferring rats. Prenatal stress antagonized the inhibitory effect of an intense stress on ethanol consumption in adult high-preferring female animals. Dotted line indicates a similar consumption of ethanol and water. Means ± SEM are given. #$P<0.05$ basal versus shock in the prenatal stress high-preferring group; **$P<0.01$, ***$P<0.001$ basal versus shock, SR1, SR2 or SR3 in the control high-preferring group.

Table 1 – Neuropeptide levels in control and prenatal stress groups divided into low or high preferring subgroups

	Control		Prenatal stress	
	Low	High	Low	High
Frontal cortex				
NPY	11.82±0.83	12.89±1.07	9.58±1.26	11.26±0.96
CGRP	0.96±0.03**	1.28±0.11	1.14±0.10	1.28±0.04
Neurotensin	2.97±0.22	2.69±0.37	2.83±0.60	2.09±0.12
CRH	1.12±0.06	1.06±0.12	0.86±0.10	1.08±0.07
Neurokinin A	10.12±0.94	11.47±1.10	9.95±1.14	10.96±0.65
Hippocampus				
NPY	6.25±0.26*	7.51±0.25	6.74±0.55	7.08±0.44
CGRP	2.24±0.32	2.52±0.25	1.96±0.23	2.41±0.25
Neurotensin	8.74±1.19	9.45±2.09	6.18±0.84	9.11±1.56
CRH	0.43±0.02	0.41±0.02	0.51±0.15	0.43±0.03
Neurokinin A	4.29±0.65	5.13±0.73	4.68±0.77	5.21±0.39

NPY, CGRP, Neurotensin, CRH and neurokinin A levels in the frontal cortex and in the hippocampus were expressed as fmol/mg. Means ± SEM are given. *$P<0.05$, **$P<0.01$ control low preferring versus control high preferring.

With respect to neuropeptide levels, no significant type (high preferring versus low preferring) or group (control versus prenatal stress) effects were found for neurotensin, CRH and neurokinin A in the frontal cortex or in the hippocampus (Table 1). High-preferring animals tended to have higher levels of NPY in the hippocampus (type effect, $F(1,23)=3.81$, $P=0.06$), whereas no significant changes were observed in the frontal cortex (Table 1). The enhanced hippocampal levels of NPY were mainly observed in the control group (planned comparisons, control high-preferring group versus control low-preferring group, $P<0.05$). In contrast, regardless of the alcohol preference, the NPY levels tended to be decreased by prenatal stress (group effect, $F(1,23)=3.42$, $P=0.07$). For CGRP, no significant changes were reported in the hippocampus, whereas high-preferring rats showed a significant increase of CGRP levels in the frontal cortex (type effect, $F(1,23)=8.96$, $P<0.01$, Table 1). Specific comparisons revealed that marked differences in the CGRP levels in the frontal cortex between high- and low-preferring animals were only detected in the control group (planned comparisons, control high-preferring group versus control low-preferring group, $P<0.01$).

Variability in the initial alcohol preference and early life stress may in part explain the conflicting results obtained in the studies conducted on the impact of stress on ethanol intake (Pohorecky, 1990). Our work shows that prenatal stress *per se* had no effect on basal alcohol preference in adult female rats. However, taking into account interindividual differences in the spontaneous pre-stress alcohol preference, we found that footshock followed by situational reminders decreased ethanol preference of high-preferring control rats, whereas

consumption remained elevated in high-preferring rats exposed to stress during the prenatal period. Furthermore, after the aversive procedure, control animals exhibited changes in their levels of CGRP and NPY in the frontal cortex and in the hippocampus respectively, whereas prenatally stressed rats were unaffected. These results suggest that prenatal stress antagonizes the inhibitory effect of an intense stress on ethanol consumption in adult high-preferring female animals.

Since prenatal stress increases the vulnerability to psychostimulants (Déminière et al., 1992; Koehl et al., 2000; Morley-Fletcher et al., 2004), our data suggest that prenatal stress could induce a differential vulnerability according to the class of drug considered. Gender difference could also be involved in the lack of effect of prenatal stress on basal ethanol intake reported here. Thus prolonged periods of maternal separation enhance voluntary ethanol intake in male (Ploj et al., 2003) but have no effect in female rats (Roman et al., 2004). Our results extend previous studies showing a differential effect of repeated stress in adult rats initially divided as high or low consuming groups (Rockman et al., 1986, 1987; Volpicelli et al., 1990). However, contrary to previous works, we did not observe any enhancement of ethanol intake in low-preferring rats, suggesting that several exposures to the inescapable stress are required to increase ethanol preference. Stress is known to induce anhedonia-like behaviors such as a decrease of the preference for palatable drink (Willner et al., 1992; Rygula et al., 2005). Since ethanol constitutes an appetitive stimulus for high-preferring animals, the decrease of the ethanol preference could reflect a decreased sensitivity to reward induced by the intense stress. High ethanol intake has been associated with self-medication in humans suffering from anxiety disorders (Carrigan and Randall, 2003). High anxiety levels have been reported in high-preferring animals (Hwang et al., 2004) and prenatal stress procedure induces an increase of anxiety-like behavior in rats (Vallée et al., 1997). In this view, the stable high preference observed in prenatal stress high-preferring group could reflect an attempt to attenuate the impact of the aversive procedure. The aversive procedure used in the present study has long lasting effect on behavior in female rats (Louvart et al., 2005b) and we recently showed that prenatal stress is associated with a sensitization of the effects of the aversive procedure, inducing a long-lasting increase of freezing behavior during exposure to situational reminders (Louvart et al., 2005a).

We did not observe marked changes between control and prenatally stress rats or between low and high ethanol groups for the neuropeptides studied in the frontal cortex and in the hippocampus. NPY overexpression in mice reduces ethanol self-administration (Thiele et al., 1998) and ethanol consumption is suppressed by NPY administration in ethanol preferring animals (Badia-Elder et al., 2001). A recent work from Primeaux et al. (2006) demonstrates that an increase of NPY in the central amygdala leads to a decrease of ethanol self-administration in anxious animals. CGRP binding sites are decreased in the central amygdaloid nucleus in high-preferring and high drinking animals (Hwang et al., 1995) and CGRP is known to play an important role in the control of ingestive behavior (Krahn et al., 1984). Thus it could be hypothesized that the NPY and CGRP increase in specific brain regions in control high-preferring rats plays a role in the suppressed ethanol preference that we observed in these animals during the aversive procedure. Analysis of neuropeptide levels in the central amygdala is necessary to clarify this issue.

Mechanisms underlying the differential response to shock in control and prenatally stressed animals are unknown. Both the aversive procedure (Louvart et al., 2005b) and prenatal stress modify the hypothalamic-pituitary adrenal (HPA) response to stress (Maccari et al., 1995; Vallée et al., 1997). Interestingly, the manipulation of the HPA function modulates the ethanol preference (Fahlke and Hansen, 1999). Indeed, the administration of a glucocorticoid receptor (GR) antagonist reduces the ethanol intake (Koenig and Olive, 2004). Prenatally stressed rats exhibit a decrease of mineralocorticoid receptors (MR) and GR (Maccari et al., 1995; Koehl et al., 1999), and low levels of MR and GR mRNA in the hippocampus (Van Waes et al., 2006). To our knowledge, potential variations in GR levels between high and low ethanol preferring animals have so far not been explored yet. The aversive procedure (footshock and situational reminders) used in the present study has no effect on GR mRNA levels in the hippocampus; in contrast, in female animals, it reduces the CRH mRNA levels in the paraventricular nucleus of the hypothalamus (Louvart et al., 2006). We have recently reported that prenatal stress induces a blunted response of the HPA axis to an ethanol challenge (Van Waes et al., 2006). Thus, a differential response of the HPA axis to stress and/or to ethanol may be involved in the resistance to stress challenge-induced suppression of alcohol consumption in prenatally stressed high-preferring rats.

In conclusion, our study indicates that an intense stress in adult female rats differently affects ethanol preference and consumption according to both a history of stress *in utero* and interindividual differences in the pre-stress preference. These results suggest that adverse experiences early in life may modify the effect of stress on alcohol preference in rats.

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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

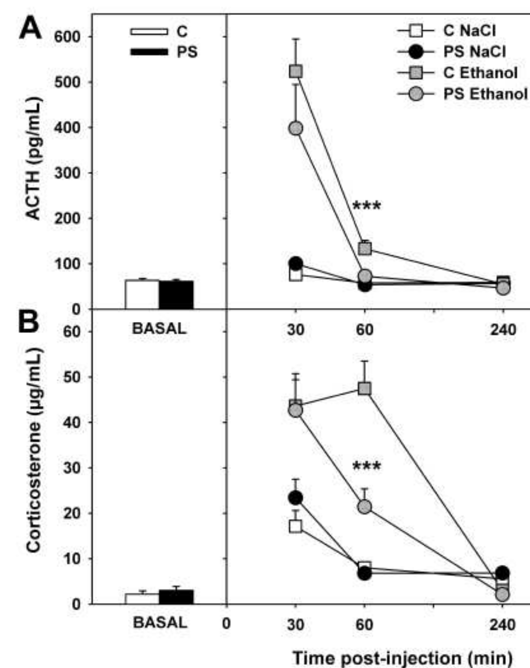

FIG. 1. (A) Plasma adrenocorticotropic hormone (ACTH) levels (pg/mL) and (B) plasma corticosterone levels (μg/dL) in control (C) and prenatal stress (PS) groups, in basal condition (left panel, $n = 19$) or 30 min ($n = 42$), 60 min ($n = 38$) and 240 min ($n = 40$) after ethanol (1.5 g/kg) or NaCl (0.9%)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

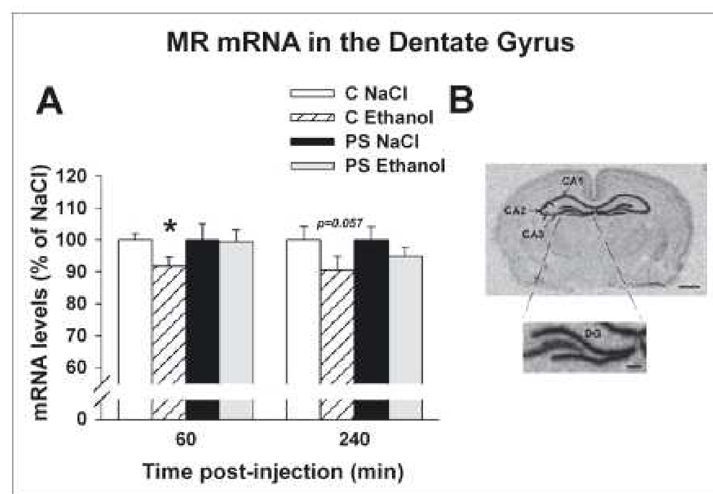

FIG. 2. (A) Semiquantitative analysis of dentate gyrus (DG) mineralocorticoid receptor (MR) mRNA levels (expressed as percentages of respective NaCl groups) in control (C) and prenatal stress (PS) groups, 60 and 240 min after the ethanol (1.5 g/kg) or the NaCl (0.9%) intraperitoneal injection ($n = 7–9$ 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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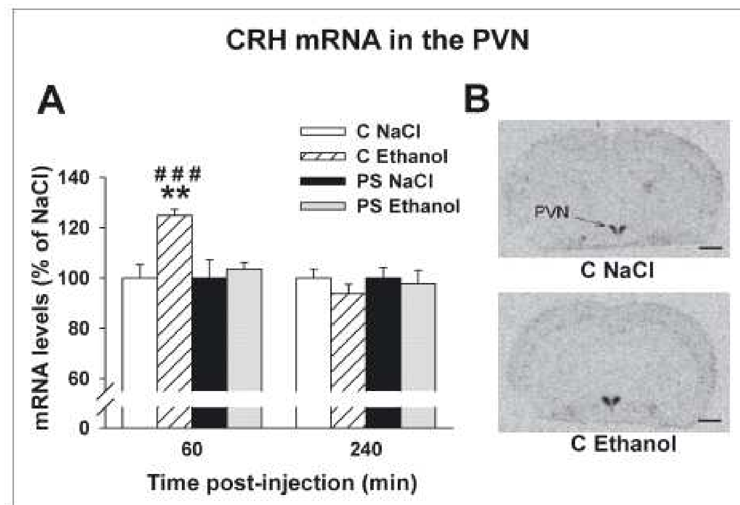

FIG. 3. (A) Semiquantitative analysis of paraventricular nucleus (PVN) corticotropin-releasing hormone (CRH) mRNA levels (expressed as percentages of respective NaCl groups) in control (C) and prenatal stress (PS) groups, 60 and 240 min after the ethanol (1.5 g/kg) or the NaCl (0.9%) intraperitoneal 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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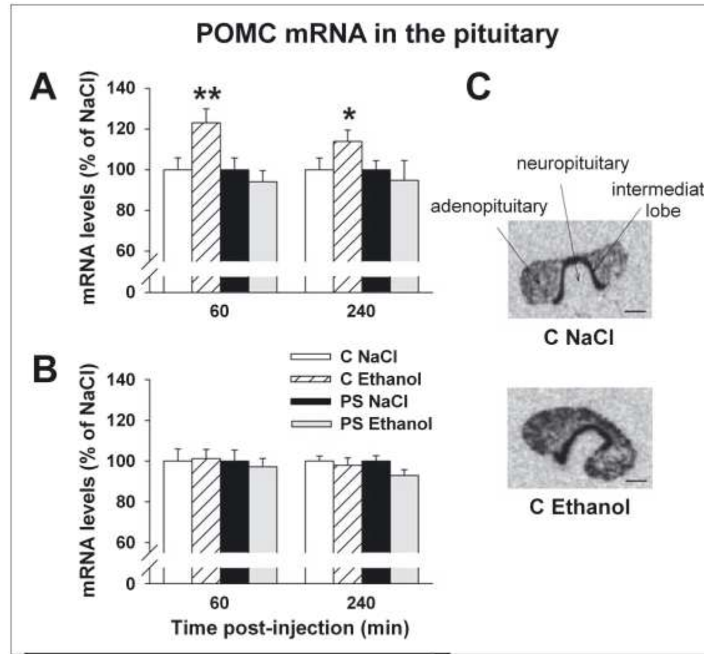

FIG. 4. Semiquantitative analysis of pro-opiomelanocortin (POMC) mRNA levels (expressed as percentages of respective NaCl groups) (A) in the anterior pituitary and (B) in the intermediate lobe of the pituitary in control (C) and prenatal stress (PS) groups, 60 and 240 min after the ethanol (1.5 g/kg) 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.

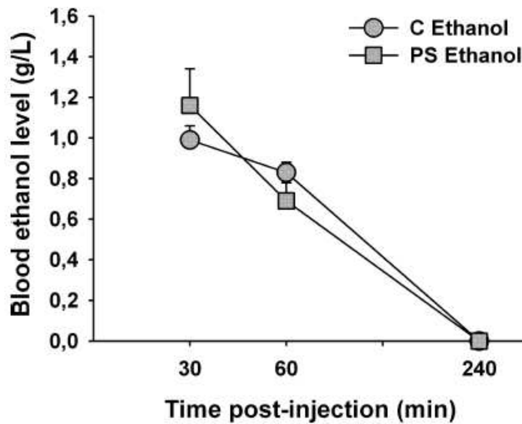

FIG. 5. Blood ethanol level (g/L) 30, 60 and 240 min after the intraperitoneal injection of ethanol (1.5 g/kg) in control (C) and prenatally stressed (PS) rats ($n = 9$–10 per group).

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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Erratum :

Page 1195, Figure 1. Plasma corticosterone levels are expressed in µg/dl .

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BRAIN RESEARCH

Research Report

Impact of an acute exposure to ethanol on the oxidative stress status in the hippocampus of prenatal restraint stress adolescent male rats

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ABSTRACT

Prenatal restraint stress (PRS) in rats is associated with hippocampal dysfunctions and several behavioural and endocrine disorders related to this brain area. Recently, we have reported that the PRS modifies the hypothalamic–pituitary–adrenal (HPA) response to an ethanol challenge in adolescent animals. Since hippocampus is particularly sensitive to the deleterious effects of ethanol during adolescence, we investigated in this study the combined effects of PRS and ethanol administration on the oxidative status in the hippocampus of 28-day-old male rats. Thirty minutes after an intraperitoneal (i.p.) injection of ethanol (1.5 g/kg), the activities of several antioxidant enzymes (superoxide dismutase, catalase and glutathione peroxidase) but also non-enzymatic antioxidant (reduced glutathione) were assayed. Thiobarbituric acid reactive substances (TBARS) levels were also measured as a marker of lipid peroxidation. Ethanol enhanced superoxide dismutase activity in control rats but not in PRS rats. At basal level, catalase activity was lower in PRS rats than in control rats, indicating a potentially higher sensitivity to oxidative damages after this early stress. However, the hippocampal TBARS levels were not significantly affected by the ethanol administration, showing that an acute ethanol exposure does not induce oxidative damage in adolescent male rats. In conclusion, our data suggest that PRS affects both basal antioxidant status in the hippocampus and antioxidant response after an acute ethanol exposure. These findings extend previous works showing that PRS leads to hippocampal dysfunctions and raise the question of the potential increase of the hippocampal oxidative damage in PRS rats after repeated exposure to ethanol.

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1. Introduction

Brain ethanol exposure can be associated with oxidative perturbation of cellular oxidant/antioxidant balance (Calabrese et al., 2000). Ethanol induces the formation of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\cdot}$) and superoxide anion radical (O_2^{-}) in different cerebral areas. The main damage to cells after ethanol results from the ROS-induced alteration of macromolecules, such as polyunsaturated fatty acids in membrane lipids, proteins, and DNA (Montoliu et al., 1994; Renis et al., 1996). Lipid peroxidations, especially in membranes, play a crucial role in tissue injury (Sahin and Gumuslu, 2004). In order to neutralize ROS, the body uses enzymatic antioxidants such as superoxide dismutase, catalase and glutathione peroxidase, but also non-enzymatic antioxidants such as reduced glutathione.

Vulnerability to ethanol in the brain is region specific and some data suggest that hippocampus exhibits a high vulnerability to oxidative stress (Renis et al., 1996; Binienda and Kim, 1997; McIntosh et al., 1998). Indeed, chronic ethanol induces specific DNA damages restricted to the cerebellum and the hippocampus. Repeated injections of ethanol (2.5 g/kg ethanol) stimulate TBARS production in the rat hippocampus, but not in the prefrontal cortex (Gonenc et al., 2005). In rats, during adolescence (postnatal days 28–48), strong behavioural changes and a number of important physiological alterations occur (Spear, 2000). During this period, the hippocampus is more sensitive to the deleterious effects of ethanol (White and Swartzwelder, 2004). Alcohol inhibits the induction of the long-term potentiation and NMDA receptor-mediated synaptic potentials, more potently in hippocampal slides from adolescent rats than in those from adults (Swartzwelder et al., 1995a, b; Pyapali et al., 1999). However, the impact of ethanol on oxidative stress status in the hippocampus remains poorly documented in adolescent animals.

Hippocampus contains the highest density of corticosteroid receptors in the brain and constitutes a key area involved in the stress response process. Chronic high corticosterone levels stimulate the formation of ROS (McIntosh and Sapolsky, 1996), enhance the toxicity of oxygen radical generators (Behl et al., 1997), and inhibit antioxidant enzymes such as Cu/Zn superoxide dismutase and glutathione peroxidase in the hippocampus (McIntosh et al., 1998). In rats, PRS leads to permanent changes of the stress response as well as of hippocampal function in the offspring. PRS induces long-lasting high levels of corticosterone after an acute psychogenic stress (Maccari et al., 1995; Barbazanges et al., 1996; Vallee et al., 1997, 1999), associated with both a reduction of the number of corticosteroid receptors in the hippocampus (Maccari et al., 1995; Barbazanges et al., 1996; Van Waes et al., 2006) and a blunted cellular activation of this area after an acute emotional stress (Viltart et al., 2007). Interestingly, prenatal stress increases the levels of neuronal nitric oxide synthase in the hippocampus during adolescence (Zhu et al., 2004), but its impact on the oxidative defenses is unknown. PRS affects the vulnerability to several drugs of abuse (Deminiere et al., 1992; Koehl et al., 2000; Morley-Fletcher et al., 2004; Yang et al., 2006; Kippin et al., 2007), including ethanol (Darnaudery et al., 2007). In contrast, few data are available on the consequences of drug of abuse on the brain of rats stressed *in utero*. Recently, we reported that PRS modulates the HPA response to an acute ethanol challenge (1.5 g/kg, i.p.) in adolescent male animals (Van Waes et al., 2006). Indeed, in 28-day-old male rats, PRS induces a blunted ACTH and corticosterone secretion after the ethanol injection. PRS animals also exhibit a lack of activation of central markers of the HPA axis after the ethanol injection, as indicated by the stable mRNA levels of the CRH in the NPV and of the POMC in the anterior pituitary. In the hippocampus, after the ethanol injection, we observed a decrease of the mineralocorticoid receptors mRNA levels in the dentate gyrus of the control animals whereas PRS rats were unaffected.

Therefore, we hypothesized that PRS could affect the oxidative response to an ethanol injection in the hippocampus of adolescent rats. To test this hypothesis, we examined in the present study the impact of PRS on the activities of ROS-scavenging enzymes, glutathione and TBARS levels in the hippocampus of adolescent male rats exposed to ethanol.

2. Results

2.1. Litters size and body weights

The size of the litters was not affected by the PRS procedure (Control: 12.2±0.7, PRS: 12.6±0.7). The ratio male/female were 1.04±0.15 in the Control group and 1.28±0.19 in the PRS group. Rats prenatally exposed to stress showed a significant reduction of their body weight at 28 days compared with control rats (Control Vehicle: 73.5±3.0 g, Control Ethanol 73.0±2.2 g, PRS Vehicle: 65.1±2.1 g, PRS Ethanol: 65.9±3.0; group effect: $F(1,31) = 9.28$, $P < 0.01$).

2.2. Blood ethanol levels

Thirty minutes after the i.p. injection of ethanol (1.5 g/kg), there was no significant difference in blood ethanol levels between control (0.94±0.07 g/l) and PRS rats (1.19±0.22 g/l) (Student's *t*-test, $t = 1.18$, $df = 13$, $P = 0.25$).

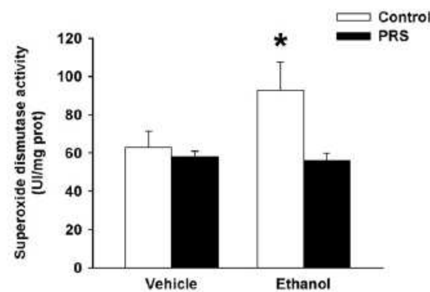

Fig. 1 – Superoxide dismutase activity (UI/mg prot) in the hippocampus of control and PRS adolescent male rats exposed to an ethanol (1.5 g/kg) or a vehicle (NaCl 0.9%) intraperitoneal injection. Ethanol significantly increased the superoxide dismutase activity in the hippocampus of control animals, whereas it had no effect in PRS rats. *$P < 0.05$ Control Ethanol versus all other groups ($n = 6$–8 per group).

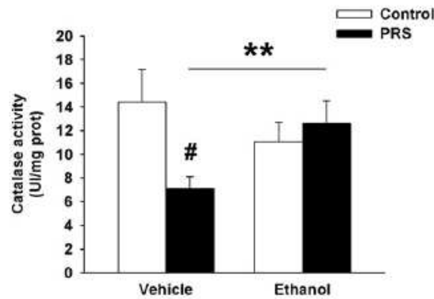

Fig. 2 – Catalase activity (UI/mg prot) in the hippocampus of control and PRS adolescent male rats exposed to an ethanol (1.5 g/kg) or a vehicle (NaCl 0.9%) intraperitoneal injection. Catalase activity was lower in PRS rats than in control rats. #P<0.05 Control Vehicle versus PRS Vehicle. After the ethanol administration, catalase activity was enhanced in PRS animals. **P<0.01 PRS Vehicle versus PRS Ethanol (n=6–8 per group).

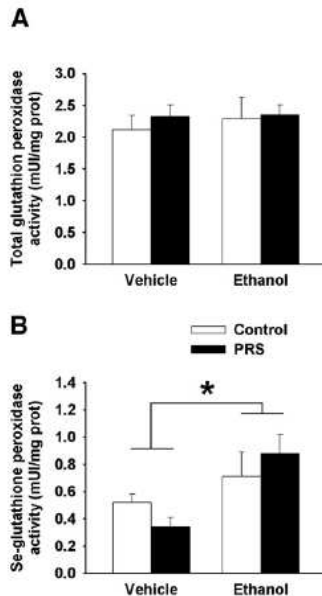

Fig. 3 – Total glutathione peroxidase activity (A) and selenium-dependent glutathione peroxidase activity (B) (mUI/mg prot) in the hippocampus of control and PRS adolescent male rats exposed to an ethanol (1.5 g/kg) or a vehicle (NaCl 0.9%) intraperitoneal injection. Ethanol significantly enhanced the selenium-dependent glutathione peroxidase activity in the hippocampus of animals (treatment effect: P<0.05) (n=6–8 per group).

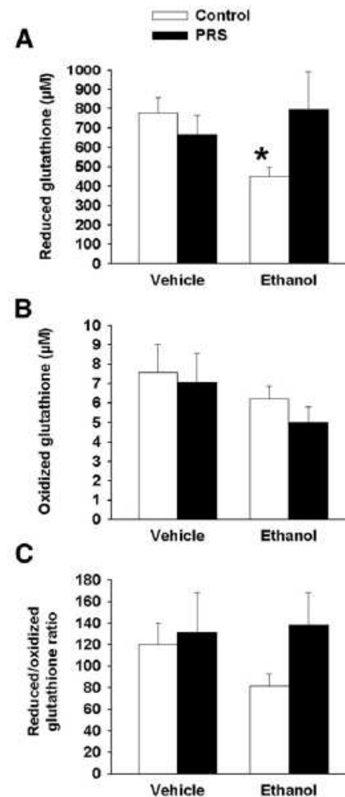

Fig. 4 – Reduced glutathione levels (A), oxidized glutathione levels (B) and reduced/oxidized glutathione ratio (C) in the hippocampus of control and PRS adolescent male rats exposed to an ethanol (1.5 g/kg) or a vehicle (NaCl 0.9%) intraperitoneal injection. Ethanol decreased the levels of reduced glutathione in the hippocampus of control animals but had no effect in PRS rats. *P<0.05 Control Vehicle versus Control Ethanol. However, PRS or ethanol had no effect on the reduced/oxidized glutathione ratio (n=6–8 per group).

2.3. *Antioxidant protective enzyme activities in the hippocampus*

2.3.1. *Superoxide dismutase activity*

Superoxide dismutase activity in the hippocampus was similar in PRS and control rats injected with vehicle. In contrast, after an acute ethanol administration, superoxide dismutase activity tended to be differently affected according to the groups (Fig. 1, group × treatment effect: $F(1,25)=3{,}63$, $P=0.07$). Ethanol enhanced superoxide dismutase in the control group whereas it has no significant impact in the PRS group (planned comparison: Control Vehicle versus Control Ethanol, $P<0.05$).

2.3.2. *Catalase activity*

As shown in Fig. 2, a group × treatment interaction was observed for the catalase activity ($F(1,26)=6.61$, $P<0.05$). Planned comparison revealed that catalase activity in the hippocampus

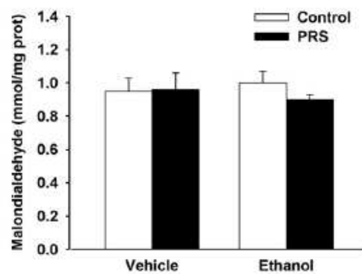

Fig. 5 – Malondialdehyde levels in the hippocampus of control and PRS adolescent male rats exposed to an ethanol (1.5 g/kg) or a vehicle (NaCl 0.9%) intraperitoneal injection. PRS or ethanol had no impact on malondialdehyde levels (n=6–8 per group).

of adolescent animals was decreased by PRS (Control Vehicle versus PRS Vehicle, $P<0.05$). Ethanol induced a significant increase of catalase activity in PRS rats but has no effect in control rats (planned comparison: PRS Vehicle versus PRS Ethanol, $P<0.01$).

2.3.3. *Glutathione peroxidase activity*

PRS and ethanol treatment had no effect on total hippocampal glutathione peroxidase activity (Fig. 3A). In contrast, whatever the group, the selenium-dependent glutathione peroxidase activity was enhanced by the ethanol administration (Fig. 3B, treatment effect: $F(1,19)=1.80$; $P<0.05$).

2.4. *Antioxidant status*

The levels of reduced glutathione were differently affected by the ethanol administration in control and PRS rats (Fig. 4A, group × treatment effect, $F(1,30)=3.92$, $P=0.05$). Ethanol decreased reduced glutathione in the control group, whereas it has no significant impact in the PRS group (planned comparison: Control Vehicle versus Control Ethanol, $P<0.05$). The levels of oxidized glutathione were altered neither by PRS nor by ethanol (Fig. 4B). However, the reduced/oxidized glutathione ratio was unchanged after the ethanol injection (Fig. 4C, group × treatment effect, $F(1,29)=0.73$, $P=0.39$).

2.5. *Oxidative damage*

As shown in Fig. 5, lipid peroxidation levels in the hippocampus were affected neither by PRS (group effect, $F(1,28)=1.11$, $P=0.30$), nor by the ethanol treatment (treatment effect, $F(1,28)=0.34$, $P=0.56$).

3. Discussion

The aim of this work was to examine the impact of a single injection of a moderate dose of ethanol on the oxidative defences in the hippocampus of control and PRS adolescent male rats. Our results reveal, independently of the ethanol injection, that stress *in utero* significantly decreased the hippocampal catalase activity. Moreover, the absence of superoxide dismutase activation after the ethanol challenge in PRS rats suggests a blunted response of the oxidative defences in these animals during adolescence. However, whatever the group, the single ethanol injection did not induce lipid peroxidation in the hippocampus, indicating that an acute exposure to a moderate dose of ethanol does not lead to oxidative damages in adolescent rats.

The effects of ethanol exposure on the activity of superoxide dismutase are controversial, with reports of an increase (Somani et al., 1996), no change (Gonenc et al., 2005), or a decrease (Ledig et al., 1981), depending on the brain region, the dose and the duration of ethanol exposure. In our study, the i.p. administration of 1.5 g/kg of ethanol in adolescent male rats significantly enhanced the superoxide dismutase activity in the hippocampus of control animals. Superoxide dismutase catalyzes the reduction of O_2^- to H_2O_2. This activation may protect the hippocampus against the ethanol-induced oxidative injury. In PRS rats, the superoxide dismutase activity remained unchanged after the ethanol administration. Since blood ethanol levels were similar between experimental groups, the blunted response of PRS animals could be related to a differential sensitivity to ethanol, rather than to a variation of ethanol metabolism between groups. Among ROS-scavenging enzymes, superoxide dismutase is particularly important because it is the first involved in ROS detoxification. In consequence, it seems that PRS rats are less responsive to the activation of oxidative defences. We previously demonstrated that adolescent male rats exposed to PRS presented a hyporesponse of the HPA axis after an ethanol challenge, leading to a blunted release of corticosterone after the ethanol injection in these rats (Van Waes et al., 2006). Here we demonstrate that oxidative stress response after acute ethanol exposure was also blunted in rats exposed to PRS, an effect that suggests an overall lower sensitivity to acute ethanol exposure in these rats. The low defences against ROS observed in rats submitted to the early stress indicate that PRS could lead to a higher sensitivity to different kind of aggression, such as repeated alcohol exposures, ischemia or traumatic brain injuries. Indeed, fetus from mothers exposed to infection during pregnancy show higher oxidative stress levels in the hippocampus (Lante et al., 2007). Furthermore, a recent study demonstrates that neonatal excitotoxic brain lesions, induced by intracerebral injection of glutamate analogs, are worsened in pups exposed to gestational stress (Rangon et al., 2007).

Zhu and collaborator have reported that PRS in middle or late pregnant stage causes an increase in the neuronal nitric oxide synthase expression in the hippocampus of adolescent male and female offspring rats. A significant increase in ROS levels was also induced by PRS in the hippocampus of female rats (Zhu et al., 2004). Together, these data suggest that PRS is able to cause oxidative stress in the offspring. Our study is the first to report that basal oxidative status is affected by PRS in male, as indicated by the reduced catalase activity reported in the hippocampus. PRS, induced by maternal contention during the gestation, provokes increased maternal corticosterone levels and decreased placental 11betaHSD2, the enzyme that inactivates the maternal corticosterone, making the fetus also more exposed to high levels of corticosterone (Mairesse et al., 2007). This phenomenon concerning maternal corticosterone levels is one of the mechanisms by which PRS could

induce long-lasting disturbance of the HPA function in the offspring (Barbazanges et al., 1996). Interestingly, a similar decrease of catalase activity in the cerebellar granule cells of offspring rat (40 days old) has been reported in a model of rat prenatal exposed to dexametasone between embryonic day 14 until birth (Ahlbom et al., 2000). In light of these data, it seems that the exposition to glucocorticoids during the late gestation contributes to reduce catalase activity in the brain of adolescent offspring. Catalase, localized to peroxisomes, is one of the enzymes responsible for converting H_2O_2 to water and oxygen. The decrease of catalase activity could consequently induce a high sensitivity to oxidative stress in rats born from stressed mother. Our results suggest that it is not the case in response to a single ethanol injection. Although PRS significantly reduced hippocampal catalase activity in adolescent male rats, this activity was increased after the ethanol exposure in stressed animals. However, the activity of the enzyme only reached the activity observed in the control animals. This implies that an adaptative response occurred to counterbalance the diminished basal catalase activity in PRS rats. It remains to determine whether in PRS rats, the catalase could detoxify, in a same efficient manner than in control animals, H_2O_2 after repeated ethanol exposure.

Glutathione peroxidase catalyzes the reduction of hydroperoxides, including H_2O_2, by reduced glutathione. Acute ethanol administration significantly enhanced hippocampal selenium-dependent glutathione peroxidase activity, both in control and PRS groups. In contrast, neither PRS, nor ethanol injection affected the total glutathione peroxidase activity in the hippocampus of adolescent rats. This finding is congruent with previous data obtained in adult animals after repeated ethanol administrations of 2.5 g/kg (Gonenc et al., 2005). Glutathione participates directly in the destruction of ROS. Repeated ethanol administrations (5 g/kg, intragastrically) for 7 days induce a decrease in reduced glutathione in several brain regions in adult rats (Calabrese et al., 2000). In the present study, we reported in adolescent animals that an acute ethanol injection with a moderate dose can also diminish the reduced glutathione levels in control animals, without affecting glutathione levels in PRS rats. However, the glutathione depletion observed in control rats did not result in a significant decrease in the reduced/oxidized glutathione ratio. Finally, the acute ethanol administration had no effect on lipid peroxidation in the hippocampus. These findings are consistent with previous studies conducted in the adult brain after ethanol administration (2 g/kg) (Pal et al., 1993), suggesting that the adolescent brain is not more sensitive than adult brain to the oxidative damages of ethanol. It is also important to consider gender differences since recent reports indicate that estrogens could protect brain against lipid peroxidation observed after ethanol withdrawal (Jung et al., 2004, 2006). However, independently of an ethanol exposure some data suggest a bigger neuronal loss and oxidative stress in the hippocampus of adolescent PRS female rats compared to male rats (Zhu et al., 2004).

In conclusion, our data demonstrate that exposure of animals to stressful experience during pregnancy decreased the catalase activity in the hippocampus of adolescent offspring. Furthermore, PRS blunted the antioxidant response to ethanol after an ethanol challenge. These findings demonstrate that a stress *in utero* may affect the developing brain and decrease the later oxidative response to ethanol. These alterations may lead to a higher vulnerability to oxidative injury in the hippocampus of PRS animals following a chronic ethanol treatment or after ethanol withdrawal.

4. Experimental procedures

4.1. *Animals and maternal restraint stress procedure*

Rats were housed in a temperature (22±2 °C) and humidity controlled animal room on a 12:12 h light–dark cycle (light exposure 7:00 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).

Twenty-two nulliparous female Sprague–Dawley rats (Harlan, France), weighing 200–225 g, were group-housed for at least 10 days in our animal facilities. Subsequently, females were individually housed overnight with a sexually experienced male rat (400 g) for mating 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 ($n=11$) were left undisturbed whereas stressed dams ($n=11$) were subjected to a repeated restraint stress procedure as previously described (Maccari et al., 1995; Van Waes et al., 2006). Restraint stress procedure consisted of restraint the pregnant dam, called PRS, under a bright light (6500 lx) 45 min three times daily in a transparent cylinder (7.5 cm diameter, 19 cm long) 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-ratio were selected for the study and housed individually. A maximum of 2 males per littermate was used for each experimental group to avoid any litter effect.

4.2. *Ethanol treatment and sacrifice*

Adolescent (28 days old) PRS ($n=18$) and control ($n=17$) male rats 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 physiologic saline and were killed by decapitation 30 min later (PRS Ethanol group: $n=8$, Control Ethanol group: $n=9$, PRS Vehicle group: $n=10$ and Control Vehicle group: $n=8$). This treatment was chosen based on our previous results showing that 1.5 g/kg ethanol injection induced a differential ethanol response between prenatal stress and control groups (Van Waes et al., 2006). Experiment was performed between 9:00 and 13:00 h. Trunk blood samples (approximately 4 ml) were collected in chilled tubes containing 40 µl of 5% EDTA (Sigma Aldrich, France), centrifuged at 2000×g for 15 min at 4 °C and stored at −20 °C until the ethanol assay. Brains were quickly removed, immediately frozen on dry ice and stored until assays at −80 °C.

4.3. *Blood ethanol levels*

Blood ethanol levels were measured using gas chromatograph coupled with a flame ionization detector (Hewlett Packard 5890 series II, Hewlett Packard, France). Acetonitrile was used as internal standard. To 100 µl of plasma was added 100 µl of NaOH (N/2), 100 µl of zinc sulfate (30%) and 50 µl of acetonitrile (pure). The whole was centrifuged at 1000×*g* for 10 min at 4 °C, the supernatant was collected and 3 µl of this one was injected into the chromatograph. The detection limit of the method was 0.01g/l.

4.4. *Tissue collections and preparation of tissue extract*

Hippocampi were dissected on an ice-cold surface and homogenized in cold 50 mM sodium phosphate buffer (pH 7.0) containing 0.1 mM EDTA to give 5% homogenate (w/v). The homogenates were then centrifuged at 6000×*g* for 10 min at 4 °C to remove nuclei and debris. The supernatants were separated, aliquoted, and stored at −80 °C until analysis.

4.5. *Biochemical measurements in the hippocampus*

The activities of antioxidant enzymes superoxide dismutase, catalase and glutathione peroxidase, the levels of reduced and oxidized glutathione as well as TBARS were determined in the whole hippocampus with commercial kits. From 20 to 200 µl of homogenate were used for each measure and the assays were done in duplicate.

4.5.1. *Superoxide dismutase activity*

Superoxide dismutase activity (UI/mg protein) was determined using a kit (Sigma-Aldrich, France). This kit uses Dojindo's highly water-soluble tetrazolium salt, WST-1 (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) that produces a water-soluble formazan dye upon reduction with a superoxide anion. The rate of the reduction with O_2 is linearly related to the xanthine oxidase activity, and is inhibited by superoxide dismutase. IC50 (50% inhibition activity of superoxide dismutase) in the supernatant was measured at 450 nm on a MRX II absorbance reader (Dynex Technologies, UK).

4.5.2. *Catalase activity*

Catalase activity (UI/mg protein) was determined using a kit (Sigma-Aldrich, France). This assay method is based on the measurement of the hydrogen peroxide substrate remaining after the action of catalase. First, the catalase converts hydrogen peroxide to water and oxygen (catalytic pathway) and then this enzymatic reaction is stopped with sodium azide. An aliquot of the reaction mix is then assayed for the amount of hydrogen peroxide remaining in the presence of 3,5-dichloro-2-hydroxybenzenesulfonic acid, 4 aminoantipyrine and horseradish peroxidase. The red quinoneimine dye (N-(4-antipyryl)-3-chloro-5-sulfonatep-benzoquinone-monoimine) formed was measured at 520 nm (Varian Cary 50 Bio UV–visible spectrophotometer, France).

4.5.3. *Glutathione peroxidase activity*

Glutathione peroxidase activity (mUI/mg protein) was determined using a kit (Sigma-Aldrich, France). The decrease in NADPH (b-Nicotinamide Adenine Dinucleotide Phosphate, Reduced) absorbance measured at 340 nm (Varian, France) during the oxidation of NADPH to NADP+ is indicative of glutathione peroxidase activity in a coupled reaction in the presence of reduced glutathione, glutathione reductase, and *tert*-butyl hydroperoxide. The presence of non-selenium enzymes was determined using cumene hydroperoxide as the substrate. The results were expressed as total glutathione peroxidase activity and selenium-dependent glutathione peroxidase activity (mUI/mg protein).

4.5.4. *Glutathione levels*

Reduced glutathione (µM) was assayed for total level as well as reduced/oxidized glutathione ratio using a colorimetric assay kit (Oxis International, USA). 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) reacts with reduced glutathione to form a spectrophotometrically detectable product at 412 nm (Varian, France). Oxidized glutathione can be determined by the reduction to reduced glutathione by NADPH catalyzed by glutathione reductase, which is then determined by the reaction with DTNB. In brief, the method utilizes the change in color development during the reaction, and the reaction rate is proportional to the reduced glutathione and oxidized glutathione concentrations.

4.5.5. *TBARS levels*

TBARS levels, as an index of lipid peroxidation, were estimated using a kit (Northwest Life Science Specialties, Canada). The NWK-MDA01 assay is based on the reaction of malondialdehyde (MDA) with thiobarbituric acid (TBA) forming an MDA-TBA2 adduct that absorbs strongly at 532 nm. Homogenates were mixed with butylated hydroxytoluene, phosphoric acid and TBA reagent. The samples were incubated at 60 °C for 60 min. After centrifugation, the absorbance was recorded at 532 nm on the UV–visible spectrophotometer (Varian, France). Using tetramethoxypropane, a standard curve was prepared and the value of the homogenate was determined from this curve. Results are expressed as MDA equivalents (nmol/mg protein).

4.5.6. *Protein assays*

Pyrogallol red assay (Total protein Pyrogallol red kit, Randox, UK) using a human albumin calibrator was tested for rat brain samples. Quantitative measurements of whole hippocampus total protein were made on the Olympus AU 600 analyzer (Olympus Optical Co., Japan) by following the respective reagent instructions for implementation on the instrument.

4.6. Statistics

All data were presented as means ± S.E.M. They were analyzed using 2-way analysis of variance (ANOVA), with group (control versus PRS) and treatment (vehicle versus ethanol) as between variables, followed by planned comparisons. Student's *t*-tests were employed to compare blood ethanol levels in control and PRS rats. Significance was set at $P < 0.05$.

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STRESS, GESTATION ET ALLOSTASIE MATERNELLE

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Prenatal stress induces intrauterine growth restriction and programmes glucose intolerance and feeding behaviour disturbances in the aged rat

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Abstract

There is growing evidence that prenatal adversities could be implicated in foetal programming of adult chronic diseases. Since maternal stress is known to disturb the foetal glucocorticoid environment, we examined the consequences of prenatal stress on foetal growth, on glucose–insulin metabolism and on feeding behaviour in the aged male rat. In foetuses at term, maternal stress reduced body, adrenal and pancreas weight as well as plasma corticosterone and glucose levels. In aged male rats (24 months of age), prenatal stress induced hyperglycaemia and glucose intolerance and decreased basal leptin levels. Moreover, after a fasting period, they showed an increased food intake. These data suggest that maternal stress induces a long-lasting disturbance in feeding behaviour and dysfunctions related to type 2 diabetes mellitus. This programming could be linked to the early restricted foetal growth and to the adverse glucocorticoid environment *in utero*.

Journal of Endocrinology (2004) **181**, 291–296

Introduction

In humans, an inverse relationship between birth weight and adult ischaemic heart disease has been described, suggesting the role of prenatal 'programming' as a determinant of adult diseases (Barker & Osmond 1986). This suggests that environmental influences acting on foetal life are reflected in altered birth size/phenotype and permanently affect structure and metabolism, thereby leading to a greater risk of developing coronary heart disease, hypertension, insulin-resistance syndromes and osteoporosis (Barker 1998, Nathanielsz & Thornburg 2003). However, while increasing epidemiological evidence supports the role of early developmental growth patterns in the development of specific adult diseases, the determinant of the foetal growth restriction remains unclear. Maternal hormonal and nutritional status may be particularly important (Holness *et al.* 2000, Lesage *et al.* 2002). In this view, in humans and in rodents prenatal glucocorticoid overexposure induced by hormonal treatment or by maternal stress during gestation has been proposed to programme an adverse adult cardiovascular, metabolic, neuroendocrine and behavioural phenotype (Seckl 2001).

In rats, prenatal stress (PS) is known to disturb the foetal environment and to programme permanently neuroendocrine and behavioural responses in adult offspring (Maccari *et al.* 2003). For example, PS increased stress-induced adrenocorticotrophin (ACTH) and corticosterone (CORT) secretion and decreased binding capacity of hippocampal glucocorticoid receptors (GRs) (Koehl *et al.* 1999). These hypothalamo–pituitary–adrenal (HPA) axis dysfunctions have been suggested to be mediated by maternal glucocorticoids during pregnancy. Indeed, adrenalectomy of pregnant dams suppressed the effects of maternal stress on the HPA axis of the offspring (Barbazanges *et al.* 1996). Even if PS constitutes a model of glucocorticoid overexposure *in utero*, few studies have explored its long-lasting consequences on metabolic parameters. It was reported that in young adult animals PS increased basal glycaemia and reduced both body weight and food intake (Vallée *et al.* 1996). However, although the vulnerability to develop certain metabolic disorders such as type 2 diabetes mellitus strongly increases with ageing (Holness *et al.* 2000), metabolic alterations in aged PS rats have never been investigated. The aim of the present study was to evaluate if PS induces an *in utero* growth restriction and increases the vulnerability to develop metabolic disorders with ageing such as altered glucose–insulin metabolism and disturbed feeding behaviour.

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Materials and Methods

Subjects

Female Sprague–Dawley rats (250 g) were mated with a male for one night. The next day was considered as day 0 of pregnancy if spermatozoa were found in the vaginal smears. Pregnant females were then transferred to individual cages. During the last week of pregnancy (from embryonic day E14 to E21), pregnant females in the stress group (n=10) were placed for 45 min in a transparent plastic cylinder in a lighted environment three times per day (0900, 1200 and 1700 h). Control females (n=10) were left undisturbed. For studies in foetuses at term (E21), the maternal stress procedure was continued until E20. This stress procedure was previously described by Maccari *et al*. (1995). After weaning, offspring were housed in groups of four animals and left undisturbed for 23 months. Then, aged male rats were individually housed for 1 month before the beginning of experiments. All rats were maintained on a 12 h light:12 h darkness cycle (lights on 0800–2000 h), with free access to food and water. Manipulation of the animals was performed following the principles of laboratory animal care published by the French Ethical Committee and the rules of the European Union Normative (86/609/EEC).

Plasma and tissue collections

On day 21 of gestation, pregnant females at term (n=6 females/group) were killed rapidly by decapitation between 1000 and 1200 h. Each litter usually contained 8–12 foetuses, which were collected by caesarean section, rapidly weighed and killed by decapitation.

Trunk blood samples of foetuses were collected after decapitation in tubes pre-rinsed with EDTA. Blood glucose was measured using a glucometer (One Touch II; Lifescan, Roissy, France). Body length was measured in foetuses and the adrenals and the pancreas were removed and weighed.

For aged male rats (24 months old), a maximum of two males from the same litter were used. After food intake measurements and oral glucose tolerance tests (OGTTs), rats were killed at rest by decapitation between 1000 and 1200 h. Trunk blood was collected and blood glucose was measured.

Adrenals, pancreas and the perirenal and perigonadal fat pads were removed and weighed. All blood samples were centrifuged at 3200 g for 10 min at 4 °C and plasma samples were kept at − 30 °C prior to CORT and leptin assays.

OGTTs

OGTTs were performed on eight or nine animals from both groups at 24 months of age after a 16 h fasting. Animals were given 2 g glucose/kg body weight with an oral cannula. Blood samples were collected from the tail vein 5 min before (time 0) and 60 and 120 min after glucose load. Blood glucose was measured and plasma aliquots were kept at − 30 °C until assayed for insulin.

Food intake measurement

Basal feeding behaviour was evaluated by measuring consumption of food in the home cages of the animals for a 24 h period. Cumulative food intake was also determined for 1, 2, 3 and 24 h periods after 24 h of fasting. Food consumption was determined by placing 150 g of chow in the home cage and weighing the residual food at indicated intervals.

RIAs

Plasma CORT levels were measured with an ^{125}I RIA kit (ICN Biomedicals, Irvine, CA, USA) using a highly specific CORT antibody and a detection threshold of 0·1 µg/100 ml.

Plasma insulin levels were measured using mono-iodinated ^{125}I-labelled porcine insulin (Sorin Biomedica, Sallugia, Italy) as a tracer, guinea pig anti-insulin antibody kindly provided by Dr Van Schravendijk (Brussels, Belgium) and purified rat insulin (Novo Nordisk, Boulogne, France) as standard. Charcoal was used to separate free from bound hormone. The sensitivity of the assay was 0·25 ng/ml.

Plasma leptin concentration was measured using a rat/mouse leptin RIA kit (LEP-R61; Mediagnost, Tuebingen, Germany). Standards and ^{125}I-labelled tracer were prepared from recombinant mouse leptin. No cross-reactivity was found with insulin and insulin-like growth factor-I. Sensitivity was 6 pg/ml in undiluted plasma samples.

Statistical analysis

All data are presented as means ± s.e.m. Morphometric and biological parameter comparisons between control and PS rats were performed using independent Student's t-tests. One-way ANOVA followed by a Newman–Keuls (NK) post-hoc test was used to compare groups for parameters with repeated measures (OGTT test and food intake measurement). $P<0·05$ was considered significant.

Results

Effect of maternal stress on physiological parameters of foetuses at term

Foetuses from stressed mothers showed reduced body weight both in males ($P<0·001$) and in females ($P<0·01$),

Table 1 Effects of maternal stress during the last week of gestation on morphometric and biological parameters of foetuses at term (day 21 of gestation). Data are means ± S.E.M. (n=29 male foetuses/group and n=31–39 female foetuses/group for body weight values; n=13–20 male foetuses/group for others parameters)

	Control	PS
Body weight (g)		
Males foetuses	6·09 ± 0·09	5·34 ± 0·10***
Female foetuses	5·85 ± 0·09	5·46 ± 0·08**
Body length (cm)	4·89 ± 0·04	4·84 ± 0·05
Adrenals (mg)	3·33 ± 0·28	2·20 ± 0·25**
Pancreas (mg)	30·78 ± 0·88	25·49 ± 0·86***
Plasma glucose (mg/dl)	77·10 ± 4·44	60·76 ± 4·24*
Plasma CORT (µg/dl)	7·07 ± 0·76	2·40 ± 0·35***
Plasma leptin (ng/ml)	2·71 ± 0·29	3·35 ± 0·28

*P<0·05, **P<0·01, ***P<0·001, PS vs control.

as well as reduced adrenal (P< 0·01) and pancreas (P< 0·001) weight in males (Table 1). Plasma glucose and CORT levels were also significantly reduced (P< 0·05 and P< 0·001 respectively) in these foetuses (Table 1). However, plasma leptin levels were not affected by maternal stress (Table 1).

Effect of PS on physiological parameters of aged male rats

PS had no effect on body, adrenal, pancreas and fat depots weights (Table 2). Basal plasma CORT levels tended to increase in PS rats (Table 2) but statistical analysis was not significant (P=0·08). In contrast, plasma leptin levels, at rest, were reduced (P< 0·05) in PS rats (Table 2).

As shown in Fig. 1, the OGTT induced an increase of plasma glucose and insulin levels (time effect, glucose, $F(2,28)=58·33$, P< 0·001; insulin, $F(2,28)=14·78$, P< 0·001). Aged PS rats had higher glucose levels than control rats at all investigated times (group effect, $F(1,14)=4·68$, P< 0·05) (Fig. 1A). In contrast, insulin secretion after OGTT was similar between experimental groups (Fig. 1B).

Table 2 Effects of PS on body and organ weights and basal plasma CORT and leptin levels in 24-month-old male rats. Data are means ± S.E.M. (n=7–8 animals/group)

	Control	PS
Body weight (g)	676·14 ± 17·53	647·25 ± 17·02
Adrenals (mg)	77·93 ± 6·92	67·21 ± 4·91
Pancreas (g)	1·07 ± 0·07	1·02 ± 0·06
Perirenal fat (g)	6·52 ± 0·72	5·44 ± 0·23
Gonadal fat (g)	4·76 ± 0·56	4·38 ± 0·23
Plasma CORT (µg/dl)	9·29 ± 2·15	16·72 ± 4·34
Plasma leptin (ng/ml)	13·55 ± 0·99	10·28 ± 0·57*

*P<0·05, PS vs control.

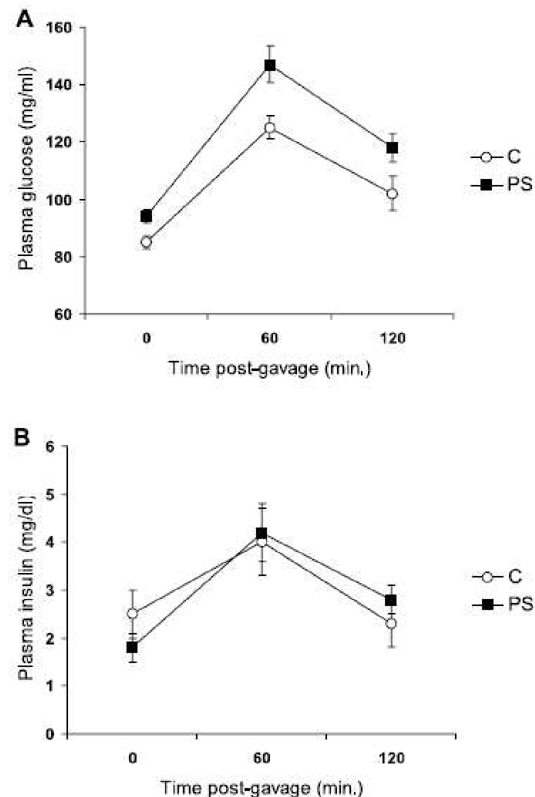

Figure 1 Effect of PS on plasma glucose (A) and insulin levels (B) during an OGTT in 24-month-old male rats. Values are means ± S.E.M. from eight or nine animals/group.

Effect of PS on feeding behaviour of aged male rats

PS had no effect on the basal food intake in aged animals (control, 25·7 ± 1·8 g; PS, 25·1 ± 0·6 g). In contrast, after 24 h of fasting, the cumulative food intake over a period of 3 h was higher in PS rats than in controls (group effect, $F(1,12)=14·59$, P< 0·01; Fig. 2). The time course of food consumption differed between control and PS rats. Indeed, PS rats continued to increase their food intake 2 h (NK post-hoc, P< 0·001 compared with the 1 h period) and 3 h (NK post-hoc, P< 0·05 compared with the 2 h period) after food was placed in their home cage, whereas in controls there was no significant increase of food intake after the first hour.

Discussion

In the present study, we report that prenatal restraint stress induces a restriction of intrauterine growth in both male

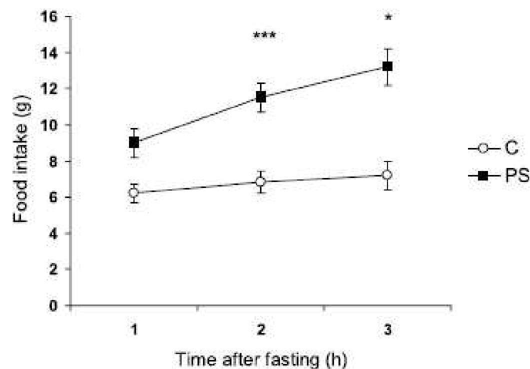

Figure 2 Effect of PS on cumulative food intake in 24-month-old male rats after 24 h of fasting. Values are means ± S.E.M. from eight or nine animals/group. ***$P<0·001$, 2 h vs 1 h values; *$P<0·05$, 3 h vs 2 h values.

and female foetuses. Interestingly, we demonstrate for the first time in aged PS animals an increase of plasma glucose levels without a change in plasma insulin concentration after an OGTT procedure. Moreover, aged rats had reduced plasma leptin levels and showed an increase of food intake after a fasting period. These results suggest that PS could increase later vulnerability to metabolic diseases with ageing.

Our study indicates that maternal stress decreases foetal body weight at term, indicating a foetal growth restriction. Previous reports on the effects of PS on body weight are conflicting. Indeed, a reduced body weight of pups has been reported in some cases (Drago *et al.* 1999, Patin *et al.* 2002), whereas other studies have not revealed any differences (Power & Moore 1986, Von Hoersten *et al.* 1993). These discrepancies could be a result of postnatal factors such as maternal milk yield. In our case, pups were removed by caesarean procedure, excluding the possibility of differences in milk secretion between control and stressed mothers (Lau 1992) or in milk intake in newborns.

Processes by which maternal stress affects pups development are unknown. However, numerous studies have shown that excessive glucocorticoids exposure *in utero* reduces birth weight and alters organ maturation in a variety of mammalian species, including primates and man (Reinisch *et al.* 1978, Novy & Walsh 1983). Maternal hypersecretion of glucocorticoids as well as exogenous administration of CORT or dexamethasone has been reported to reduce both foetal adrenal growth and activity in parallel with a drastic reduction of both hypothalamic corticotrophin-releasing hormone content and plasma ACTH concentration (Dupouy *et al.* 1987, Lesage *et al.* 2001). Chronic restraint stress is known to increase CORT levels in pregnant dams (Barbazanges *et al.* 1996). We report here a reduction of CORT secretion and an atrophy of adrenal glands in the foetus at term. These modifications confirm the foetal glucocorticoid overexposure, since adrenal atrophy is a physiological adaptation in the foetus to attenuate high glucocorticoids levels. Our present results also indicate that maternal stress reduces both plasma glucose levels and pancreas weight in foetuses. In a previous study, we showed that the development of pancreatic islets and β-cells is extremely sensitive to glucocorticoids from both maternal or foetal adrenal glands (Blondeau *et al.* 2001). Indeed, inhibition of foetal steroid production drastically increases both islet number and β-cell mass, whereas overexposure to maternal glucocorticoids has opposite effects (Blondeau *et al.* 2001). The reduction of plasma glucose levels in foetuses from stressed mothers could result from numerous disturbances such as a reduced efficiency in placental (maternal-to-foetal) glucose transfer, or an increased foetal glucose metabolism.

When they were 24 months old, PS rats had no alterations in their body or organs weights. However, PS rats exhibited hyperglycaemia under basal conditions and after a glucose load, whereas insulinaemia was not affected. This is the first experimental proof suggesting that maternal stress could programme type 2 diabetes mellitus in aged offspring. These results extend other reports in the literature. Indeed, it has been shown that PS rats have high plasma glucose levels at the age of 5 months (Vallée *et al.* 1996). Present data suggest a persistent effect of PS on glycaemia. Administration of synthetic glucocorticoids during late gestation causes hyperglycaemia and glucose intolerance in adult rat offspring (Nyirenda *et al.* 1998). Moreover, inhibition of placental 11β-hydroxysteroid dehydrogenase type 2, which protects the foetus from an excess of glucocorticoids of maternal origin, reduces birth weight and leads to impaired glucose tolerance in adult rats (Saegusa *et al.* 1999). So, our results reinforce the hypothesis that prenatal programming of glucose metabolism may be mediated by the glucocorticoid environment during foetal life. It was reported that early stress paradigms reduce the food intake in young adult rats (Vallée *et al.* 1996, Penke *et al.* 2001). We show that basal food intake was not altered in aged PS rats. We also report an increase of food intake after a fasting period in aged PS rats, suggesting an alteration of the feeding behaviour during stressful situations in these animals. PS reduces leptin secretion in aged offspring. Leptin is well documented to activate hypothalamic proopiomelanocortin/cocaine-amphetamine-regulated transcript anorexigenic neurons and to inhibit NPY/AgRP orexigenic ones, resulting in a decreased food intake (Schwartz *et al.* 2000). So, it could be hypothesised that low leptin levels in PS rats could be involved in the increase of food intake. However, in PS rats this increase of food intake is only triggered after a fasting period. PS has been well described to provoke HPA axis hyperactivity in response to stress through life (Vallée *et al.* 1999). As CORT is implicated in the feeding behaviour after fasting (Castonguay 1991, Hamelink *et al.* 1994), it

could be hypothesised that a differential corticosteroid response to the stress of fasting in PS rats could be implicated in the differences in food intake. The reduction of plasma leptin levels in aged PS rats, in spite of an unchanged weight of several adipose tissues, is quite surprising. However, an altered adipocyte metabolism may be involved since recent data demonstrate that prenatal dexamethasone exposure is associated with increased GR expression and attenuated fatty acid uptake in adult visceral adipose tissue (Cleasby *et al.* 2003).

Several data in humans suggest an early programming of adult diseases including an increased risk for developing a type 2 diabetes mellitus with ageing in subjects with a low body weight at birth. We demonstrate here that maternal stress induced an intrauterine growth restriction in rat foetuses and programmes a type 2 diabetes mellitus and eating disorders in aged offspring. These data support the concept of a prenatal programming of chronic adult diseases and demonstrate that stress during perinatal life may have a profound impact on health throughout life.

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Maternal stress alters endocrine function of the feto-placental unit in rats

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[1]*Perinatal Stress Unit, University of Lille 1, Villeneuve d'Ascq;* [2]*Institut National de la Santé et de la Recherche Médicale, Cordeliers Biomedical Research Institute, Paris, France;* [3]*Institute of Histology and Embryology, University of Graz, Graz, Austria;* [4]*Department Physiology/Endocrinology, Institute Neuroscience, The Sahlgrenska Academy, Gothenburg University, Gothenburg, Sweden;* [5]*Endocrinology Unit, Queen's Medical Research Institute, University of Edinburgh, Edinburgh, United Kingdom; and* [6]*Department of Experimental Medicine and Pathology, University Rome 1 "La Sapienza", Rome, Italy*

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Mairesse J, Lesage J, Breton C, Bréant B, Hahn T, Darnaudéry M, Dickson SL, Seckl J, Blondeau B, Vieau D, Maccari S, Viltart O. Maternal stress alters endocrine function of the feto-placental unit in rats. *Am J Physiol Endocrinol Metab* 292: E1526–E1533, 2007. First published January 30, 2007; doi:10.1152/ajpendo.00574.2006.—Prenatal stress (PS) can cause early and long-term developmental effects resulting in part from altered maternal and/or fetal glucocorticoid exposure. The aim of the present study was to assess the impact of chronic restraint stress during late gestation on feto-placental unit physiology and function in embryonic (E) *day 21* male rat fetuses. Chronic stress decreased body weight gain and food intake of the dams and increased their adrenal weight. In the placenta of PS rats, the expression of glucose transporter type 1 (GLUT1) was decreased, whereas GLUT3 and GLUT4 were slightly increased. Moreover, placental expression and activity of the glucocorticoid "barrier" enzyme 11β-hydroxysteroid dehydrogenase type 2 was strongly reduced. At E21, PS fetuses exhibited decreased body, adrenal pancreas, and testis weights. These alterations were associated with reduced pancreatic β-cell mass, plasma levels of glucose, growth hormone, and ACTH, whereas corticosterone, insulin, IGF-1, and CBG levels were unaffected. These data emphasize the impact of PS on both fetal growth and endocrine function as well as on placental physiology, suggesting that PS could program processes implied in adult biology and pathophysiology.

prenatal stress; placenta; adrenal; testis; pancreas; glucose; growth hormone; adrenocorticotropic hormone

SUBSTANTIAL EPIDEMIOLOGICAL FINDINGS and experimental studies have emerged associating low birth weight with an increased prevalence of cardiovascular and metabolic disorders in adult life (2–4, 18, 20, 38). In humans, exposure to deleterious environmental factors in utero reduces birth weight and predicts the subsequent occurrence of hypertension, ischemic heart disease deaths, hyperlipidemia, insulin resistance/type 2 diabetes mellitus, and neuroendocrine alterations in adulthood (4, 18, 44). Of the prenatal challenges that might underpin these long-term effects, variations in nutrition and/or glucocorticoid exposure (1, 13, 20, 49, 53, 57) as well as variations in maternal behavior (10, 40) have been proposed as key mediators of developmental programming of adult pathophysiology.

Importantly, exposure of the pregnant rat dam to chronic stress reduces offspring weight at birth and produces long-term metabolic, behavioral, and neuroendocrine changes (35, 39, 46, 65) consistent with a prenatal programming of the adult biology and pathophysiology. Thus, the adult offspring of rat dams exposed to chronic restraint stress during the last week of gestation (40, 62) display metabolic changes, including hyperglycemia (56), altered sensitivity of the cardiovascular system (27), and increased food intake after fasting (35). Prenatal stress (PS) also induced emotional and cognitive disturbances in adult and aged animals including, "anxiety," depressive-like behavior, and altered reactivity to stress of the hypothalamic-pituitary-adrenal (HPA) axis (11, 37, 39, 41, 55, 60). Collectively, these findings indicate that maternal stress "signals" the developing fetus to adjust multiple facets of its tissue development to alter the adult phenotype. These changes mirror those seen in rats, sheep, and other species prenatally exposed to glucocorticoids (51) and broadly parallel the human phenotype associated with low-birth-weight populations (4).

It has been postulated that the origin of such altered developmental plasticity is the in utero exposure to glucocorticoids, which increased during maternal chronic stress (1), and/or the early postnatal maternal environment (10, 40). However, placental dysfunctions and/or fetal endocrine disturbances could also be implicated.

Maternal-to-fetal transfer of glucocorticoids is predominantly regulated by a placental enzyme, 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2). In the placenta of rats (61) and humans (54), 11β-HSD2 catalyses the conversion of active corticosterone (cortisol in humans) into inert 11-dehydrocorticosterone (cortisone). This enzyme normally protects the fetus from relatively high levels of maternal glucocorticoids. In contrast, placental 11β-HSD type 1 is expressed in decidua and other maternal components of the rat placenta and acts in the reverse (reductase) direction, increasing local glucocorticoid levels (61). It has been hypothesized (13) that variations in the activity of placental 11β-HSD2 may be the physiological equivalent of exposure of the fetus to synthetic glucocorticoids, which are poor substrates for the enzyme. In line with this, placental 11β-HSD2 activity correlates with birth weight in rats (6) and humans (54). However, its sensitivity to maternal stress has never been described, although both glucocorticoids (57) and catecholamines (50), some well-known endocrine

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mediators of stress, alter trophoblast cell 11β-HSD2 expression in vitro.

Fetal growth is also dependent on adequate placental nutrient transfer. A primary nutrient for the developing fetus is glucose, which crosses the placental barrier through facilitated transporters down a maternal-fetal concentration gradient (31). Three high-affinity isoforms of these protein transporters have been identified in both human and rodent placentas: glucose transporter (GLUT)1, GLUT3, and GLUT4 (32, 33). GLUT1 mediates glucose transport into both the placenta and fetus (31, 67), whereas GLUT3 is restricted to cells on the fetal surface of the maternal-fetal barrier (8). GLUT4 expression is very low in rodent placentas but highly expressed in humans (66). To our knowledge, although PS was shown to reduce birth weight of offspring (35), the effect of maternal stress on placental nutrient transfer capacity has never been reported.

This study was undertaken to explore the effects of a prenatal restraint stress on early physiological processes in vivo. For this purpose, we assessed whether chronic maternal stress in late pregnancy alters body weight and food intake of the dams, affects placental 11β-HSD2 and transplacental glucose transfer, and has immediate effects upon fetal growth, pancreatic function, glucose homeostasis, and endocrine functions.

MATERIALS AND METHODS

All experiments were approved by the Institutional Animal Care and Use Committee in accordance with the principles of laboratory animal care (European Communities Council Directive of 1986, 86/609/EEC) and following the Institute for Laboratory Animal Research "Guide for Care and Use of Laboratory Animals."

Housing conditions. Adult virgin Sprague Dawley female rats (Charles River, L'Arbresle, France) weighing 240 g were housed in groups of five per cage for 2 wk before mating to coordinate their estrous cycles ($n = 20$). They were then housed overnight separately with a sexually experienced male (400 g). Copulation was determined by detection of sperm, and this was designated as E1. Pregnant females were then divided to PS ($n = 10$) or control (CTL; $n = 10$) groups, individually housed in plastic cages, allowed ad libitum access to food (regular rat chow no. 113, containing 22% protein, 5% fat, and 53% carbohydrate; UAR, Villemoisson-sur-Orge, France) and water and maintained on a 12:12-h light-dark cycle (lights on at 0700) with constant temperature and humidity.

In another experiment related to the pregnant females, 12 PS and 12 CTL females were weighed at the beginning and at the end of gestation (*day 1* and *day 21*), and their food intake was monitored during the last week of gestation. Basal feeding behavior was evaluated by measuring consumption of food in the home cages of the animals during each 24-h period from E15 to E21. Food intake was expressed as the weight of the mean daily intake from E15 to E21 (Fig. 1*B*).

Maternal restraint stress procedure. The stress procedure was performed every day for the last 11 days of gestation according to a previous description (40, 42). Pregnant females were subjected to restraint stress for 45 min three times a day in a transparent plastic cylinder (7 cm in diameter and 19 cm long) exposed to a bright light (650 lux). Stress sessions were conducted during the light phase, but the schedule of sessions was not fixed to reduce a possible habituation to repeated restraint stress (approximate stress exposures were performed around 900, 1200, and 1700). Control females were left undisturbed in their home cages. The offspring were raised by their biological mothers until weaning, 21 days after birth. Only litters of 8–13 pups with similar sex ratio were kept for the study, and the other ones were eliminated to rule out extra stressors such as removal of the pups. Only male fetuses were used, and a maximum of two males per litter were put away in this study.

Tissue collection. On the last day of the stress protocol (*day 21* of gestation), females were killed rapidly by decapitation between 1130 and 1300 (corresponding at the time when they were usually submitted to restraint stress). The adrenals and thymus of mothers were dissected and weighed. The uterus was quickly removed, fetuses were carefully separated from the placenta, and both were cleaned and weighed. All placentas were frozen in liquid nitrogen. The ano-genital distance of the fetuses was measured to determine sex to keep only the litters that had a similar sex ratio (1 ± 0.25). Trunk blood of male fetuses was rapidly collected after decapitation (~500 ul) in tubes prerinsed with EDTA. Blood glucose was measured using a glucometer (One Touch II; Lifescan, Roissy, France), and then blood samples were centrifuged at 3,500 *g* for 10 min at 4°C. Plasma aliquot fractions were kept at −30°C until the day of the assay (RIA).

The fetal adrenals, pancreas, liver, thymus, and testis were dissected and rapidly weighed. All organs except pancreas were frozen in liquid nitrogen and stored at −80°C until analysis.

Placental GLUT levels. Placental protein levels of glucose transporters (GLUT1, GLUT3, and GLUT4) were measured after SDS-PAGE and Western Blotting, as previously described (36). Control blots were incubated with antisera preadsorbed with the corresponding oligo-peptide sequences (10 μg/ml; Pichem, Graz, Austria) used for the immunization of the antibody-generating rabbits.

11β-HSD1, 11β-HSD2, and glucocorticoid receptor mRNA levels. Total RNA was extracted from placenta and fetal liver tissues with TRIzol reagent (Gibco BRL, Strasbourg, France) according to the

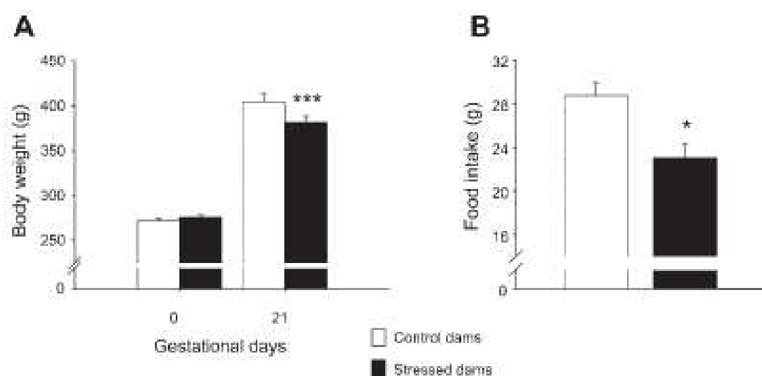

Fig. 1. Chronic restraint stress during gestation provoked a decrease in the body weight gain and food intake of dams (control dams: $n = 12$; stressed dams: $n = 12$). *A*: maternal body weight before and at the end of gestation. *B*: mean of the daily maternal food intake from gestational *days 15* to *21*. $*P < 0.05$; $***P < 0.001$.

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manufacturer's instructions. The quality of the total RNA was assessed by determining the 260:280 absorbance ratio and by gel electrophoresis in agarose. The semiquantitative RT-PCR analysis method used here has been described and validated previously (9). Briefly, 3 μg of total RNA were reverse transcribed into complementary DNA (cDNA) using 3 μg of random hexamers and 200 U Moloney murine leukaemia virus RT (Gibco BRL, Strasbourg, France). Semiquantitative PCR was performed with one-thirtieth (1 μl) of the first-strand synthesis reaction in 50 μl volume containing 5 μl of 10× buffer (500 mM KCl, 100 mM Tris-HCl, Triton 1×), 4 μl of $MgCl_2$ (25 mM), 5 μl of dNTP (2.5 mM each), 1 μl (20 μM) of forward and reverse primer, 33.8 μl of H_2O, and 0.2 μl of *Taq* polymerase (1 U) (Qbiogen, Illkirch, France). The cycling parameters were 94°C for 90 s, 60°C for 90 s, and 72°C for 120 s. Negative control RT-PCR reactions were performed by omitting RT from the reaction mixture or by adding H_2O instead of template. In the four 25-mer primer pairs, the priming sites were separated by a large intron, thus preventing amplification of any contaminating genomic DNA. For rat glucocorticoid receptor (GR; accession no. M14053), 11β-HSD1 (accession no. NM017080), and 11β-HSD2 (accession no. NM017081) amplification, the forward primer corresponded to the region encoding residues 336–344, 37–45, and 80–88, and the reverse primer was complementary to the region encoding residues 418–426, 180–188, and 255–263, respectively. The predicted size of amplification was 272, 453, and 551 bp, respectively. As a control for the RT-PCR amplification of rat cyclophilin B (accession no. AF071225), a forward primer corresponding to the region encoding residues 45–53 and a reverse primer complementary to the region encoding residues 189–197 were used. The predicted size of the cDNA product was 456 bp. Preliminary experiments allowed us to determine the optimal cycle numbers for each primer pair for linear semiquantitative amplification. Each experiment was performed in triplicate and gave similar results. After amplification, the samples were separated on a 1% gel agarose, visualized by ethidium bromide, and analyzed with a Bio-Rad GS-700 densitometer using the Multi-Analyst software (Bio-Rad Laboratories, Hercules, CA).

11β-HSD1 and -2 activities. 11β-HSD1 and -2 activities in placenta were assayed after homogenization, as previously described (28). The reaction included 0.2 mg/ml protein, 10 nM tritiated corticosterone as substrate, and an excess (400 μM) of the 11β-HSD1- or 11β-HSD2-specific cofactors (NADP or NAD, respectively) as cosubstrate. Reactions were within the linear portion of the relationships between product formation, time, and protein concentration. After 6 h for 11β-HSD1 or 50 min for 11β-HSD2 incubation at 37°C, steroids were extracted with ethyl acetate, separated by TLC, identified by migration compared with standards, and quantified with a phosphorimager tritium screening. 11β-HSD1 reductase activity was expressed as the percentage of radioactive corticosterone over the total dehydrocorticosterone added to the radioactive corticosterone. 11β-HSD2 dehydrogenase activity was expressed as the percentage of radioactive dehydrocorticosterone over the total dehydrocorticosterone added to the radioactive corticosterone.

Pancreatic β-cell mass. Pancreases were fixed and processed for immunohistochemistry as previously described (16). Pancreatic β-cells were detected using a polyclonal guinea pig anti-insulin antibody (Dako, Trappes, France), and the β-cell fraction was measured using a Leica DMRB microscope equipped with a color video camera coupled to a Quantimet 500MC computer (16). β-Cell mass was obtained by multiplying the β-cell fraction by total pancreatic mass.

Plasma hormone levels. All the measures were done in individual fetuses. In E21 fetuses, plasma ACTH levels were measured in unextracted plasma using a commercial kit (ACTHK-PR; Cis Bio International). The sensitivity of the assay was 10 pg/ml. Plasma corticosterone levels were measured using a commercial kit (Corticosterone DA; MP Biomedicals, Strasbourg, France). The minimum level of detection of the assay was 7.7 ng/ml. Plasma corticosteroid-binding globulin (CBG) binding capacity was determined as previously described (34). The apparent maximum binding capacity and dissociation constant of CBG for corticosterone were individually evaluated from Scatchard plots. Plasma insulin levels were measured using a previously described RIA (16). The sensitivity of the assay was 0.25 ng/ml. IGF-I plasma levels in E21 fetal blood were measured with a commercial kit IGF-R20 from Mediagnost (D-72770; Reutlingen). The assay sensitivity was 0.16 ng/ml. Growth hormone (GH) plasma levels analysis was made using the assay kit RPA 551 (Amersham Biosciences). The sensitivity of the assay was 0.16 ng/ml.

Statistical analysis. Data are expressed as means ± SE. Two-way ANOVA with repeated measures followed by Neumman-Keuls post hoc test was used to analyze data from rat dams (body weight and food intake). For placental and fetuses parameters, comparison between groups was performed using Student's *t*-tests. In each case, $P < 0.05$ was considered statistically significant.

RESULTS

Effects of restraint stress on the dam physiology. Restraint stress altered maternal pregnancy. Stressed dams showed a decrease in body weight gain [$F_{(4-88)} = 5.25$, $P < 0.001$; Fig. 1*A*]. Although both stressed and control females progressively increased food intake during gestation (data not shown), stressed females had lower overall food intake than controls [$F_{(1-12)} = 10.19$, $P < 0.005$; Fig. 1*B*]. At term, stressed dams had increased adrenal weight (stressed: 93.1 ± 7.4 mg; CTL: 68.2 ± 2.7 mg; $t_{14} = 3.17$, $P < 0.05$), whereas their thymus weight was reduced (stressed: 223 ± 11 mg; control: 295 ± 12 mg; $t_{14} = 4.44$, $P < 0.05$).

Effects of PS on placental functions. PS did not affect the weight of the placenta [PS rats: 95.9 ± 3.59 mg/g body wt; CTL rats: 93.6 ± 3.65 mg/g body wt; $t_{14} = 0.67$, not significant (NS)]. PS significantly modified glucose transporter protein levels in the placenta (Fig. 2). GLUT1 was strongly reduced in PS placentas ($t_{18} = 8.37$, $P < 0.001$; Fig. 2). In

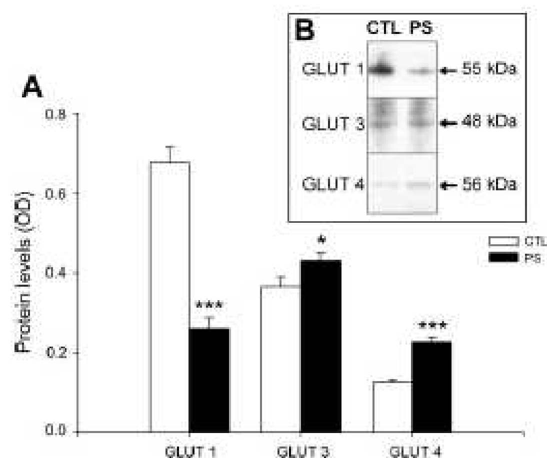

Fig. 2. In the rat E21 placentas, prenatal stress significantly modified glucose transporter (GLUT) proteins [control rats (CTL): $n = 10$; prenatal stress (PS) rats: $n = 10$]. *A*: protein levels of GLUTs measured by optical density (OD). *B*: representative Western blot of the GLUT1, GLUT3, and GLUT4 proteins in rat term placentas. GLUT1 was strongly reduced in PS placentas. In contrast, levels of GLUT3 and GLUT4 were slightly were augmented by PS. *$P < 0.05$; ***$P < 0.001$.

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contrast, levels of GLUT3 and GLUT4 were augmented by PS (GLUT 3: t_{18} = 2.13; P < 0.05; GLUT4: t_{18} = 7.64, P < 0.001).

PS strikingly decreased placental 11β-HSD2 mRNA levels (t_8 = 8.06, P < 0.001; Fig. 3*A*) and 11β-HSD2 activity (t_{11} = 4.14, P < 0.05; Fig. 3*B*). PS was also associated with a small decrease in placental 11β-HSD1 mRNA (t_8 = 2.26, P < 0.05), but this was not linked to any change in NADP-driven activity (Fig. 3*B*).

Effects of PS on the E21 fetuses physiology. In Table 1, the weight of male PS fetuses (n = 7–9) at term (E21) was significantly lower than CTL (n = 7, t_{13} = 2.98, P < 0.01). E21 PS fetuses had reductions in adrenal (t_{14} = 2.98, P < 0.01), pancreatic (t_8 = 2.37, P < 0.05), and testis (t_{14} = 2.83, P < 0.05) weights, whereas liver (t_{13} = 0.42, NS) and thymus (t_{13} = 0.80, NS) weights were similar to CTL. Moreover, the ano-genital distance of male PS fetuses (n = 16) was shorter (t_{36} = 5.72, P < 0.01) than CTL (n = 22).

Associated with the decreased weight of the pancreas, PS induced a significant decrease in pancreatic β-cell mass (t_8 = 4.05, P < 0.01; Fig. 4*A*).

However, in Table 2 (CTL: n = 7; PS: n = 5–11), plasma insulin levels were not changed in PS fetuses (t_{18} = 0.26, NS), although they had lower plasma glucose levels (t_{20} = 2.32, P < 0.05). In terms of growth and counterregulatory hormones, E21 PS fetuses had lower plasma levels of GH, although without change in circulating IGF-I (t_{17} = 0.36, NS). Plasma ACTH was decreased (t_{12} = 4.22, P < 0.01), whereas corticosterone and CBG were unchanged (t_{17} = 0.35, NS, and t_8 = 0.85, NS, respectively).

Although PS tended to increase hepatic 11β-HSD1 mRNA levels (t_8 = 2.11, P = 0.06; Fig. 4*B*), no changes in liver GR mRNA levels were observed (t_8 = 0.49, NS).

Table 1. *Mean characteristics of the E21 male fetuses*

	CTL	PS
Fetus body weight, g	5.76±0.03	5.48±0.08†
Adrenals, mg/g body wt	0.72±0.07	0.51±0.04†
Pancreas, mg/g body wt	5.19±0.22	4.58±0.27*
Liver, mg/g body wt	57.82±2.63	55.94±3.52
Thymus, mg/g body wt	2.28±0.15	2.05±0.23
Testis, mg/g body wt	1.21±0.08	0.91±0.04*
Ano-genital length, mm	3.90±0.15	2.92±0.15†

Values are means ± SE [control rats (CTL): n = 7; prenatal stress (PS) rats: n = 7–9]. The weight of PS fetuses was significantly lower than CTL rats. Reduced adrenal, pancreatic, and testis weights were noted in PS fetuses, whereas their liver and thymus weights were similar to controls. The ano-genital length was measured on 22 CTL and 16 PS fetuses. *P < 0.05; †P < 0.01.

DISCUSSION

We demonstrate that chronic maternal stress affects the growth and organ development of the fetus. Specifically, PS reduces fetal growth, targeting some organs more than others, strikingly attenuates placental 11β-HSD2 and GLUT1 expression, and diminishes fetal plasma glucose, GH, and ACTH levels. We suggest that PS induces early feto-placental unit dysfunction that may contribute to the development of the long-term behavioral and metabolic alterations seen previously in the adult offspring (39).

The present study shows that PS alters fetal growth, resulting in a decrease of body weight at term, in accord with our previous observations (35). Furthermore, we reported here that maternal stress affected mothers' physiology during pregnancy, extending our previous observations (10) showing that gestational stress has long-lasting effects on emotional reactivity of dams. The mechanisms by which PS affects pup growth remain largely unknown. However, it has been postulated (1, 13, 53) that overexposure to the catabolic effects of maternal glucocorticoids in utero could underlie such alteration. Indeed, maternal administration of dexamethasone, a synthetic glucocorticoid that readily crosses the placenta, reduces fetal growth in rats and in other mammals (7, 43, 53). This hypothesis is in accord with our previous data (1) showing that elevation of plasma corticosterone levels in stressed pregnant female rats decreased HPA axis feedback mechanism in adult offspring and is reinforced in our study with the adrenal hypertrophy that these females presented at term. Alternatively, the low body weight of E21 PS fetuses could also result from a reduction of maternal food intake during late gestation. Consistent with this, we showed that chronic restraint stress decreases both maternal body weight gain and food intake of the dams, two factors that are well described to restrict fetal growth in rats (34), although whether the ~20% reduction in maternal food intake found here is sufficient per se to affect fetal weight is uncertain. These two mechanisms are not necessarily distinct since fetal nutritional restriction reduces placental 11β-HSD2 levels in rats and thus allows increased access of active glucocorticoids to the fetus (33, 34). It could be hypothesized that the effects of PS to restrain fetal growth relate to the excess glucocorticoid action on the feto-placental unit due to a combination of elevated maternal corticosterone

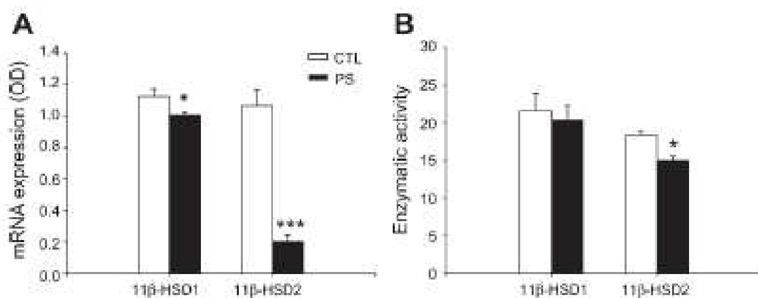

Fig. 3. Quantitative analysis of 11β-hydroxysteroid dehydroxygenase type 1 (11β-HSD1) and type 2 (11β-HSD2) in the E21 placentas (CTL: n = 5–7; PS: n = 5–6). PS strikingly affects the 11β-HSD2 mRNA levels and activity. *A*: mRNA expression of 11β-HSD1 and 11β-HSD2 measured by semiquantitative RT-PCR. *B*: enzymatic activities of 11β-HSD1 and 11β-HSD2 with assays lasting 6 h and 50 min, respectively. The enzymatic activity is expressed as the %radioactive dehydrocorticosterone over the total dehydrocorticosterone added to the radioactive corticosterone *P < 0.05; ***P < 0.001.

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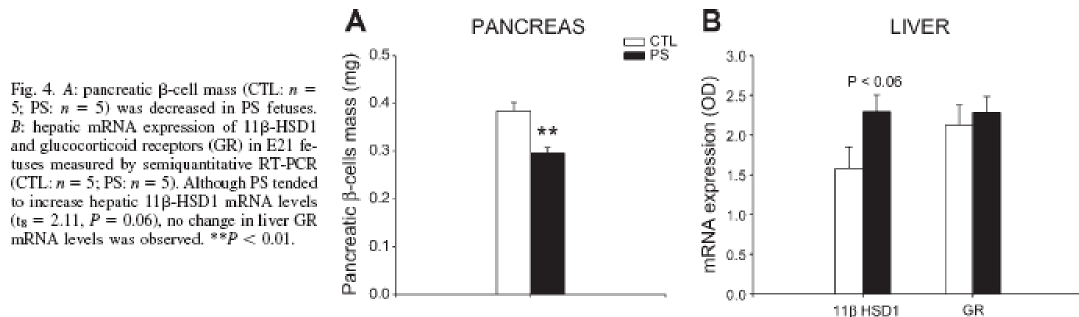

Fig. 4. *A*: pancreatic β-cell mass (CTL: $n = 5$; PS: $n = 5$) was decreased in PS fetuses. *B*: hepatic mRNA expression of 11β-HSD1 and glucocorticoid receptors (GR) in E21 fetuses measured by semiquantitative RT-PCR (CTL: $n = 5$; PS: $n = 5$). Although PS tended to increase hepatic 11β-HSD1 mRNA levels ($t_8 = 2.11$, $P = 0.06$), no change in liver GR mRNA levels was observed. $^{**}P < 0.01$.

levels, as we have previously shown (1), and reduced placental 11β-HSD2 activity per se and/or to reduced maternal food intake and/or other mediators of stress. However, recent crosses of mice heterozygous for a null allele of the 11β-HSD2 gene show that within the same mother, birth weight correlates closely with feto-placental 11β-HSD2 genotype (lowest in 11β-HSD2$^{-/-}$ offspring) (25). Thus, the reduced placental 11β-HSD2 in PS rats is compatible with increased transfer of corticosterone from the maternal to the fetal compartment and may be sufficient to reduce birth weight and program offspring physiology. Indeed, in mice, rats, and humans (6, 52, 54), reduced body birth weight is associated with deficient placental 11β-HSD2 (52) allowing more corticosterone to cross the placenta barrier. Perhaps as a consequence of the increased fetal glucocorticoid exposure, PS fetal HPA axis function is attenuated since we found atrophied adrenals and reduced plasma ACTH levels without change in the plasma corticosterone in E21 PS fetuses. The maintained corticosterone levels in PS fetuses are also compatible with the increased transplacental glucocorticoid transfer (from mother to fetus), as their adrenals are atrophied. In late gestation, the development and activity of the fetal adrenals depends mainly on the secretion of pituitary ACTH, which in turn is controlled by hypothalamic CRH (21). It was shown that maternal hypersecretion of glucocorticoids (34) as well as exogenous administration of dexamethasone (12) reduce both fetal adrenal growth and activity in correlation with drastic reduction of hypothalamic CRH and suppression of plasma ACTH. Thus, the present results suggest that the HPA axis alterations observed in PS fetuses can be related to the negative feeback control exerted by high levels of maternal glucocorticoids on the fetal HPA axis.

Present data show that maternal stress induced a decrease in testis weight as well as a reduction of the ano-genital length. These alterations are in accord with the observations made by Ward (64) in 1983. Ward et al. (63) have suggested that these early gonadal disturbances could be responsible for the reduction of the adult sexual behavior of male PS rats and could be the result of a depression in early gonadal steroidogenesis, the origin of which remains to be found.

During late gestation, fetal growth is also mainly dependent on the utero-placental unit to deliver oxygen and nutrients as well as the activity of the fetal GH-IGF endocrine system (26). A primary nutrient for the developing fetus is glucose, which crosses the placental barrier through GLUTs that mediate the transport of glucose across plasma membranes by facilitated diffusion (30). We report here that chronic maternal restraint stress reduces placental GLUT1 protein levels but slightly increases GLUT3 and GLUT4 protein levels at term. GLUT1 is the highest-expressed GLUT in rodent placentas (32) and is considered rate limiting for glucose transport from mother to fetus (5). Decreased placental GLUT1 therefore suggests reduced glucose transfer across the PS placenta, a notion in accord with the observed hypoglycemia in PS fetuses. The small increases in the minor placental transporters GLUT3 and GLUT4 might reflect an adaptation to increase placental and/or fetal glucose supply in response to the downregulation of GLUT1, although proof of this notion requires direct studies to measure glucose flux in these animals. How PS alters placental GLUT protein levels remains unknown. However, because glucocorticoids regulate expression of GLUT transporters in rat placenta (22), the increased maternal corticosterone secretion induced by chronic stress may play a role. Additionally, as maternal undernutrition alters placental GLUT transporters (34), the reduced food intake in PS dams might be involved. In accord with our data, intrauterine growth retardation in humans is also associated with alterations in placental nutrient transport (29). Systemic levels of glucose production are powerfully regulated in the liver by glucocorticoids, whose action is determined by intracellular regeneration of active corticosterone by 11β-HSD1 and the density of GRs. In our study, since hepatic GR and 11β-HSD1 gene expression remain unaffected in the PS fetuses, the fetal hypoglycemia is not due to deficient glucocorticoid action on the fetal liver gluconeogenesis. The

Table 2. *Plasma levels of endocrine parameters in the E21 fetuses*

	CTL	PS
ACTH, pg/ml	141±9	89±9†
Corticosterone, ng/ml	157±16	149±12
CBG, B_{max}-nM	225±45	238±22
Glucose, mg/dl	77±5	61±5*
Insulin, μU/ml	132.0±11.0	132.0±8.8
GH, ng/ml	20.9±3.8	8.1±4.4*
IGF-I, ng/ml	47.5±3.2	49.1±2.9

Values are means ± SE (CTL: $n = 5$–11; PS: $n = 5$–11). CBG, corticosteroid-binding globulin; GH, growth hormone. Plasma insulin levels were not changed in PS fetuses, even if they had lower plasma glucose levels. PS fetuses had lower plasma levels of GH without change in circulating IGF-I. Plasma ACTH was decreased in PS fetuses, whereas corticosterone and CBG were unchanged. $^*P < 0.05$; $^†P < 0.001$.

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observed hypoglycemia in PS fetuses is likely to be due to placental failure and/or GLUT1 deficiency.

Maternal restraint stress provoked a decrease in fetal plasma GH levels without circulating plasma IGF-I levels being altered. Abnormalities in the GH-IGF axis are commonly described (24, 52, 59) in growth-retarded fetuses and neonates as well as in many adult diseases associated with low birth weight. Before birth, GH has been thought to have only a minor role in regulating fetal growth, contrary to the IGFs (both IGF-I and IGF-II) that are the principle regulators of fetal growth. Interestingly, it has been described (15, 45) that IGF-I levels are highly regulated in fetuses by nutritional factors. In late gestation, even if fetal levels of both IGF-I and liver GH receptor are low, GH-inducible genes are expressed in fetal liver, suggesting that GH may be physiologically active before term (47). The development of the fetal somatotrophic axis during late gestation is dependent on the plasma glucocorticoid levels, since the surge of fetal corticosterone (or cortisol) is essential to initiate the switch from GH-independent local production of IGFs in utero to GH-dependent hepatic production of endocrine IGF-I (15). Thus, fetal overexposure to glucocorticoid may prematurely activate the fetal growth axis, inducing an altered somatic development. Here, although the fetal plasma GH levels were diminished, the IGF-I levels remained surprisingly unaffected. One can speculate that PS reduces pituitary GH secretion and/or its hypothalamic control through glucocorticoid effects or undernutrition (48). Interestingly, in E21 fetuses, Fowden et al. (14) reported that IGF-I production is controlled by paracrine factors rather than by GH alone. Consequently, both nutrient restriction and glucocorticoid overexposure of PS fetuses could have disturbed hepatic sensitivity to GH and/or the local control of IGF-I production. Future experiments will be needed to clarify this point as well as to investigate the effects of PS on IGF-II, which is an important factor for early feto-placental unit development.

Associated with the low birth weight of PS fetuses, gestational stress reduced fetal pancreatic weight and impaired β-cell development at term. A similar decrease of pancreatic β-cell mass is observed in rat model of maternal food restriction in late pregnancy and implies a rise of both maternal and fetal plasma corticosterone levels (7). Moreover, treatment of pregnant rats with dexamethasone or with an 11β-HSD2 inhibitor, carbenoxolone, also produced a reduction in β-cell number in fetuses at term (7). Since glucocorticoids play a pivotal role in pancreatic endocrine cell lineages at specific developmental windows (17), the increased glucocorticoid levels induced by PS can give rise to impairment of β-cell development and differentiation. Such early effects of PS on pancreatic β-cells could contribute to long-term alterations, as we showed (35) that PS programs glucose intolerance in aged rats. Collectively, these data emphasize how some of the late alterations observed in humans born with intrauterine growth retardation may result from fetal disturbances such as altered β-cell development, as initially suggested by Hales and Barker (23).

In conclusion, our data emphasize the impact of PS on fetal endocrine functions as well as on placental physiology. Besides HPA axis alteration, PS fetuses also exhibit altered somatotopic and endocrine activities. Mechanisms involved in this altered fetal phenotype could be identified, at least in part, on *1*) maternal food intake that is diminished, *2*) GLUT system that is disrupted, and *3*) maternal corticosterone levels that are increased during restraint stress and by the decreased feto-placental 11β-HSD2, which usually protects the fetus from maternal glucocorticoid excess. Since the action of glucocorticoids on homeostasis is widespread, affecting most of the body compartments as well as the brain, one can speculate that the chronic stress applied to mothers can generate a deleterious environment for the fetus development. The early-altered phenotype obtained in this study may subsequently be at the origin of the adult pathophysiology programmed in PS offspring.

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Research report

Stress during gestation induces lasting effects on emotional reactivity of the dam rat

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Abstract

Human and animal studies indicate that repeated stress during pregnancy can produce long-term biological and behavioural disorders in the offspring. In contrast, although maternal stress is supposed to induce an increase of maternal anxiety, few studies have been conducted to demonstrate it. Therefore, in the present study we examined the emotional reactivity in stressed (chronic restraint stress applied 3× 45 min per day during the last week of pregnancy) and unstressed females rats after the weaning of their pups. Restraint stress procedure reduced the body weight gain both during pregnancy and up to four weeks after the stress period. Stressed dams presented a reduction of exploration and of corticosterone levels when exposed to a novel environment (25 and 49 days post-stress). They spent less time in the open arms of the elevated plus-maze (26 days post-stress). Finally, they showed no increase in the time spent in immobility after a second exposure to the forced-swim test (35–36 days post-stress). In the contrary, such differences were not observed when the chronic stress procedure was applied on virgin females. Overall, our results show that, chronic stress during gestation induces lasting effects on emotional reactivity of the dams, thus indicating that gestation constitutes a critical period in the vulnerability to stressful events also for the mother.
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Keywords: Gestation; Rat dams; Chronic restraint stress; Anxiety; Novelty; Forced-swim test; Exploration; Corticosterone

1. Introduction

Stressful events such as pregnancy, labour and lactation occur simultaneously with marked fluctuations in plasma levels of steroid hormones, known to be involved in the vulnerability to emotional disorders. The first days postpartum are often associated with the onset of major emotional vulnerability. However, pregnancy and lactation are generally described as hyporesponsive periods to stress [15,34,53]. Thus, it remains unclear if pregnancy constitutes a critical period involved in the vulnerability to stress. In rats, chronic maternal stress during pregnancy has been used to study the impact of early stress on development and later vulnerability to stress in offspring. Several paradigms of stress including daily subcutaneous injection [8], water immersion [9], overcrowding [14] or restraint stress [24,49] induce long-term behavioural [7,17,48,55] and neurobiological [19,24,28] abnormalities in the offspring. Processes mediating the effect of prenatal stress on the offspring have been proposed to involve both prenatal [3] and postnatal [38,42] maternal factors.

While the impact of maternal stress during pregnancy on physiology and behaviour of the offspring has been well documented (for review, see [23,50]), up to date few studies have explored the effect of gestation stress on the maternal behaviour [25,29,35,36] and none on the behaviour of the dams after weaning. Measurement of maternal anxiety during lactation is difficult to validate since it involves the reactivity to pup's separation [43]. In the present study, we evaluate reactivity of stress in several paradigms, 1–5 weeks after the weaning of the pups. Our results demonstrate that stress during pregnancy durably decreases maternal body weight growth, increases anxiety-like behaviour and affects reactivity to stress in primiparous females more than one month after the stress procedure. Finally, in another experiment we show that chronic stress has no effect on virgin females suggesting that gestational state is a period of particular vulnerability to stress.

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2. Methods

2.1. Subjects

Virgin female Sprague Dawley rats weighing 250–260 g. were housed individually for a whole oestrus cycle in the presence of sexually experienced males weighing 440–460 g (Iffa Credo, France). Pregnant rats were then individually housed in breeding cages and randomly assigned to stress and control groups. Control group ($n = 10$) was left undisturbed in the home cage. Stress group ($n = 7$) was submitted to restraint stress under a bright light during the last week of pregnancy (3× 45 min per day) as previously described [19,24]. Animals were allowed ad libitum access to food and water and maintained on constant temperature (22 °C) and light/dark cycle (light on: 08:00–20:00 h).

2.2. Body weight growth

Females body weight was measured each week during pregnancy and after weaning.

2.3. Locomotor reactivity to novelty

Four days after weaning (25 days after the end of the stress period), dams were exposed for 10 min to a novel environment consisting in transparent Plexiglas cages (18 cm × 30 cm × 18 cm high) (Fig. 1). Activity was automatically monitored by photocell beams and recorded via a computer system (Imétronic, France).

2.4. Elevated plus-maze test

According to the specifications of Pellow et al. [37], the apparatus made in wood, consisted of two open arms (50 cm × 10 cm), alternating at right angles with two arms enclosed by 40 cm high walls. The fours arms delimited a central area of 10 cm^2. The whole apparatus was placed 60 cm above the floor. Twenty-six days post-stress, each dam was placed in the centre of the elevated plus-maze, facing a closed arm, and was allowed to freely explore the maze for 5 min (Fig. 1). The rat behaviour was videotaped. Then, the number of entries and the time spent in open and enclosed arms were recorded and a four-paw criterion was used for arm entries. Percentages of exploration of the open arms (time spent and entries) with respect to total exploration (time and entries) in both open and closed arms were calculated.

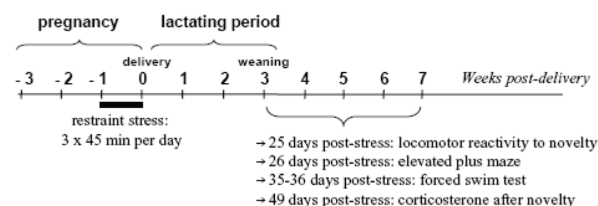

Fig. 1. Experimental design. Stressed females were exposed to chronic restraint stress three times per day (45 min) throughout the last week of pregnancy. Control females were left undisturbed. During the lactating period stressed and control dams were left undisturbed until the weaning of the pups. Then, females were submitted to behavioural tests for anxiety (elevated plus-maze) and reactivity to inescapable stress (novel environment and forced swimming). Forty-nine days post-stress, blood samples were taken after novelty exposure.

2.5. Forced-swim test

Two weeks after weaning (35–36 days post-stress), dams were tested in an adapted version of the forced-swim test described by Porsolt et al. [41] (Fig. 1). On the first day, dams were introduced for 15 min into a transparent cylindrical glass tank (35 cm high, 19 cm diameter) containing water (25 °C) up to a level of 23 cm. Twenty-four hours after the first trial, the animals were exposed to the same experimental conditions outlined above, for a 5-min period only. Time spent in immobility during the first 5 min of test (days 1 and 2) was scored from the videotape by a trained observer.

2.6. Blood samples after novelty exposure for corticosterone assay

Four weeks after the weaning (49 days post-stress), dams were exposed to a new home cage (Fig. 1). Blood samples were quickly collected in heparinised tubes from the tail vein immediately after 10 min of novelty exposure. Blood samples were centrifuged at 4 °C (3600 rpm, 20 min) and plasma aliquots were stored at −20 °C until assay. Corticosterone levels were determined by radioimmunoassay kit (ICN Biomedicals, France).

2.7. Behavioural measurement on virgin females

A behavioural study was conducted using non-pregnant control and stressed females ($n = 10$ per group). The procedures and the time course were similar to that described in Fig. 1 for pregnant females.

2.8. Statistical analysis

Differences between groups for body weight growth and immobility in the forced-swim test were assessed by two-way ANOVA with repeated measures, followed by Newman Keuls (NK) post hoc comparisons. Values of locomotor reactivity, exploration of open arms in the plus-maze and corticosterone secretion after novelty were compared between groups using independent Student t-tests. Correlation between corticosterone levels and behavioural measures was conducted by Pearson's test. P value <0.05 was accepted as significant.

3. Results

3.1. Long-term effect of gestational restraint stress on body weight growth

Chronic restraint stress procedure during the last week of pregnancy affected the body weight growth of dams (ANOVA, group × time effect, $F_{(1,60)} = 7.29$, $P < 0.0001$). As shown in Fig. 2, dams stressed during pregnancy exhibited a lower body weight growth at the end of pregnancy (post hoc analysis by Newman Keuls, $P < 0.0001$), but also three (NK, $P < 0.001$) and four weeks (NK, $P < 0.01$) after the stress period. Daily water and food intake were similar between control and stressed dams (water: control dams 37.9 ± 2.9 ml, stressed dams, 39.1 ± 4.7 ml; food: control dams, 22.3 ± 1.4 g, stressed dams, 22.8 ± 1.5 g).

3.2. Long-term effect of gestational restraint stress on reactivity to a novel environment

Females exposed to chronic restraint stress during pregnancy exhibited lower locomotor reactivity (Student's *t*-test, $t_{(15)} = 2.29$, $P = 0.03$; Fig. 3A) and lower plasma corticosterone levels ($t_{(15)} = 2.56$, $P = 0.02$) after novelty (determined respectively 25 and 49 days after parturition) than control females (Fig. 3B). Moreover, a significant positive correlation was observed between corticosterone levels and locomotor activity after novelty (Pearson's correlation, $r = 0.51$, $P = 0.04$).

3.3. Long-term effect of gestational restraint stress on exploration in the elevated plus-maze

As shown on Fig. 4, chronic stress during pregnancy reduced the percentage of time spent in the open arms of the elevated plus-maze 26 days after the end of stress period ($t_{(15)} = 2.22$, $P < 0.05$). No significant reduction was found in the percentage of visits in the open arms in stressed dams ($t_{(15)} = 1.65$, $P = 0.12$, data not shown).

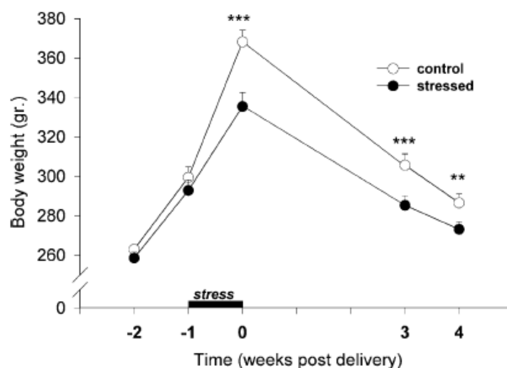

Fig. 2. Body weight growth during pregnancy and after lactating period in control and stressed females. Chronic restraint stress during the last week of pregnancy (black bar) reduced body weight gain until four weeks after delivery. Values are presented as mean±S.E.M. ** $P < 0.01$, *** $P < 0.001$ control vs. stressed females.

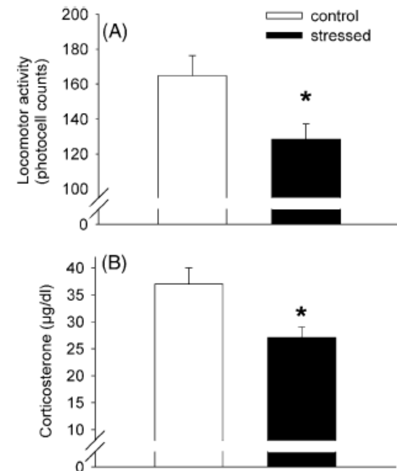

Fig. 3. Locomotor activity (A) and plasma corticosterone levels (B) in response to ten minutes of exposure to a novel environment. Chronic restraint stress during the last week of pregnancy decreased locomotor reactivity and corticosterone secretion after novelty exposure. Values are presented as mean ± S.E.M. * $P < 0.05$ control vs. stressed females.

3.4. Long-term effect of gestational restraint stress on immobility in the forced-swim test

The two-way ANOVA analysis revealed a significant interaction between the group and the day of testing for the time spent in immobility in the forced-swim test (ANOVA, $F_{(1,15)} = 4.97$, $P < 0.05$). In control rats, the immobility increased after a second exposure (NK, $P < 0.01$, first versus second exposure), whereas no significant variation between the two days of testing was observed in stressed dams (Fig. 5). During the second exposure, immobility behaviour was higher in control than in stressed females (NK, 0.01). Moreover, the percentage of increase between first and second exposure was 29.9 ± 6.2% in the control females whereas the variation was 2 ± 13.4% in stressed females.

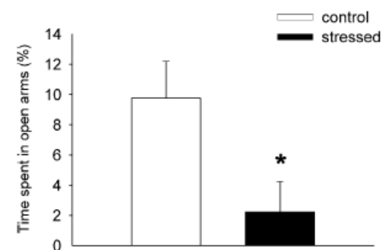

Fig. 4. Effect of chronic stress during pregnancy on females anxiety. Chronic restraint stress during the last week of pregnancy decreased the percentage of time spent in open arms. Values are presented as mean ± S.E.M. * $P < 0.05$ control vs. stressed females.

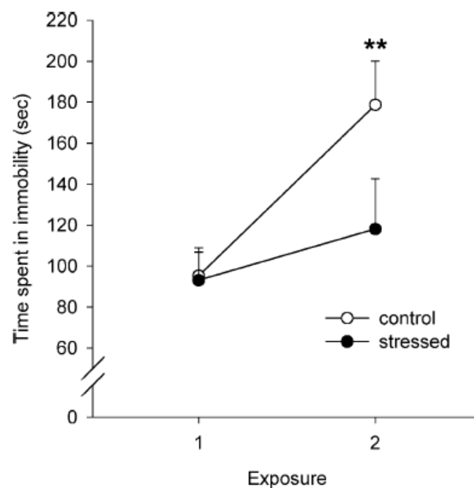

Fig. 5. Effect of chronic stress during pregnancy on females immobility in the forced swim test. Chronic restraint stress during the last week of pregnancy suppressed the increase of immobility on the second exposure to the test that was observed in control females. Values are presented as mean ± S.E.M. ** $P < 0.01$ control vs. stressed females; and first exposure vs. second exposure in control females.

Table 1
Effects of chronic restraint stress on virgin females using the same temporal schedule as in the first study (see Fig. 1)

	Virgin control	Virgin stressed
Body weight after stress (g)	261.90 ± 2.58	260.20 ± 2.88
Locomotor reactivity (cell counts)	174.70 ± 14.74	156.40 ± 13.28
Corticosterone after novelty (μg/dl)	55.72 ± 2.18	53.48 ± 5.63
Time spent in the open arms (%)	10.44 ± 3.32	7.00 ± 2.96
Immobility (% 1st/2nd exposure)	33.70 ± 7.73	32.20 ± 13.20

Chronic restraint stress had no effect on body weight growth (measured 7 days after the end of the stress period), on behavioural parameters determined 3–4 weeks after the end of the stress period and on corticosterone levels after exposure to novelty.

3.5. Long-term effect of restraint stress on behaviour in virgin females

Stress and testing were applied with the same time frame as the first study (Fig. 1). However, as shown on Table 1, chronic restraint stress conducted on virgin females had no effect on their body weight. Furthermore, virgin females exposed to chronic stress exhibited the same locomotor reactivity to novelty and the same behaviour in the elevated plus-maze or in the forced-swim test than virgin control females. Finally, corticosterone levels after exposure to a novel cage were similar between stressed or control virgin females.

4. Discussion

The aim of the present study was to evaluate if chronic restraint stress conducted during pregnancy could affect later female's anxiety and their reactivity to stress. We demonstrated for the first time that gestational stress disturbed female's behaviour more than one month after chronic stress procedure, increasing their anxiety-like behaviour and affecting their reactivity to inescapable stress such as novelty or forced-swim test. Interestingly, such differences were not observed when the chronic stress procedure was applied on virgin females. Previous findings in the literature report strong alterations in the offspring of dams stressed during gestation [23,50]. Our results indicate that, pregnancy constitutes a period of high vulnerability to the effects of chronic stress also for the dam itself.

The long-term effect of chronic restraint stress on maternal physiology was revealed by a reduction of the body weight growth in stressed females, that was maintained until four weeks after the end of the stress period. Our results extend previous works showing a reduction of body weight growth in pregnant rats exposed to restraint stress [18]. Low body weight gain has been previously described in several stress paradigms both in male and female rats [4,5]. A majority of the studies has focused on the acute effect of stress [21,27]. However, some reports demonstrated a decrease of the body weight in stressed animals compared to control rats even 30 days after a single social defeat [30] or long episodes of restraint stress (3 h) [11]. The long-term effect of stress did not seem related to a reduction of food intake in the stressed females. Indeed, a previous study showed that restraint stress produced a transient hypophagia, but a permanent down-regulation of body weight in rat [44].

Dams that experienced chronic restraint stress during pregnancy differed from control females when they were exposed to an escapable stress such as exposure to the elevated plus-maze, where they can explore protected or exposed environments. Stressed dams avoided the exposed environment as shown by the reduced percentage of time spent in open arms, that demonstrated an increase of their anxiety-like behaviour. Virgin females exposed to chronic restraint stress were no different from control virgin rats. Chronic stress has been demonstrated to increase anxiety. However, these perturbations were mainly observed few days after the stress procedure [39]. Our results support previous findings from Maestripieri et al. [25] showing that repeated restraint of pregnant mice increased their anxiety in the black and white box test, six days postpartum. Moreover, our data were the first to show that chronic stress lead to an increase of anxiety-like behaviour, observable several weeks after the last stressful event, suggesting that stressed dams exhibited a permanent increase of their anxiety throughout the pregnancy and the lactating period.

When they were exposed to an inescapable stressful situation, such as novelty or forced swimming, stressed dams also differed from control females. Stress during pregnancy decreased exploration of a novel environment. The hypoactivity of the stressed dams was associated to a reduced corticosterone secretion in response to novelty. Previous work indicated that chronic stress during pregnancy increased plasma corticosterone levels in pregnant dams

[19,36]. Our results suggested that this maternal corticosterone response to subsequent stress was blunted. Interestingly, periods following cessation of chronic stress and postpartum depression are associated with decreased hypothalamo–pituitary–adrenal axis activity [47,54]. The blunted response to novelty observed in our study may reflect an alteration of the coping strategy. In this view, exploration in a novel environment is an adapted response that leads to a reduction of the stress induced by the novelty of the stimulus in absence of menace such as a predator. We reported that females with the lower behavioural response exhibited the lower corticosterone levels. Corticosterone increase in a stressful situation is an adaptive physiological response that helps to cope with stress, by promoting, for example, gluconeogenesis and glycogenolysis. Usually, when exposed to the forced-swim test, rats exhibit first a marked escape behaviour characterised by struggling, while after a second exposure to the test, animals show a decrease of struggling and an increase of the time spent in immobility. Here, females exposed to stress during pregnancy exhibited a similar time spent in immobility between the two exposures in the forced-swim test. Moreover, they showed higher activity than control dams during the test. These results were in agreement with previous works showing that chronic restraint stress or chronic mild stress reduced immobility in the forced-swim test [10,12,13,40]. However, they contrasted with the high immobility reported in the offspring of stressed dams [1,2,33]. In this regard, the increase of immobility during the second testing has been alternatively interpreted as “behavioural despair” [41,45] or learning to conserve energy [6,16,51,52]. Processes mediating these effects are unclear. However, the increase of immobility between first and second exposure involves in the animal, the learning that the situation is inescapable. Interestingly, memory of the stressful event in this task is mediated by the increase of plasma corticosterone levels in response to the first exposure to the forced-swim test [32]. In our study, we could hypothesise that, as observed after novelty exposure, stressed dams also present a poor corticosterone secretion in response to the forced-swim test that could mediate a memory impairment in this task. Further studies should be conducted on maternal stress and learning performances in dams.

In conclusion, our study demonstrates that chronic stress during pregnancy induces long-term impairments in females behaviour lasting more than one month after the end of the chronic stress. Most of the procedures of chronic stress used in animal models produces a rapid adaptation of the animal [22,31,46]. Thus, physiological and behavioural disturbances induced by the majority of stress procedures do not persist longer after the cessation of stress [20,26]. In this view, it could be hypothesised that pregnant females are more vulnerable to stress than non-pregnant females or male rats. Moreover, alterations of dams behaviour observed after weaning should also be present during lactating period. Thus, such behavioural perturbations may be involved in the long-term effects of maternal stress on the offspring.

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Early motherhood in rats is associated with a modification of hippocampal function

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Summary
The transition to motherhood results in a number of hormonal, neurological and behavioral changes necessary to ensure offspring survival. However, little attention has been paid to changes not directly linked to reproductive function in the early mother. In this study, we demonstrate that spatial performances during the learning phase were impaired after the delivery in rats, while spatial retention ability was improved 2 weeks later. In addition, we also report that early motherhood reduced the cell proliferation in the dentate gyrus of the hippocampus without inducing a decrease in the newborn cells 2 weeks later. The decrease of estradiol levels and high levels of glucocorticoids after delivery could in part explain the changes in the hippocampal function. In summary, our findings suggest that early postpartum period is associated with a modification of hippocampal function. This may reflect a homeostatic form of hippocampal plasticity in response to the onset of the maternal experience.
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1. Introduction

The delivery and the onset of maternal experience constitute one of the greatest challenges experienced by female mammals. In rats, motherhood involves a myriad of new behavioral responses such as retrieving, crouching over and licking the newborn pups (Rosenblatt and Snowdon, 1996). The peripartal period is associated with marked fluctuations of several hormones, which play a significant

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role in the onset of maternal behavior (Rosenblatt et al., 1988). Many works have explored the neurobiology of maternal behavior in rodents and it is known that motherhood produces strong brain modifications in particular in the hypothalamic, ventral striatum and olfactory circuitries (Freund-Mercier et al., 1994; Fleming et al., 1999; Theodosis, 2002; Shingo et al., 2003; Numan, 2007). Furthermore, some of the hormones affected by parturition, such as estrogens and glucocorticoids (Atkinson and Waddell, 1995; Escalada et al., 1996) can also have drastic effects on systems and functions not directly linked to maternal behavior. The hippocampus plays a crucial role in spatial learning and memory processes (Squire et al., 2004). This structure shows a remarkable plasticity and is strongly influenced by hormonal and environmental challenges (McEwen, 2001; Ciriza et al., 2004; Li et al., 2004). It also constitutes one of the few neurogenic areas of the adult brain (Gould and Gross, 2002). Neurogenesis occurs in the olfactory bulbs and in the hippocampus of the mammal adult brain throughout life. This phenomenon takes place continuously, but the rate of proliferation and the fate of new cells may be affected by several factors (Gould et al., 2000; Abrous et al., 2005). Although, the exact function of the neurogenesis in the adult hippocampus remains to be clarified, several works suggest a possible implication in cognitive processes (Shors et al., 2001; Kee et al., 2007).

In humans, the period around parturition is a critical phase that is sometimes associated with cognitive disturbances (Eidelman et al., 1993; Brett and Baxendale, 2001; De Groot et al., 2006). In contrast, in rats, the reproductive experience seems to have a beneficial impact on learning and memory. The majority of the studies concerning rodents, however, are conducted after the lactating period (Kinsley et al., 1999; Gatewood et al., 2005; Lambert et al., 2005; Lemaire et al., 2006; Pawluski et al., 2006a, b), while the peripartal period remains poorly explored.

The dramatic changes in the mother's hormonal milieu that occur just before and during the first 24 h following birth (Rosenblatt et al., 1988) could lead to marked changes of the hippocampal function and plasticity. The aim of this study, therefore, was to examine the hippocampal function after parturition in female rats. We assessed spatial performances in the water maze and neurogenesis in the hippocampus of mothers. Finally, corticosterone and estradiol levels, known to modulate both cognitive function and hippocampal neurogenesis were also measured.

2. Material and methods

2.1. Subjects

Nulliparous female Sprague–Dawley rats (Charles River, France) were housed by pairs for 1 week with a male for mating (mother group) or with a female (virgin group). Female rats were single-housed and not disturbed until the beginning of the study. Dams gave birth to 13.47 pups$\pm$1.47 (range 6–20 pups). Animals were maintained in a 12-h light/12-h dark cycle (lights on at 08:00 h) with free access to food and water. Manipulation of the animals was performed following the principles of laboratory animal care published by the French Ethical Committee and the rules of the European Union Normative (86/609/EEC).

2.2. Water maze

The water maze task has been validated as an accurate index of hippocampal-dependent spatial learning in rodents (Morris et al., 1982). Spatial performances of virgin ($n = 10$) and mother ($n = 9$) groups were evaluated in a circular pool. The water maze task was chosen to measure spatial performances because it allows a learning/memory assessment with minimal litter separation (5–10 min per session). Furthermore, it does not require food deprivation nor painful stimuli as several others paradigms. Apparatus consisted of a black plastic tank, 2 m in diameter and 0.6 m in height (Darnaudery et al., 2006). The tank was filled with water (22$\pm$2 °C) to a depth of 35 cm. The platform (15 cm diameter) was 3 cm below the surface of the water during spatial learning and 2 cm above the surface of the water during the visible session. Extra-maze visual cues around the room remained in a fixed position throughout the experiment. Each session consisted in six successive trials (inter-trial interval, 30 s) and each trial began with the rat placed pseudo-randomly in one of six starting locations. Before the first initial trial, animals were trained to stay 30 s on the platform. Four learning sessions (six trials per day) were conducted in virgins and mothers (postpartum days 1–4) to examine spatial reference memory learning. If the rat had not found the platform after a maximum of 90 s, it was gently guided to the platform. Animals were then given 10 days of retention time and were tested for long-term retention during a 60 s probe trial without the platform. During the probe trial, the latency to reach the exact platform location (diameter = 15 cm), as well as the distance covered (cm) and the time spent (s) in the platform area (diameter = 50 cm) were recorded. One day after the probe trial, virgin females and mothers (postpartum days 16) were submitted to a transfer test consisting in three sessions (six trials per day) with a new platform location to assess their cognitive flexibility and their ability to solve a new spatial problem. To control any difference between experimental groups in the visuomotor abilities or motivation, latencies to reach the platform were evaluated with a visible platform at the end of the transfer (six trials for 1 day). In addition, to rule out the possibility that the spatial learning difference reported at the beginning of the lactating period was related to confounding variables, the latencies to reach a visible platform (six trials for 2 days) were also assessed in additional groups of naïve virgins ($n = 10$) and mothers ($n = 10$) at postpartum days 1–2. The cue version of the water maze allows the comparison of procedural memory abilities between virgins and mothers after delivery. After each session, animals were carefully dried and immediately replaced in their home-cage. Behavior was recorded by an automated system (Viewpoint, Lyon, France).

2.3. Bromodeoxyuridine administration, blood sampling and sacrifice

Virgins ($n = 10$) in diestrus (when the cell proliferation is low in the dentate gyrus, Tanapat et al., 1999) and mothers

24 h after the parturition ($n = 11$) were intraperitoneally (300 mg/kg) injected with bromodeoxyuridine (BrdU, Sigma, France). Immediately after the injection, animals were returned to their home-cage (the separation between mother and her pups lasted less than 5 min). Animals were then sacrificed (between 09:00 and 14:00 h), 2 h (proliferation study) or 14 days (survival study) later. Before sacrifice, blood samples were quickly taken by cutting the tail to assess plasma corticosterone and estradiol levels. Animals were deeply anaesthetized with pentobarbital; adrenal glands were removed and weighed. Rats were then perfused intracardially with saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were removed and immersed in the same fixative for 4 h at 4 °C and then rinsed in phosphate buffer.

2.4. Immunohistochemistry

For each brain, coronal sections (50 μm thick) of the entire dentate gyrus of the right cerebral hemisphere were obtained with a Leica Vibratome (Heidelberg, Germany). Endogenous peroxidase activity was quenched by incubating brain sections for 30 min at room temperature, in 0.1 M phosphate buffer, with 10% methanol and 3% hydrogen peroxide. To ensure detection of BrdU-labeled nuclei, DNA was denatured by incubating the sections for 15 min at 37 °C, in 2 N HCl. After this step, sections were rinsed twice in 0.1 M borate buffer (pH 8.5), followed by a rinse in 0.1 M phosphate buffer, and then incubated for 1 h at room temperature, with 0.1 M phosphate buffer with 0.3% Triton X-100 and 0.3% bovine serum albumin. This buffer solution was used in the following washes and incubations. Sections were then incubated overnight, at 4 °C, with mouse anti-BrdU antibody (1:5000; Hybridoma Bank, Iowa City, IA). After rinsing in buffer, sections were incubated for 90 min at room temperature in biotinylated goat anti-mouse IgG (1:1000; Pierce, Rockford, IL), rinsed and transferred to the peroxidase avidin biotin complex (1:250; Pierce) for 45 min at room temperature. Peroxidase was detected using diaminobenzidine as chromogen. For double immunofluorescence, sections were incubated overnight, at 4 °C, with the primary antibodies rat anti-BrdU (1:2000, Accurate Scientific, USA) and mouse anti-β III tubulin (1:10,000, Promega, USA). Sections were then washed in buffer and incubated for 2 h at room temperature with the fluorescent secondary antibodies: goat anti-rat IgG labeled with Alexa 488 and donkey anti-mouse IgG Alexa 594 (1:1000; Molecular Probe).

2.5. Morphometric analysis of BrdU-immunoreactive cells

The morphometric analysis of BrdU-labeled cells was performed on coded sections. For each animal, BrdU-positive cells were counted on every sixth section (300 μm apart) throughout the rostral-septal half of the dentate gyrus (from the rostral extreme of the hippocampus, at −1.80 mm from bregma, to the caudal end, at −6.80 mm from bregma). The same areas and number of sections were studied for all the animals and all the experimental groups. Sixteen sections were analyzed from each animal. All BrdU-positive cells were counted with a 100× microscope objective. Cell counts were restricted to the granular cell layer (GCL) and the subgranular zone (SGZ) of the dentate gyrus. The SGZ was defined as a two-nucleus-wide band below the apparent border between GCL and the hilus. The total number of BrdU-labeled cells was estimated as previously described (Kempermann et al., 1997). Briefly, all BrdU-immunoreactive nuclei that come into focus while focusing down through the thickness of the section were counted, according to the optical disector principle (Coggeshall and Lekan, 1996), whereas BrdU-immunoreactive nuclei located in the uppermost focal plane were disregarded. We considered BrdU-positive nuclei as those completely filled with DAB product or fluorescent marker or showing patches of variable intensity. The number of BrdU-immunonreactive nuclei counted in the GCL/SGZ was multiplied by 6 (because every sixth section was used) to estimate the total number of BrdU-immunoreactive cells in the hippocampus. The number of BrdU-immunoreactive cells that were also labeled with βIII tubulin, a marker of immature neurons, was assessed on every sixth section and the percentage of BrdU-positive cells immunostained for βIII tubulin was calculated.

2.6. Hormonal assays

Plasma corticosterone and estrogen levels were measured with radioimmunoassay kits (corticosterone kit: ICN, Biomedical, Orsay, France; estrogen kit: Diagnostic Products Corporation, Los Angeles, CA). The minimum levels of detection were 0.2 μg/dl for corticosterone and 1.4 pg/ml for estradiol. Intra-assay and inter-assay coefficients of variation were, respectively, 7% and 8%, for estradiol and 4% and 8%, for corticosterone.

2.7. Statistics

Data are presented as means ± S.E.M. Data were analyzed by ANOVAs followed by Newman–Keuls post hoc tests (NK). Correlations were calculated using Pearson's test. $p<0.05$ was considered statistically significant.

3. Results

3.1. Early motherhood impairs spatial performances but enhances long-term retention of the spatial learning

Both virgin and mother rats exhibited a decrease in the latency (session effect, $F_{(3,51)} = 12.16$; $p<0.0001$, Fig. 1A) and in the distance covered (session effect, $F_{(3,51)} = 20.11$; $p<0.0001$, Fig. 1B) to reach the hidden platform across the four sessions of learning (six trials each) in the water maze task (group × session × trial effect, latency, $F_{(15,255)} = 0.45$; $p = 0.95$; distance, $F_{(15,255)} = 0.44$; $p = 0.94$). During the learning phase, however, lactating rats showed impaired performances as indicated by their high latencies (group effect, $F_{(1,17)} = 12.54$; $p = 0.002$, Fig. 1A) and distances (group effect, $F_{(1,17)} = 6.21$; $p = 0.023$, Fig. 1B) to retrieve the hidden platform. Fig. 2A and B shows the females'

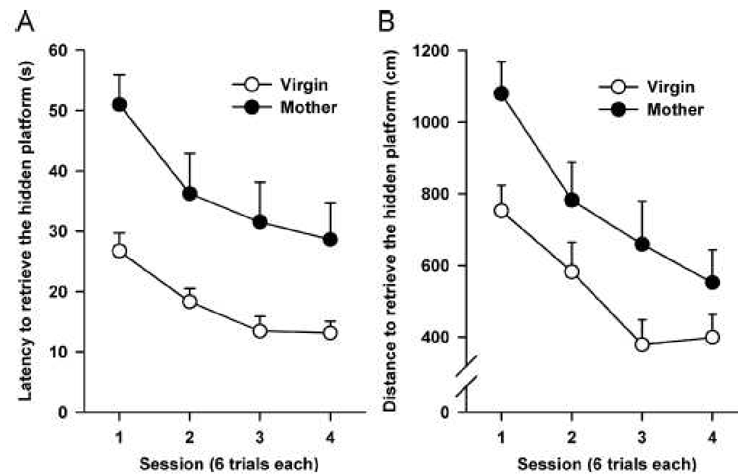


Figure 1 Spatial performances during postpartum period in the hidden platform version of the water maze. (A) Mother rats (postpartum days 1–4) showed significantly longer latencies and (B) covered significantly longer distances to reach the platform than virgin females throughout the four sessions of test (six trials each). $n = 9–10$ per group.

performances during a probe trial test conducted after 10 days of retention. Virgin and mother animals were differently affected by the retention period (last learning trial and probe test trial, group × time effect, $F_{(1,17)} = 4.21$; $p = 0.055$, Fig. 2A). In virgins, the latency to reach the platform location during the probe trial was higher than the latency reported for the last trial at the end of the initial learning (NK, $p = 0.013$). In contrast, mothers' performances were unaltered after 10 days of retention and tended to be better than those observed in virgins (NK, $p = 0.058$). Furthermore, MANOVA conducted on the percentages of the time spent and the distance covered in the platform area showed that the mother group explored the platform area significantly more than the virgins (group effect, $F_{(2,16)} = 10.13$; $p = 0.001$, Fig. 2B). Significant negative correlations were reported between the latency to reach the platform location during the probe trial and the percentages of time spent in the platform area ($r = -0.54$; $p = 0.002$) and the distance covered herein ($r = -0.57$; $p = 0.001$). In contrast, the total distance covered by the animals during the probe trial was not correlated with the percentage of exploration of the platform area (time spent, $r = -0.06$; distance covered, $r = -0.10$). One day after the probe trial, we tested the ability of the animals to localize a hidden platform in a new location in a transfer situation. The latency and the distance covered to retrieve a hidden platform during the transfer were similar between virgins and mothers (group effect, latency, $F_{(1,17)} = 3.09$; $p = 0.096$, distance, $F_{(1,17)} = 0.34$; $p = 0.56$). However, performances across trials differed between groups (group-session × trial effect, latency, $F_{(10,170)} = 2.35$; $p = 0.012$, Fig. 2C; distance, $F_{(10,170)} = 1.81$; $p = 0.06$, data not shown). Post hoc analysis revealed that compared to virgins, the mother group had a higher latency (NK, $p = 0.015$, Fig. 2C) and tended to cover a higher distance (NK, $p = 0.055$, data not shown) to retrieve the hidden platform during the first trial of transfer. In contrast, both groups exhibited similar performances in reaching an emerged platform during the visible session conducted 1 day later (group effect, latency, $F_{(1,17)} = 2.37$; $p = 0.142$; distance, $F_{(1,17)} = 0.73$; $p = 0.402$, data not shown). Correlations were performed to assess possible links between the number of pups by litter and the spatial performance of the mothers. The number of pups did not correlate with any measures of spatial learning, transfer or visible platform session (data not shown). In contrast, a negative correlation was observed between the number of pups and the latency to reach the platform location during the probe test ($r = -0.75$; $p = 0.019$), indicating that mothers with the larger litter exhibited the better memory performances (Fig. 2D). To exclude the possibility that the impaired performances reported during the initial learning phase were related to confounding variables due to delivery (motor dysfunctions, fatigue, motivational alterations, etc.), we subsequently trained a new set of female rats in a cue version of the task (Fig. 3). Both virgins and mothers (postpartum days 1–2) quickly learned to reach a visible platform during the two sessions of six trials (session × trial effect, latency, $F_{(5,90)} = 15.71$; $p < 0.0001$; distance, $F_{(5,90)} = 12.09$; $p < 0.0001$, Fig. 3A and B). The mother group displayed the same latencies and distances as virgin rats to retrieve the visible platform throughout the two sessions, suggesting that mothers did not have procedural memory deficits after delivery (group effect, latency, $F_{(1,18)} = 1.86$; $p = 0.107$; distance, $F_{(1,18)} = 1.22$; $p = 0.283$; group × session × trial effect, latency, $F_{(5,90)} = 1.11$; $p = 0.358$; distance, $F_{(5,90)} = 1.48$; $p = 0.202$).

3.2. Early motherhood decreases cell proliferation in the dentate gyrus of the hippocampus but not cell survival

Cell proliferation and survival were differently affected by motherhood (Fig. 5A and B). The number of BrdU-labeled cells in the dentate gyrus decreased between 2 h and 14 days

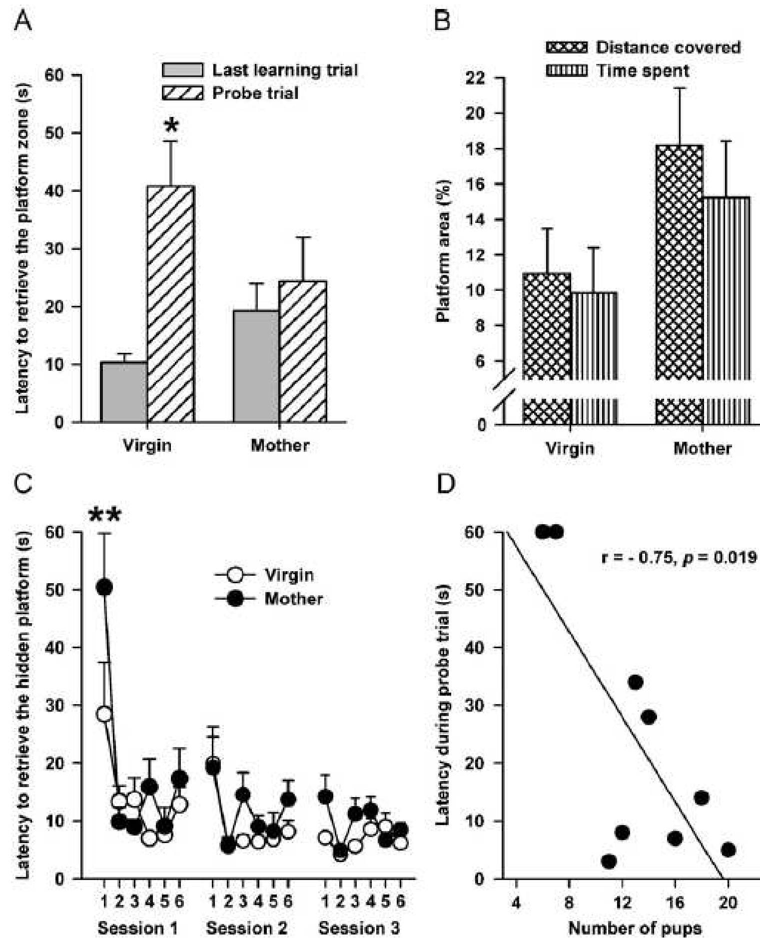


Figure 2 Spatial memory and transfer performances during postpartum period in the water maze. (A) In comparison to their last learning trial, following 10 days of retention, virgin rats exhibited an increase in their latency to reach the platform location during the probe trial, whereas mothers' performances (postpartum day 15) were unaffected. $^{*}p = 0.013$ last learning trial versus probe trial. (B) During the probe trial, the percentage of exploration of the platform area (time spent and distance covered) was higher in mothers than in virgins (MANOVA, group effect, $p = 0.001$). (C) During the transfer sessions (hidden platform with a new location), virgins and mothers (postpartum days 15–17) performances differed across trials. Mothers showed a higher latency during the first trial. $^{**}p = 0.015$. (D) A negative correlation was observed between the number of pups and the latency to reach the platform location during the probe test. $n = 9$–10 per group.

post-BrdU administration in both virgin and mother rats (time effect, $F_{(1,17)} = 51.78$; $p < 0.00001$; NK, virgin, $p = 0.0001$, mother, $p = 0.010$). This decrease, however, differed across groups (time × group effect, $F_{(1,17)} = 6.11$; $p = 0.024$). The proliferation was strongly impaired in mothers in comparison to virgin animals (NK, $p = 0.0002$, Figs. 4,5A and B), whereas the number of BrdU-labeled cells did not significantly differ between virgins and mothers 14 days after the BrdU administration (NK, $p = 0.158$). Phenotype analysis (Fig. 5C and D) showed that motherhood had no effect on neuronal differentiation (Student's t-test, $t_{(4)} = 0.57$; $p = 0.595$). Fourteen days after BrdU administration, 84±4% and 79±8% of the BrdU-positive cells in the virgin and mother groups, respectively, were immunoreactive for the neuronal marker βIII tubulin (Fig. 5C and D).

3.3. Early motherhood decreases plasma estradiol levels and increases hypothalamic–pituitary–adrenal (HPA) axis activity

One day after parturition, mother rats had lower plasma estradiol levels than virgins (group effect, $F_{(2,21)} = 3.55$; $p = 0.046$; NK, mother postpartum day 1 versus virgin, $p = 0.046$, Fig. 6A), and higher plasma corticosterone levels (group effect, $F_{(2,17)} = 4.03$; $p = 0.036$; NK, virgin versus

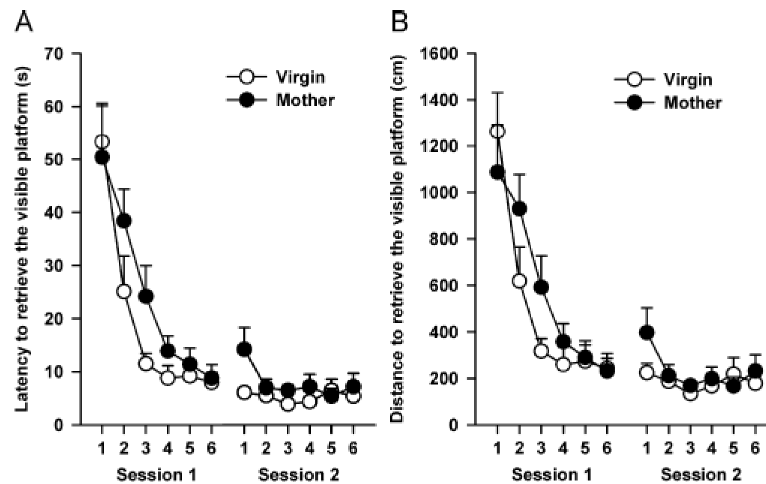


Figure 3 Spatial performances during postpartum period in the visible platform version of water maze. (A) Mother rats tested after delivery (postpartum days 1–2) did not show significant differences in the latency or (B) the distance covered to reach a visible platform compared to virgin rats. $n = 10$ per group.

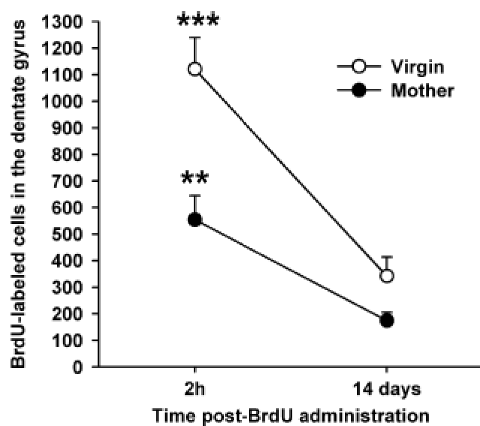


Figure 4 Cell genesis changes in the hippocampus during postpartum period. BrdU-labeled cells in the dentate gyrus of virgin and mother rats, 2 h or 14 days after BrdU administration ($n = 5$–6 per group). At postpartum day 1, proliferation was reduced in mothers compared with virgins. The number of BrdU cells decreased over time in both groups, but the decrease was greater in virgin rats. ***$p < 0.0002$ virgin 2 h post-BrdU versus mother 2 h post-BrdU or virgin 14 days post-BrdU; **$p = 0.010$ mother 2 h post-BrdU versus mother 14 days post-BrdU.

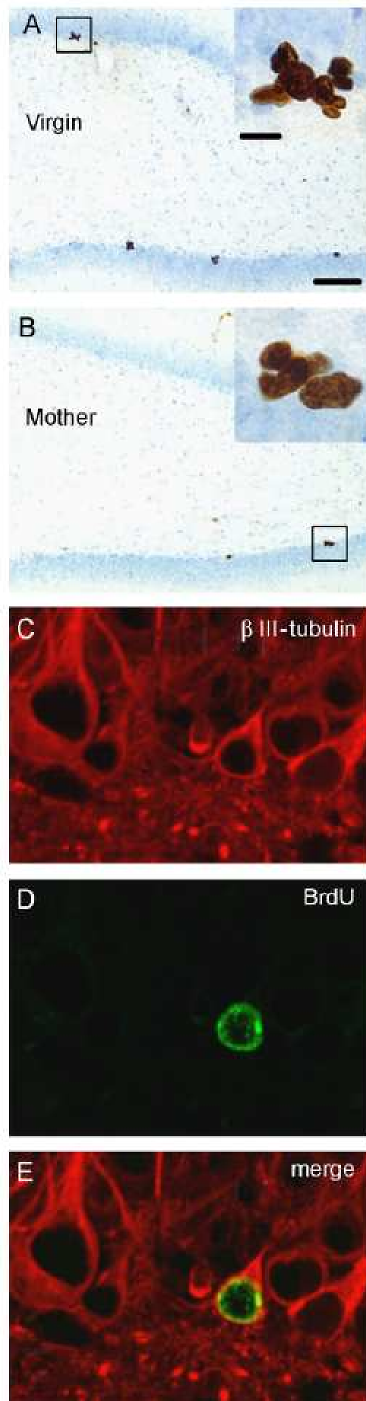
mother postpartum day 1, $p = 0.035$, Fig. 6B). These hormonal changes were attenuated at postpartum day 15 (NK, virgin versus mother day 15, estradiol, $p = 0.069$; corticosterone, $p = 0.140$). Higher adrenals weight was also noted 1 day and 15 days after the delivery (group effect, $F_{(2,15)} = 4.78$; $p = 0.024$; NK, virgin versus mother postpartum day 1 or versus postpartum day 15, $p = 0.001$ and 0.032, respectively, Fig. 6C). Adrenal gland mass positively correlated with plasma corticosterone levels ($r = 0.59$, $p = 0.013$, data not shown). In the mother group, the adrenals weight was negatively correlated to the cell proliferation in the hippocampus ($r = -0.89$; $p = 0.040$, data not shown).

4. Discussion

Despite severe changes inherent to the transition from pregnancy to early postpartum, little attention has been paid to possible cognitive alterations during this period (Galea et al., 2000; Bodensteiner et al., 2006). Our work provides the first evidence of plasticity of the hippocampal function after delivery in rats. We show that spatial performances during learning phase were reduced after the delivery, while spatial retention ability was improved. In addition, early motherhood was associated with a marked decrease in the hippocampal cell proliferation rate without significant changes in neurogenesis 2 weeks later.

Previous observations have reported that motherhood produces lasting improvement in spatial learning performances after weaning (Kinsley et al., 1999; Gatewood et al., 2005). However, the link between motherhood and memory appears complex. For instance, it has been documented that the intensity of maternal behavior is inversely related with the mothers' reference memory performances assessed after the lactating period (Pawluski et al., 2006a). Despite a general positive effect of parity on spatial learning, our work suggests that mothers' performances are initially reduced at the beginning of the postpartum period. During the first 2 days postpartum, mothers performed as well as virgins to retrieve a visible platform. Thus, the decrease in the spatial performances in the water maze task during the early postpartum was not related to fatigue, motor or motivational alterations. The processes underlying the behavioral changed observed during the spatial learning in early mothers are yet to be explored. Indeed, impairment to develop efficient spatial search strategy and/or higher thigmotaxic behavior could explain the poor mothers'

performances (Graziano et al., 2003). Given that the water maze task is stressful, especially during the first trials, it cannot be excluded that an increase in the sensitivity to stress may also be involved in the mothers' performances impairment. However, several studies have demonstrated that lactating females are less responsive to stress (Lightman



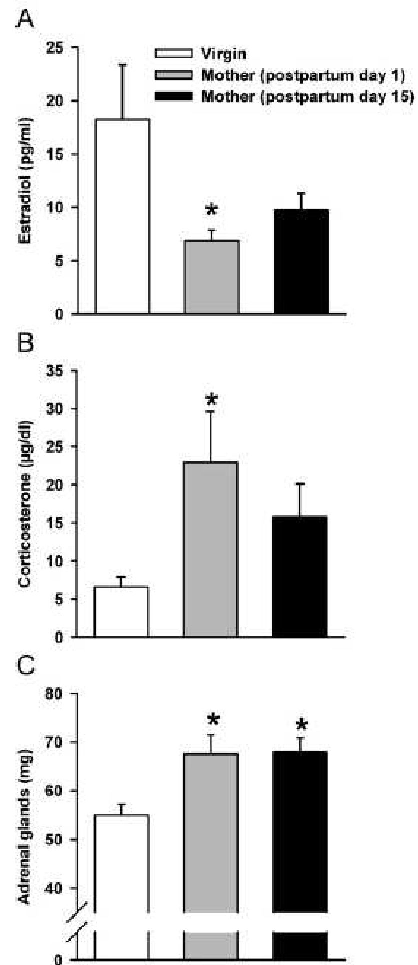


Figure 6 Endocrine changes during postpartum period. (A) Plasma estradiol levels (pg/ml), (B) plasma corticosterone levels (µg/dl) and (C) adrenal glands weight (mg) in virgin and mother groups at postpartum days 1 and 15. At postpartum day 1, mothers showed lower estradiol and higher plasma corticosterone levels in plasma than virgin animals. Lactating females showed hypertrophied adrenal glands in comparison to virgins. $n = 5$–8 per group. *$p < 0.05$ from virgin.

Figure 5 Immunohistochemical staining of BrdU-labeled cells in the dentate gyrus: 2 h after BrdU injection in (A) virgin and (B) mother rats (postpartum day 1). Scale bar, 100 µm; scale bar for the inset, 10 µm. (C–E) Identification by confocal microscopy of the cell phenotype in the granular cell layer of the dentate gyrus 14 days post-BrdU: (C) βIII tubulin labeling (red stain), (D) BrdU-labeled cells (green stain) and (E) BrdU-labeled cells double-stained with the neuronal marker βIII tubulin.

and Young, 1989; Neumann, 2001; Leuner and Shors, 2006). Surprisingly, despite their poor initial performances, the mothers had sound retention abilities and showed better retention performances than the virgins after a delay of 10 days. The number of pups by litter was negatively correlated with the latency to localize the platform area during the probe trial. This suggests that the pups could constitute an environmental enrichment improving the mothers' memory performances. This result extends previous findings, which demonstrate that the exposure of mother to pups enhances her spatial performance in a food foraging test (Lambert et al., 2005). Our work indicates that even if postpartum females exhibited marked performance deficits during the initial training, they are able to memorize spatial information. This leads to the question of the strategy used by the mothers to retrieve the hidden platform during the learning phase. Learning abilities are not necessarily predictive of long-term memory performances during a probe test or during the transfer (Chou et al., 2001; Karhunen et al., 2003; Choi et al., 2006). Indeed, rats with hippocampal lesions well trained in the water maze tend to persevere in exploring the platform area during a probe test (Pearce et al., 1998). Usually, rats use multiple allocentric cues in this task to build a complex spatial map (O'Keefe, 1990). Perhaps, due to their impairments, mothers only used few allocentric cues (the most salient visible cues in the testing room) to retrieve the hidden platform during learning. In this context, it could be hypothesized that after a long lasting retention period, it is easier to remember some cues rather than a complex spatial map (Pearce et al., 1998; Choi et al., 2006). This hypothesis is, in part, supported by the observation that mother rats also show a high latency to reach the hidden platform during the first transfer trial (i.e. retrieve the platform in a new location). Interestingly, straight after the first trial mothers performed as well as virgins, indicating that mothers are not strongly impaired to transfer the rule previously learnt in the task to a new situation.

The consequences and the potential adaptive function of the change of the spatial learning in the young mother are unknown. After delivery, several new behaviors appear such as lactation, retrieving and nursing of the pups (Rosenblatt and Snowdon, 1996). During the first days, the female's activity is focused on pups (Champagne et al., 2003). As result, some aspects of the behavior are reinforced and others, such as feeding (Shirley, 1984), transiently inhibited. We cannot exclude the possibility that an inhibition of some kind of learning may be adaptive to support the initial pup-directed behaviors. In contrast, simple learning such as classical conditioning seems to be preserved in early postpartum females (Leuner et al., 2006). Furthermore, we reported that the mothers' learning abilities were unaffected in a cue version of the water maze task. Thus, despite an impairment of reference memory, the procedural memory is maintained immediately after the delivery. Further studies are required to determine, whether some types of learning, important for maternal care are improved during this period.

Mechanisms underlying the changes of the hippocampal function during early motherhood remain to be explored. We reported, according to previous works (Atkinson and Waddell, 1995; Escalada et al., 1996), that early motherhood induced an increase of corticosterone and a decrease of estradiol levels in plasma. Corticosterone and estradiol are potent modulators of the hippocampal function (McEwen, 2001; Li et al., 2004) and thus may affect the spatial learning performances. High estrogen levels enhance hippocampal cell proliferation (Tanapat et al., 1999), while glucocorticoids have an inhibitory action on cell proliferation and survival in the hippocampus (Cameron and Gould, 1994). Furthermore, it has been demonstrated that hippocampal cell proliferation is quickly modulated by hormonal changes such as those occurring during the estrus cycle for estradiol (Tanapat et al., 1999) or after stress exposure for corticosterone (Gould et al., 1997). We showed here that cell proliferation in the dentate gyrus of the hippocampus was decreased 1 day after delivery. However, despite this reduced cell proliferation, there was no difference in the number of new neurons in both groups 2 weeks later. Although our data did not demonstrate a causal link between spatial learning/memory and hippocampal neurogenesis, it suggests that these two functions were affected in a similar way after delivery. In contrast, an enhancement of the neurogenesis in the olfactory bulbs has been reported during the pregnancy and the early postpartum, in mice (Shingo et al., 2003) and in rats (Furuta and Bridges, 2005). However, the functional significance of this change has yet to be explored. We confirm that the adrenal gland mass, which can reflect a hyperactivity of the HPA function, was negatively correlated to the cell proliferation in the hippocampus (Darnaudery et al., 2006). In contrast, no significant correlation was noted between plasma estradiol levels and hippocampal cell proliferation. Nevertheless, it has been recently demonstrated that the proliferation of hippocampal cells requires local estrogen synthesis (Fester et al., 2006), which does not necessarily correlate with gonads production. Several other factors related to the lactating period, such as IGF-1 and oxytocin could also mediate some variations of the hippocampal function (Hapon et al., 2003; Tomizawa et al., 2003; Butovsky et al., 2006; Darnaudery et al., 2006). Further investigations are required to determine the role played by different hormonal factors on the effects reported. The role of neurogenesis on behavior has been widely debated and until now, studies focusing on the impact of neurogenesis on behavior have been inconclusive (Leuner et al., 2006). Spatial learning may exert bidirectional effects on the proliferation of granule neurons (Abrous et al., 2005); both increases (Gould et al., 1999) and decreases (Mohapel et al., 2006) in neurogenesis processes have been reported after learning. It could be hypothesized that the reduced hippocampal cell proliferation immediately after delivery may help to reinforce the stability of the hippocampal system and may contribute to the enhanced retention performances at postpartum day 15. Thus, Presenilin-1 knockout mice exhibited better retention of 2-week-old contextual fear which is associated with a significant deficiency in enrichment-induced neurogenesis (Feng et al., 2001).

In conclusion, this work shows that the early postpartum period is associated with changes in cognitive function and hippocampal plasticity. Together with previous findings, our work suggests that dynamic changes occurring with pregnancy and lactation are dependant on the stage of lactation

as well as a number of other factors including previous pregnancies, the number of pups, etc. Further studies are needed to determine whether these changes may have long-term effects and to understand the exact mechanisms underlying the modification of the hippocampal function after delivery. Finally, our findings support the idea that motherhood constitutes a remarkable naturalistic model for the study of behavioral and neural plasticity in mammals.

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Funding for this study was provided by the University of Lille 1, the University has no further role in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication.

Conflict of interest

None declared.

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