Implications of Prenatal Exposure to Synthetic Corticosteroid in the Mesolimbic Reward Pathway

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DECLARAÇÃO

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Aos meus pais, irmão e avós…

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Abstract

In the field of clinical obstetrics, glucocorticoids (GCs) have gained an important role in promoting fetal lung maturation, and are used in about 10% of risk pregnancies. The fetus is highly sensitive to perturbations of its chemical environment along critical developmental windows and, thus, it is not surprising that stress exposure or GC treatment during pregnancy cause notable changes in the physiology and behaviour of the offspring in adulthood. In fact, lifelong implications of sustained deleterious perinatal experiences, such as elevated GCs, include anxiety, mood and cognitive disorders as well as the development of addictive-like behaviors. It seems that during these crucial periods, several brain regions might be programmed by prolonged stressful insults, such as exposure to high doses of GCs. However, less is known about the potential effects of short-term therapies with these steroids during late gestation, a crucial stage of the neurodevelopment; as a consequence, very little is known about the underlying mechanisms of these detrimental effects.

In this work we show that prenatal exposure to dexamethasone (DEX) at E18-19 leads to the development of drug-seeking behaviors in the adult progeny. DEX-exposed animals displayed increased preference for the morphine-paired compartment in the conditioned place preference teste (CPP, non-contigent paradigm) and increased voluntary alcohol consumption (AC) using a two bottle free-choice paradigm (contingent paradigm). These animals also displayed increased locomotor activity after morphine administration in the open field (OF) test, suggesting a hypersensitive behavioural response to drugs of abuse. Considering the importance of the dopaminergic mesolimbic system for the development of addiction, we performed herein an extensive structural, neurochemical and molecular analysis of the nucleus accumbens (NAcc) and the ventral tegmental area (VTA). At 3 days of postnatal life, we found a significant reduction in the rate of cell proliferation in DEX-exposed animals in the NAcc and VTA. These changes seem to be sustained in time since in adulthood, DEX animals presented considerable reduced volume and cell numbers in the same brain regions. Because of well-known plastic events in the central nervous system, we underwent a morphometric analysis of dendrites and synapses in adult offspring of DEX-treated dams. We found significant changes in the number

of spines and spine density of NAcc-shell, most likely a compensatory mechanism, while in the NAcc core there was no sign of synaptic plasticity in DEX animals. The VTA also presented significant differences in the number of spines, spines density and dendritic branching.

Our neurochemical analysis demonstrated that the dopaminergic input was de-regulated in DEX-exposed animals as they displayed significantly less dopamine and an impoverishment of dopaminergic fibers in the NAcc, while an increase in dopamine levels was observed in the VTA. In parallel, a significant up-regulation of the dopamine receptor 2 (Drd2) in the NAcc of DEX-exposed animals was found, which could reflect a compensation mechanism due to the lack of the ligand. Importantly, after a dopamine-releasing stimulus (drug exposure), the levels of Drd2 were significantly reduced in DEX-exposed animals. This phenomenon was associated with an increased methylation pattern of the promoter region of Drd2 in the NAcc.

In an attempt to revert the hypodopaminergic status of DEX-exposed animals in the NAcc, we administered L-dopa/carbidopa by oral gavage. Interestingly, restoring dopamine levels in the NAcc had striking effects in the behavior of DEX-exposed animals, since the behavioral phenotype was completely rescued, i.e., DEX animals no longer displayed increased conditioning in the CPP, increased AC or enhanced locomotor activity in the OF. Of notice, these effects were observed in short- and long-term administration treatments and, at least for the long-term treatment, the therapeutic effect was sustained in time.

In summary, we show that prenatal DEX exposure programs the mesolimbic dopaminergic system at a morphological, neurochemical and molecular level and alters the threshold for the rewarding effects of drugs of abuse. Interestingly, the addictive-like phenotype seems to be dependent on dopamine levels and can be modulated by dopamine replacement, thus opening new perspectives in the treatment/prevention of addictive behaviors.

Resumo

Na clínica obstétrica, os glucocorticóides (GCs) são usados em cerca de 10% das gravidezes de risco de forma a promoverem a maturação pulmonar fetal. Existem períodos críticos de susceptibilidade do feto a perturbações do seu ambiente químico e, por isso, não é surpreendente que o stress ou tratamento com GCs durante a gravidez cause alterações na fisiologia e comportamento da descendência aquando adultos. Sabe-se que experiências prenatais adversas tais como níveis de GCs elevados, estão associadas com uma maior propensão para a ansiedade, hiper-emocionalidade e défices cognitivos, bem como com o desenvolvimento de comportamentos aditivos. Apesar de se supor que estas alterações comportamentais advém de uma "reprogramação" de determinadas áreas do cérebro por experiências nocivas, pouco se sabe acerca dos efeitos deletérios do tratamento com GCs no último trimestre da gravidez.

Neste trabalho, demonstrámos que a exposição ao GC sintético dexametasona (DEX) no dia 18 e 19 de gestação, conduz ao aparecimento de comportamentos do tipo aditivo na prole adulta. Os animais DEX têm preferência aumentada para o compartimento associado à morfina no *conditioned place preference test* (CPP, paradigma não contingente) e aumento do consumo voluntário de álcool (AC, paradigma contingente). Estes mesmos animais também apresentam maior actividade locomotora no teste de *open field* (OF) após administração de morfina, sugerindo uma resposta exacerbada aos efeitos desta droga. Considerando a importância do sistema dopaminérgico mesolimbico para o desenvolvimento de comportamentos aditivos, nós efectuámos uma caracterização extensiva a nível estrutural, neuroquímico e molecular do nucleus accumbens (NAcc) e da área tegmental ventral (VTA). Aos 3 dias de vida pos-natal, os animais DEX apresentam uma redução significativa da proliferação celular no NAcc e VTA. Estas alterações têm efeitos a longo prazo, uma vez que em adultos, estes animais têm uma hipotrofia volumétrica destas regiões. Devido à plasticidade conhecida do sistema nervoso central, efectuámos uma análise morfométrica das dendrites e sinapses nos animais adultos. Os animais DEX apresentam alterações no número e densidade de espinhas na subdivisão shell do NAcc, enquanto que o core não apresenta sinais de ter

sofrido plasticidade sináptica. O número e densidade de espinhas, bem como o número de ramos dendríticos também se encontra alterado na VTA.

A nossa análise neuroquímica mostra que o *input* dopaminérgico se encontra desregulado nos animais DEX, uma vez que eles apresentam uma diminuição de dopamina e uma redução de fibras dopaminérgicas no NAcc e uma elevação da dopamina na VTA. Em paralelo, encontrámos uma sobreexpressão do receptor de dopamina Drd2 no NAcc destes animais, sugerindo um potencial mecanismo de compensação devido à baixa quantidade de dopamina. De notar que, após um estímulo que induz a libertação de dopamina (exposição à morfina), os níveis de Drd2 estavam significativamente diminuídos nos animais DEX. Este fenómeno parece estar associado a um aumento do padrão de metilação na região promotora do Drd2 no NAcc.

Numa tentativa de reverter o estado hipodopaminérgico dos animais DEX no NAcc, administramos L-dopa/carbidopa por *gavage* oral. Ao repor os níveis de dopamina no NAcc, o fenótipo comportamental foi completamente revertido, i.e., os animais DEX deixam de apresentar condicionamento preferencial no CPP, aumento do consumo de álcool ou aumento da actividade locomotora no OF após administração de morfina. É importante referir que estes efeitos foram observados com regimes de tratamento de curta e longa duração, e que, pelo menos no caso do tratamento de longa duração com L-dopa, os efeitos terapêuticos são mantidos durante 3 semanas.

Em suma, nós demonstrámos que a exposição prenatal de DEX "reprograma" o sistema dopaminérgico mesolimbico a nível morfológico, neuroquímico e molecular; alterando também o limiar dos efeitos recompensadores das drogas de abuso. Importante mencionar que o comportamento aditivo parece ser dependente dos níveis de dopamina, e que pode ser modulado pela reposição de dopamina no NAcc, o que abre novas perspectivas para o tratamento/prevenção de comportamentos deste tipo.

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Abbreviations

11ß-HSD2: 11ß-hydroxysteroid dehydrogenase
AC: alcohol consumption
ACTH: adrenocorticotrophin hormone
AVP: arginine vasopressin
CAH: congenital adrenal hyperplasia
CB: cannnabinoid system
CB1-R: cannabinoid 1 receptor
CORT: corticosteroid
CPP: conditioned place preference
CRH: corticotropin-releasing hormone
DA: dopamine
DEX: dexamethasone
DOPAC: 3,4-dihydroxyphenylacetic acid
Drd: dopamine receptor
DST: dexamethasone supression test
GCs: glucocorticoids
GR: glucocorticoid receptor
HPA: hypothalamus-pituitary-adrenal axis
HVA: 4-hydroxy-3-methoxuphenylacetic acid
L-Dopa: levodopa
LDT: laterodorsal tegmental nucleus
MR: mineralocorticoid receptor
NAcc: nucleus accumbens
OF: open field
PBP: parabrachial pigmente area
PFC: prefrontal cortex
PN: paranigral nucleus
POMC: pro-opiomelanocortin
PPTg: pedunculopontine tegmental nucleus
PVN: paraventricular nucleus
RDS: respiratory distress syndrome
RT-PCR: real time - polymerase chain reaction
SN: substantia nigra
TH: tyrosine hydroxylase
VTA: ventral tegmental area
VTT: ventral tegmental tail
WB: western blot

Chapter 1

General Introduction

1. INTRODUCTION

1.1 Use of dexamethasone (DEX) in prenatal medical practice

Synthetic glucocorticoids (GC), such as dexamethasone (DEX) are commonly used in the clinical practice (Crane et al., 2003; Crowley, 1995) for a wide spectrum of disorders. Of direct relevance for this thesis, synthetic GCs are routinely administered to pregnant women at risk of preterm delivery and corticosteroids, in general, are also frequently prescribed in the perinatal and neonatal period.

Preterm delivery occurs in around 7-10% of all pregnancies and still is the most serious cause of neonatal mortality and morbidity. Half of all newborn under 32 weeks are affected by respiratory distress syndrome (RDS) (National Institutes of Health Consensus Development Panel, 2001). RDS is a serious complication of preterm birth and is amongst the primary causes of early neonatal death and disability. Given the efficacy of treatment, with DEX or betamethasone, in improving lung function, there is a significant reduction in the occurrence of RDS in premature babies (Liggins and Howie, 1972); thus it has become a routine in many centers to administer weekly treatment with these GCs in every pregnancy of preterm delivery (Burton and Waddell, 1999; Cadet et al., 1986). Moreover, corticosteroid treatment is also correlated with a significant reduction in the risk of intraventricular haemorrhage associated with prematurity.

Until recently, multiple courses of GCs therapy were the established regimen in Australia, Europe and North America, if the risk of preterm delivery persisted (Brocklehurst et al., 1999; Smith et al., 2000). As a result, a very large cohort of children has been exposed to GCs and many of these children were born at normal term. Both prospective animal studies and retrospective human studies have shown that multiple courses of GCs can have long-term effects on brain structure, behaviour (vulnerability to drug abuse) and endocrine function (glucose intolerance, dyslipidaemia, and hypothalamo-pituitary-adrenal axis disfunction) in both childhood and in adulthood (Banjanin et al., 2004; Owen and Mathews, 2007).

In an attempt to evaluate the efficacy of a single course weekly administration of antenatal corticosteroids compared with a single course in

reducing neonatal morbidity, a randomized controlled trial was undertaken (Guinn et al., 2001). Although the study concluded that weekly courses were no better than a single course in reducing composite neonatal morbidity, the study lacked the statistical power to establish potential negative or positive effects on measurable longer-term outcomes.

Another application of antenatal DEX is congenital adrenal hyperplasia (CAH). CAH is the common name of a constellation of diseases that impair cortisol synthesis in the adrenal cortex. Antenatal DEX for the treatment of fetus with CAH was introduced in 1978 (Forest, 2004), and has been shown to prevent genital masculinization of affected girls. CAH is a group of autosomal recessive disorders resulting from the deficiency of one of the five enzymes required for the synthesis of cortisol in the adrenal cortex. The most frequent is steroid 21-hydroxylase deficiency, accounting for more than 90% (Speiser and White, 2003) of cases, and promoting overproduction of adrenal androgens.

In summary, corticosteroids have become a mainstay of prophylactic treatment in preterm birth. However, there still remain a number of important issues/side effects to be determined regarding the use of antenatal corticosteroids. Synthetic GCs, such as the highly GR-specific ligand, DEX, are not metabolized by 11ß-hydroxysteroid dehydrogenase (11ß-HSD2), and therefore pass across the placenta from mother to fetus potentially affecting the highly plastic and sensitive developing brain (Clark, 1998). In fact, it is well known that increased antenatal exposure to synthetic GCs can result in permanent modification of HPA function in animal models but also in humans (Entringer et al., 2009).

1.2 Hypothalamic-pituitary-adrenal axis (HPA axis)

1.2.1 Overview

Any imbalance in an organism's physical or psychological wellbeing creates a stress response that involves multiple systems. The activation of these systems allows appropriate adaptation to the disturbance (homeostasis). Stress exposure can be beneficial, in that it can create a situation of increased arousal

and emotional salience enabling the organism to appropriately respond to the stressor and ensure survival. However, under states of inappropriate response, stress exposure can be maladaptive and can place the body in a state of increased susceptibility to illness. During neurodevelopment, disability to inhibit the stress response once it is initiated also increases the vulnerability to diseases and results in permanent effects on growth and differentiation of a number of developing systems, including the central nervous system (CNS).

Briefly, in the presence of a stressor, the HPA axis is activated through afferent sympathetic, parasympathetic and limbic circuits that stimulate the hypothalamic paraventricular nucleus (PVN). The PVN controls pituitary–adrenocortical activity (Herman et al., 1995). Parvocellular neurons in the PVN project to the median eminence and release corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP), which are then released into the hypophysial portal circulation (Plotsky et al., 1993). CRH and AVP stimulate the synthesis and release of adrenocorticotrophin hormone (ACTH) from pro-opiomelanocortin (POMC) in the corticotrophs of the anterior pituitary gland (Plotsky et al., 1993). At the adrenal cortex, ACTH promotes both the synthesis and release of GCs from the zona fasciculata into the systemic circulation (Checkley, 1996) (Fig.1).

GCs trigger a plethora of actions. In general, they increase hepatic glucose production and release (stimulation of glycogenolysis), as well as cardiovascular muscle tone, but suppress “nonessential systems” for immediate survival, such as the immune, muscle-skeletal and reproductive systems (Herman et al., 1995; Sapolsky et al., 1986). It is important to remember that exposure to various stressors enhances production and secretion of GCs into the peripheral circulation. To avoid the detrimental actions of elevated GCs, the body has evolved a tightly regulated feedback control over GCs production and release (Charmandari et al., 2003). The major sites of GCs feedback inhibition are the hippocampal formation, the medial prefrontal cortex (PFC), the hypothalamic PVN, and the anterior pituitary gland.

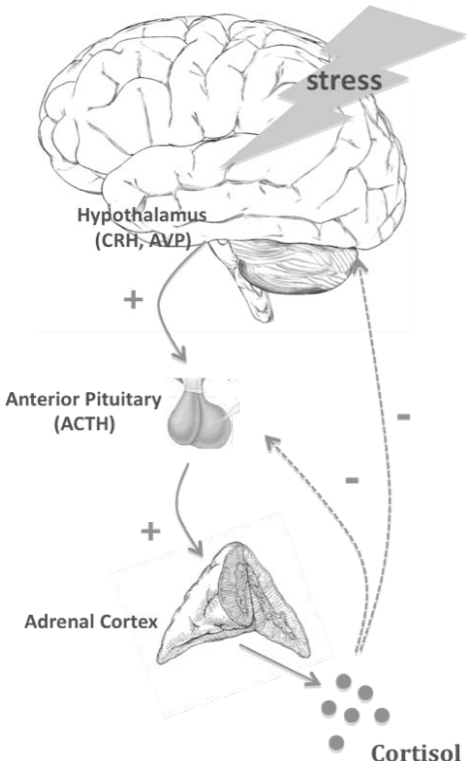

Figure 1. In the presence of a stressor, the HPA axis is activated through limbic circuits that stimulate the hypothalamic paraventricular nucleus (PVN) that controls pituitary–adrenocortical activity. Parvocellular neurons in the PVN project to the median eminence and release corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP), which are then released into the hypophysial portal circulation. CRH and AVP stimulate the release and synthesis of adrenocorticotrophin hormone (ACTH) in the anterior pituitary glan. At the adrenal cortex, ACTH promotes both the synthesis and release of GCs from the zona fasciculata into the systemic circulation.

1.2.2 GR/MR receptors

Previous studies demonstrate the existence of two adrenal steroid receptors in the brain (de Kloet et al., 1975). One, called the type I receptor or MR, has a high affinity for corticosterone, the principle GC in rodent (Beaumont and Fanestil, 1983; Krozowski and Funder, 1983). In the rat, MR are most abundant in the hippocampus and in the hypothalamus, although also present in the amygdala, the septum and the cerebral cortex (Ahima et al., 1991; Kawata et al., 1998). MR bind both physiological GCs (cortisol, cortiscoterone) and mineral corticosteroids (aldosterone) with equal high affinity. The other receptor, known as the type II receptor or glucocorticoid receptor (GR), has a much lower affinity

for corticosterone than that of MRs (Funder and Sheppard, 1987; Ratka et al., 1989; Reul and de Kloet, 1985). The GR are widely distributed in brain neurons, including hippocampus, hypothalamus and nucleus accumbens (NAcc), but also in glial cells and pituitary cells (Ahima and Harlan, 1990; Fuxe et al., 1985; Kawata et al., 1998; Morimoto et al., 1996).

The developmental pattern of GR and MR is quite different in species that give birth to mature offspring. In the rat, GR and MR expression in the brain are low throughout gestation, but increase rapidly around birth. This is consistent with the postnatal nature of brain and HPA development in this species. However, there are distinct ontogenetic patterns for GR and MR expression in the rat fetal brain (Diaz et al., 1998). Importantly, GR mRNA is present in the hippocampus, hypothalamus and pituitary by gestational day 13, and the levels of GR mRNA increase around term. In contrast, MR is not present in the hippocampus until gestational day 16–17 (Diaz et al., 1998; Gass et al., 2000). The presence of GR has been documented in the early human embryo (Muneoka et al., 1997; Ohkawa et al., 1991). Specific expression of GR mRNA has been identified in the metanephros, gut, muscle, spinal cord and dorsal root ganglia, periderm, sex cords of testis, and adrenal by 8–10 weeks of life (Condon et al., 1998). High levels of GR mRNA are present in the human lung by 12 weeks of gestation. Unfortunately, there is no information regarding developmental changes in GR expression in the human fetus later in gestation or at any stage in the developing fetal brain.

The fetal brain is, thus, endowed with GR and as such represents a key target for GCs (Andrews and Matthews, 2000; Owen and Matthews, 2003; Owen et al., 2005). Given their small size and lipophilicity, GCs can access the brain; the magnitude of their damaging effects in the brain depends largely on the particular stage of development when the exposure occurs, indicating that there are “critical windows” of sensitivity to the detrimental effects of GCs on the brain tissue. Studies have shown that prenatal exposure to GCs can lead to lifelong alterations in regulation of HPA function in the offspring of several species including the rat, and non-human primate (Levitt et al., 1996; Sloboda et al., 2002). In a number of studies, alterations in HPA function have been associated

with modification of corticosteroid receptor expression at sites of GC feedback (hippocampus, hypothalamus and pituitary) in the juvenile and adult brain.

When circulating GC concentrations exceed the levels which are controlled through binding to MR, negative feedback along the HPA axis is maintained through activation of the GR in the several areas of the brain and pituitary gland. This observation leads to an attractive theory in which predominant MR activation is responsible for feedback regulation of the HPA axis activity during basal conditions, while GR activation becomes important in mediating GC feedback during periods of stress (de Kloet et al., 1998). However, it is noteworthy that evidence in adult rats has indicated that hippocampal GR may also exert a facilitatory, rather than purely inhibitory effect, on plasma GC levels, an effect which involves the disinhibition of the tonic MR-mediated suppression over basal GC secretion (Van Haarst et al., 1997). When high GCs concentrations are present, MR receptors saturate, and the GR receptors appear to 'take over' to ensure the return of homeostasis.

1.2.3 Programming the HPA axis

Prenatal stress or GCs are able to program HPA axis activity and behavior in offspring, although the mechanisms are not entirely clear. Stress will modify cardiovascular and endocrine parameters in the mother, including an increase in ACTH, GC, ß-endorphin, and catecholamines. Under normal circumstances, the placenta forms a barrier to many of these maternal factors, though some, including synthetic GCs, will pass and act on the fetus. Besides these effects, there may be indirect effects on the fetus via modification of placental function. For example, catecholamines can constrict placental blood vessels and cause fetal hypoxia (Ohkawa et al., 1991), which will activate the fetal HPA axis (Challis et al., 2000).

Under normal circumstances, access of maternal endogenous GC to the fetus is low due to the expression of 11ß-HSD in the placenta (Burton and Waddell, 1999). 11ß-HSD interconverts cortisol and corticosterone to its inactive products (cortisone, 11-dehydrocorticosterone). There are two isoforms; 11ß-HSD type 1 which is bi-directional and type 2 which is uni-directional (cortisol to cortisone). The efficiency of placental 11ß-HSD2 varies amongst species;

however, it is generally accepted that placental 11ß-HSD2 is of primary importance in excluding maternal GC from the fetus. Further support for the "GC reprogramming hypothesis" has been provided by studies in which 11ß-HSD2 activity has been modified. Pharmacological blockade of 11ß-HSD2 during pregnancy, and consequently an increased transfer of GC from mother to fetus, results in offspring that exhibit elevated basal and stress-stimulated HPA activity (Seckl et al., 2000). Adult offspring born to mothers treated with carbenoxolone (which inhibits the activity of 11ß-HSD2) throughout pregnancy have persistently low body weight, increased basal plasma corticosterone levels, increased CRH and reduced GR mRNA in the hypothalamic PVN (Welberg et al., 2000), indicative of HPA axis hyperactivity. Importantly, this enzyme has no strong affinity for synthetic GCs such as DEX, suggesting that even low doses of synthetic GCs can pass the placenta and affect the fetus (Oliveira et al., 2006).

The mechanisms that underlie programming of adult HPA function are likely to be dependent on the type of GC, dose and timing of exposure (Welberg and Seckl, 2001). Adult male rat offspring whose mothers were exposed to synthetic GC in the last week of gestation exhibit reduced hippocampal GR and MR expression and no change in GR expression in the PVN (Welberg et al., 2000). The reduced hippocampal GC feedback sensitivity in these animals could be connected to the increased levels of CRH mRNA in the PVN, and to an overactivation of HPA activity. In addition, in the offspring born to mothers treated daily with synthetic GC throughout pregnancy there is an increase in MR and GR mRNA in the basolateral nucleus of the amygdala. Previous descriptions that central amygdaloid GR occupation triggers an excitatory influence over the HPA activity (Herman and Cullinan, 1997), it seems plausible to admit that long-term exposure to synthetic GC *in utero* may facilitate activation of the HPA axis (Welberg and Seckl, 2001).

In summary, there is increasing evidence showing that fetal exposure to GCs can effectively program HPA function. HPA axis de-regulation can lead to a series of changes in brain circuitry and affect specific neurobiological pathways. Several studies have shown that HPA axis hyperactivation results in neurodegeneration in hippocampus (Sousa et al., 2002), PFC (Cerqueira et al.,

2007), bed nucleus of stria terminalis, amygdala (Pêgo et al., 2008), and dorsal striatum (Dias-Ferreira et al., 2009). However, less is known about the impact of stress/GCs on the mesolimbic circuity, in particulary as a result of perinatal exposure to stress/GCs. The evidence available sugests that the mesolimbic system is also vulnerable to stress/GCs during development and this may explain why these subjects display increased susceptibility to addictive like behaviors.

1.3 Mesolimbic system

1.3.1 Structural organization

The anatomical concept of the mesolimbic system has been used differently by distint researchers. In 1971, Ungerstedt, originally reported the observation, that the A10 cell group (dopamine nucleus), projects primarily to the NAcc and olfactory tubercle; this nucleus was later called VTA. This projection was named the mesolimbic dopamine system, to contrast with the nigrostriatal dopamine system, projecting from the A9 cell group to the "striatum" (which is now referred to as the "dorsal striatum"). Subsequent studies employing more sensitive methods showed that VTA dopaminergic neurons project not only to the NAcc and olfactory tubercle but also to other limbic related region including the septum, hippocampus, amygdala and PFC (Fallon and Moore, 1978; Swanson, 1982), although these regions receive much less dopaminergic innervation from the VTA than does the ventral striatum. So, the mesolimbic dopamine system not only includes the limbic striatum but also other limbic forebrain regions that receive dopaminergic inputs from the ventral midbrain. However, in this thesis, we will focus on the projection from the VTA to the NAcc.

The VTA lies medial to the substantia nigra and ventral to the red nucleus in the midbrain. It is not well-defined cytoarchitectonically, and its boundaries are determined largely by those of adjacent structures. The VTA can be divided into four major regions: paranigral nucleus (PN), parabrachial pigmente area (PBP), parafasciculus retroflexus area and ventral tegmental tail (VTT). The PN and PBP are dopaminergic cell-body-rich zones, whereas the parafasciculus

retroflexus area and VTT are dopaminergic cell-body-poor zones. The parafasciculus retroflexus area consists of a low density of tyrosine hydroxylase (TH)-positive cell bodies, which are small to medium in size and light to moderate in staining. The TH-positive cell bodies in the parafasciculus retroflexus area are continuous with those in the posterior hypothalamic area including the supramammillary nucleus, which contains TH-positive cell bodies centered in its medial part (Swanson, 1982). The VTT contains a low density of TH-positive cell bodies, localized in the zone just posterior to the PN and lateral to the posterior interpeduncular nucleus.

The NAcc, a brain region located within the ventral aspects of the basal ganglia, has long been conceptualized as an essential integrator of "motivation and action". Mogenson first proposed this forebrain structure as a key element in the integration of affective and cognitive processing with voluntary motor actions (Mogenson et al., 1980). He emphasized the connectivity of the nucleus NAcc, in that it received a convergence of information from brain regions involved in emotional learning, memory, and complex cognition, such as amygdala, hippocampus, thalamus, and PFC. This region contains two functionally distinct subcompartments, termed the shell and core (Kelley, 2004) (Fig.2).

The shell, a region extending medially, ventrally and laterally around the core, is strongly interconnected with the hypothalamus and the VTA and is important in regulating ingestive behaviors (Kelley, 2004; Robinson and Berridge, 1993). The reciprocal dopamine innervation from the VTA to the shell is important in modulating motivational salience and contributes to establishing learned associations between motivational events and concurrent environmental perceptions (Bassero and Di Chiara, 1999; Sellings and Clarke, 2003).

In contrast, the core (tissue surrounding the anterior commissure) (Fig. 2) compartment is anatomically associated with the anterior cingulate and orbitofrontal cortex, and appears to be a primary site mediating the expression of learned behaviors in response to stimuli predicting motivationally relevant events (Di Ciano, 2001; Kelley, 2004). Importantly, however, the obligatory

involvement of the core in expressing adaptive behavior depends not on dopaminergic afferents but, rather, on glutamatergic afferents from the PFC (Di Ciano, 2001). Although not an obligatory event, dopamine is released into the core in response to stimuli predicting a rewarding event and, likely, modulates the expression of adaptive behaviors (Cheng et al., 2003; Ito et al., 2000).

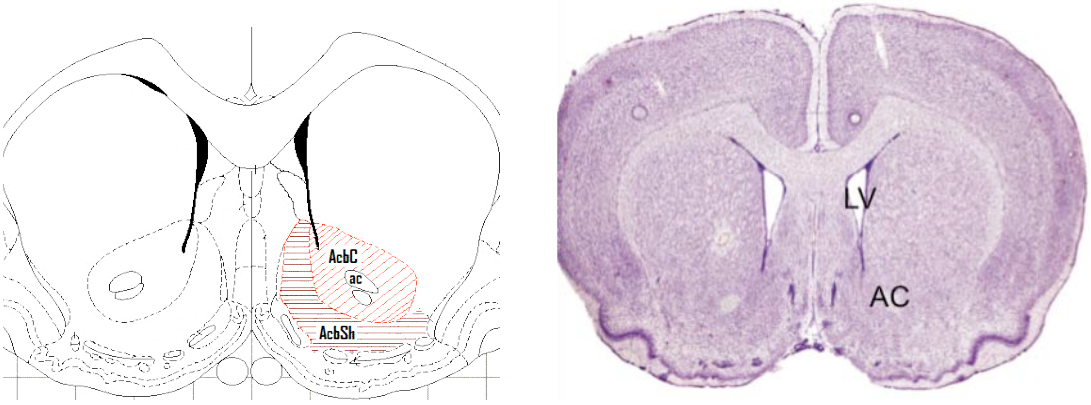

Figure 2. 'The basement of the brain'. These ventrally and medially located areas include the ventral striatum and in particular the nucleus accumbens, typically divided into two major subdivisions, the shell (AcbSh) and the core (AcbC). In the basement of the brain this dismissed merchandise corresponds to behavioral functions and response sets essential for the survival of the self and of the species. The lateral ventricle (LV) and anterior comissure (AC) are labeled for reference.

1.3.2 Main connections of NAcc and VTA

As described above, the NAcc is implicated in the interface between emotion and action. Besides the dopaminergic inputs from VTA, there are limbic areas including the amygdala, hippocampus and medial PFC (mPFC) sending major glutamatergic projections to the NAcc and the VTA (Heimer et al., 1997) (Fig. 3).

The NAcc has two main GABAergic outputs, to the ventral pallidum and VTA/substantia nigra. Both the ventral pallidum and VTA send GABAergic efferents to the medial dorsal thalamus, where glutamatergic projections originate to end in the mPFC (thus closing this limbic circuit) (Groenewegen and Uylings, 2000; Heimer, 2003; Pierce et al., 1997). Importantly, the dopaminergic

input from the VTA to the NAcc, amygdala, hippocampus, mPFC and ventral pallidum, play a critical role in modulating the flow of information through this limbic circuit, that is highly interconnected (Sesack et al., 2003; Wise, 2002).

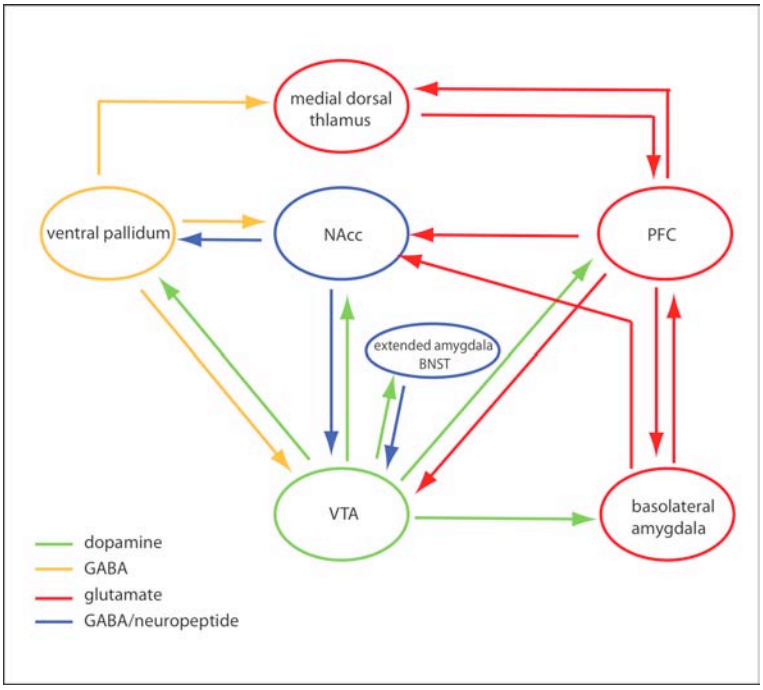

Figure 3. Neurobiology of adaptative behavior has focused on three brain regions in the activation of behavior: the amygdala, prefrontal cortex (PFC), and nucleus accumbens (NAcc). The amygdala is involved in fear-motivated behaviors, while the NAcc was identified from a connection with reward-motivated behaviors. The PFC is less involved in establishing whether a stimulus is positive or negative; rather, it regulates the overall motivational salience and determines the intensity of behavioral responding. The NAcc has dense projections of GABA and neuropeptides to the ventral pallidum that are critical for the expression of motivated behaviors. Another GABA/neuropeptide component of the circuit is to the extended amygdala, which is a cluster of interconnected nuclei, including the central amygdala nucleus, bed nucleus of the stria terminalis, and shell of the NAcc, that is in part a conduit for environmental and interoceptive stressors.

Two groups of mesopontine tegmental area neurons provide a major input to the VTA: the pedunculopontine tegmental nucleus (PPTg) and the more lateral and slightly more posterior laterodorsal tegmental nucleus (LDT) (Paxinos and Watson, 1998). Although these two nuclei receive largely overlapping inputs, including those from the lateral hypothalamus, the LDT

receives a strong input from the PFC, whereas the PPTg has a larger input from the amygdala (Semba and Fibiger, 1992). Both nuclei provide significant numbers of glutamatergic and cholinergic as well as GABAergic projections to the VTA (Oakman et al., 1995). Whereas the LDT projects primarily to the VTA, the PPTg projects to both the VTA and the substantia nigra compacta (Oakman et al., 1995). Other, presumably GABAergic, inputs to the VTA arise from the ventral pallidum (Geisler and Zahm, 2005) and the NAcc (Conrad and Pfaff, 1976). The NAcc projections arise from medium spiny neurons that contain peptides as dynorphin and endogenous κ opioid receptor-selective peptide wich play a modulatory role over the VTA (Fig. 4). Importantly, the VTA also receives projections from the noradrenergic locus coeruleus and the serotonergic dorsal raphe nucleus (Geisler and Zahm, 2005; Phillipson, 1979).

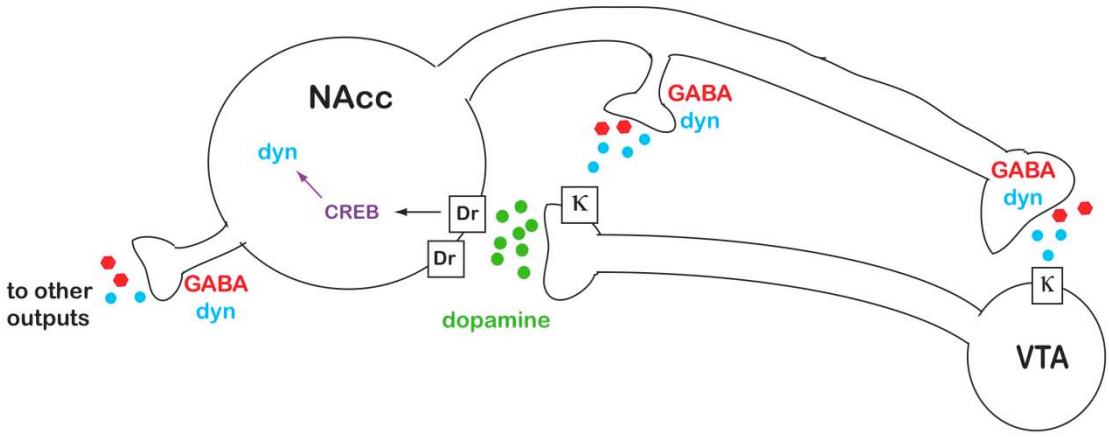

Figure 4. Mesolimbic circuitry. The figure shows a simplified scheme by which dopamine release in the synaptic cleft induces the production of dynorphin that in turn, inhibits VTA dopamine release. CREB is activated by D1 dopamine receptors, which leads to increased expression of dynorphin (dyn). Dyn feeds back on opioid receptors located on the terminals and cell bodies/dendrites of VTA dopamine neurons.

As already mentioned, the VTA projects densely and largely ipsilaterally to the ventromedial striatum, primarily the NAcc core and shell. Other limbic areas receiving large inputs from VTA are the PFC, the amygdala, and the lateral hypothalamus (Ungerstedt, 1971). The hippocampus, entorhinal cortex, and lateral septal area receive smaller projections (Beckstead et al., 1979; Swanson, 1982). Studies using different retrograde markers injected into pairs of VTA

forebrain target sites demonstrate a relatively small percentage of double labeled neurons (Margolis et al., 2006), indicating that each target receives input from a distinct group of VTA neurons. Although attention has focused on dopaminergic neurons, Swanson (1982) first demonstrated that VTA projections to different targets consist of variable proportions of dopaminergic neurons. The projection to the NAcc is richest in dopamine neurons (65%–85% dopaminergic), followed by those to the lateral septal area (72%), amygdala (53%), entorhinal cortex (46%), PFC (30%–40%), and hippocampus (6%–18%) (Margolis et al., 2006; Swanson, 1982).

1.3.3 Dopaminergic mesolimbic system

Although motivation and addiction are influenced by several neurobiological pathways, the dopaminergic mesolimbic circuit seems to play predominant role (Fig. 4). Thus, the knowledge of the distribution of dopamine receptors on these circuits is of great importance. It is known that dopamine receptors have a well-defined distribution in the mesolimbic system. They are divided into two major receptor subfamilies, the D1 subfamily (D1 and D5 subtypes) and the D2 subfamily (D2, D3, and D4 subtypes) (hereafter referred to as D1 and D2 receptors) (Sibley and Monsma, 1992). In dorsal striatum, anatomical studies have shown that striatonigral medium spiny neurons contain high levels of D1 receptors (together with substance P and dynorphin), whereas striatopallidal, medium spine neurons predominantly express D2 receptors (together with enkephalin) (Gerfen and Young, 1988). The distribution of dopamine receptors in the projections from NAcc are more complex than in dorsal striatum (Zahm, 2000). Whereas D2 receptors and enkephalin are highly expressed in neurons projecting from the NAcc to the ventral pallidum, D1 receptors and substance P are found equally distributed in projections to ventral pallidum and VTA (Lu et al., 1998). Studies of agonists and antagonists selective for D1 or D2 receptors showed that both are required for psychostimulant-dependent behavioral changes (Caine et al., 1999; Koob et al., 1987). In fact, cocaine-taking and –seeking behaviors are strongly regulated by D1 and D2 classes of dopamine receptors. Systemic pretreatment with either D1 or D2

receptor agonists reduces cocaine self-administration in rats, whereas pretreatment with either D1 or D2 receptor antagonists increases intake when access to cocaine is relatively unrestricted (Caine et al., 1999; Koob et al., 1987), suggesting that both receptors provide inhibitory feedback regulation of cocaine intake during self-administration. Relapse to cocaine-seeking behavior is also strongly regulated by both D1 and D2 dopamine receptor classes, but here D1 and D2 receptors mediate differential effects. Selective stimulation of D2 receptors strongly induces, or reinstates, cocaine-seeking behavior after cocaine-seeking responses have been extinguished in withdrawal, whereas selective D1 receptor stimulation produces relatively weak reinstating effects and attenuates cocaine seeking elicited by cocaine-related environmental cues when agonists are administered systemically (Alleweireldt et al., 2002; Dias et al., 2004). In addition, pretreatment with D1 and D2 agonists produces opposite effects on cocaine's ability to reinstate this behavior, since D1 agonists attenuate and D2 agonists facilitate cocaine seeking induced by cocaine priming injections (Alleweireldt et al., 2003; Self et al., 1996). A similar D1/D2 dichotomy regulates cocaine seeking in non-human primates (Khroyan et al., 2000), and also may "respectively" suppress and stimulate craving responses in humans (Haney et al., 1998, 1999).

Together, these studies suggest that D2 receptors could play a major role in eliciting relapse to drug seeking when environmental stimuli such as drug-related cues or stress activate the mesolimbic dopamine system (Phillips et al., 2003), while D1 receptor tone may provide inhibitory regulation over drug seeking. In contrast to agonists, both D1 and D2 receptor antagonists block reinstatement of cocaine seeking in rats (Schenk and Gittings, 2003; Vorel et al., 2002), and indiscriminately block the reinstating effects of NAcc agonist administration (Bachtell et al., 2005), consistent with well-characterized enabling and synergistic interactions between D1 and D2 receptors on behavioral responses.

It has been suggested that, the transition from non-addicted to addicted states could reflect differential adaptations in the sensitivity of these D1 and D2 receptor-mediated responses. For example, sensitization in D2-mediated responses may trigger drug seeking, while tolerance in D1-mediated responses

may inhibit drug seeking elicited by cues or drug priming. Moreover, higher preferred levels of drug intake in addicted animals could reflect a compensatory response to reduced D1 receptor function. Furthermore, such differences could emerge or be exacerbated during withdrawal from drugs, since the propensity for drug seeking increases in a time dependent manner from early to late withdrawal times (Tran-Nguyen et al., 1998).

1.4 The neurobiology of addiction and the role of mesolimbic pathway

The conceptualization of drug addiction as a brain pathology has profound social reflections because it mitigates moral connotation, and thus, a drug abuser is not a ‘‘criminal’’ but simply a ‘‘patient’’ who needs treatment, irrespective of the causes that triggered the drug-taking behavior. Drug addiction is a chronically relapsing disorder that has been characterized by a compulsion to seek and take the drug, loss of control in limiting intake, and emergence of a negative emotional state (e.g., dysphoria, anxiety, irritability) that reflects a motivational withdrawal syndrome when access to the drug is prevented (defined as Substance Dependence by the Diagnostic and Statistical Manual of Mental Disorders [DSM] of the American Psychiatric Association; Koob and Le Moal, 1997).

Substantial evidence suggests that activation of the midbrain dopamine system has multiple roles to give incentive salience to stimuli in the environment (Robinson and Berridge, 1993) and to promote goal-directed behaviors (Le Moal and Simon, 1991). More recently, the hypothesis has been raised that the time course of dopamine signaling is a key factor, with the fastest time course predominantly having a preferential role in reward and valuation of predicted outcomes of behavior, whereas the steady activation of dopamine release has a preferential role in providing an enabling effect on specific behavior-related systems (Schultz, 2007). Work in the domain of the acute reinforcing effects of drugs of abuse supports this hypothesis in which the mesolimbic dopamine system is critical for the acute rewarding effects of psychostimulant drugs but has a less role in the enabling effect (Nestler, 2005).

The dopaminergic transmission in the mesolimbic circuit appears to be drastically reduced in its tonic activity when measured in animal models of drug addiction, and in the available human studies of addicted subjects. This "hypodopaminergic state" is viewed as one of the main causes that triggers drug-seeking, even after prolonged drug-free periods, perpetuating the vicious cycle. As a result of its reduced activity, the system becomes hyper-responsive to drugs of abuse, which confers a long-lasting vulnerability to the system. It is believed that decreased dopamine function in addicted subjects results in a decreased interest to non drug-related stimuli and increased sensitivity to the drug of choice. Targeting the dopamine system with pharmacological agents, not necessarily classic receptor-oriented drugs, aimed at restoring dopamine transmission may therefore reveal useful new avenues in the treatment of this socially debilitating brain pathology.

1.5 The interplay between stress/GCs and drug susceptibility - *The state of the art at the beginning of this thesis*

Exposure to either drugs of abuse or stress produces similar alterations in the electrophysiological state of neurons in the mesolimbic dopamine reward pathway in animals. Enhanced excitatory synaptic transmission, as evidenced by an increase in glutamate receptor activation, occurs in VTA dopamine neurons following exposure to either stress or drugs of abuse (Saal et al., 2003). Both stress and drugs of abuse also cause alterations in dendrites (Listen et al., 2006; Robinson et al., 1999). Rats subjected to chronic stress exhibit decreases in dendritic branching in the hippocampus (Sousa et al., 2000) and in the medial PFC (Bessa et al., 2009; Cerqueira et al., 2005; Listen et al., 2006) but no studies have been performed in the NAcc or VTA. Alterations in dendritic branching are also observed following exposure to addictive drugs, namely with an increase in branching occurring following exposure to cocaine and amphetamine, and a reduction in branching after exposure to morphine (Robinson and Kolb, 1999).

Most physiological stressors exert their effects on the HPA axis, the primary endocrine stress pathway. Dysfunction of HPA and the peripheral stress circuit contributes to various stress-related neuropsychiatric diseases, including

addiction. Similar to the mesolimbic dopamine pathway, the HPA axis is activated in rodents and nonhuman primates following acute administration of many addictive substances — including cocaine, amphetamine, ethanol, opiates, and nicotine — and causes increased ACTH and corticosterone levels in plasma. Chronic administration of drugs of abuse in the same animal models results in either a sustained increase in HPA axis function, in the case of cocaine and amphetamine, or a reduced effect of the initial activating effects of the drug, in the case of morphine, nicotine, and alcohol (Borowsky and Kuhn, 1991; Ignar and Kuhn, 1991). Human studies demonstrate similar perturbations following illicit drug use even though with slight differences. As in animal models, acute administration of cocaine, alcohol, and nicotine, increases cortisol levels, whereas acute exposure to opiates decreases it (Allolio et al., 1987). Activation of the HPA axis is maintained in cocaine addicts, whereas following chronic opiate use, HPA responses are reduced over time a more typical response to repeated exposure to a stressor (Kant et al., 1985). However, it is unclear whether the irregularities observed in the HPA axis following drug administration indicate a vulnerability to addiction or are the result of prolonged drug exposure.

1.6 Aims

Considering the deleterious impact that elevated GCs during pregnancy have in the central nervous system, we decided to evaluate the long-term consequences of the administration of DEX at gestational day 18-19, with a special focus in the mesolimbic circuit and its related behaviors.

The research project herein presented intends to:

1. Evaluate the potential impact of antenatal (E18-E19) synthetic GC (DEX) administration in the dopaminergic mesolimbic pathway at system, cellular and molecular levels;

2. Correlate neurobiological alterations in the mesolimbic system triggered by prenatal DEX exposure with the development of addictive-like behaviours.

3. Characterize the response of the mesolimbic system in a basal state and after drug-exposure.

4. Search for a pharmacological modulation of dopaminergic circuits as a potential intervention for addictive behavior.

Chapter 2

Research Project: Technical considerations

2.1 Technical considerations

2.1.1 Animal model

Given the widespread use of synthetic corticosteroids, it is of extreme importance to analyze the molecular, endocrine, morphological and behavioural consequences of using such drugs. To do so, we decided to create a model in which we administered dexamethasone (DEX, 0.1mg/kg) or vehicle (sesame oil) at day 18 and 19 of pregnancy in Wistar rats. At this point, some methodological considerations should be made. First, we administered DEX instead of corticosterone because i) it is more commonly used in the clinical practice, ii) unlike corticosterone, DEX crosses the placental barrier because it is not metabolized by 11ß-HSD2 and passes to the fetus, iii) DEX is 25 times more potent than endogenous corticosteroids and has long-term biological effects (Goodman, 2005; White et al., 1997). Second, although it might seem that the period of treatment (G18-19) is equivalent to the last trimester of pregnancy in humans, this might not be exactly true given the low maturation of newborn rats, which continue to develop during the first week of life.

2.1.2 Behavioural analysis

Even though synthetic GCs can trigger effects in all organs, the developing brain is highly sensitive to the deleterious effects of these drugs, and GC-induced changes in specific brain regions are usually translated in an abnormal behavior later in life. Stress alters the sensitivity to drugs of abuse and is, therefore, considered an important contributory factor to the development of drug-seeking behavior and addiction (Sinha, 2001, 2008).

In this work, we were particularly interested in the programming effects of DEX in the mesolimbic circuit and how this was correlated with susceptibility to addictive-like behavior. Thus, in order to address this question, we used three different behavioral tasks, the open field (OF), the conditioned place preference (CPP) and ethanol consumption in a two-bottle paradigm (EC).

The OF test is pervasively used to assess locomotor activity, exploratory behavior and anxiety. Furthermore, it is known that there is a positive correlation between the locomotory response to novelty and the susceptibility to drug addiction (Piazza et al., 1991b). High responder animals, i.e., those who have

higher activity scores will develop drug self-administration while low responders usually do not (Piazza et al., 1991b). Therefore, besides analyzing these animals in a basal situation, where only saline solution was injected, we evaluated the influence of subcutaneous morphine administration at a dosage of 10 mg/kg in the locomotory activity of DEX-treated animals.

CPP is a commonly used test to evaluate the preference for an environment that has been associated with a positive or negative reward. In general, this procedure involves several trials where the animal is presented with the positive stimulus (e.g. food or a drug of abuse) paired with placement in a distinct environment containing various cues (e.g. tactile, visual, and/or olfactory). When later tested in the normal state, approaches and the amount of time spent in the compartments previously associated with the positive stimulus serves as an indicator of preference and a measure of reward learning. We used 10mg/kg of morphine to promote preference of the animals and to avoid aversion as it might occur with higher other dosages of morphine (Stohr et al., 1999).

Besides analyzing these animals in a non-contingent paradigm, we used the two-bottle choice protocol as described previously (Belknap et al., 1993; Blednov et al., 2001), where animals may choose between a 5% sucrose or 10% ethanol solution. Given that this test is not experimenter-dependent, it gives us an indirect measurement of the additive-like behavior of these animals.

Because GCs administration may lead to increased anxiety in the dams and thus, affecting their maternal behavior , we decided to evaluate these parameters in DEX-treated mothers. Regarding the maternal behavior, records were made every two days during 30min per mother until weaning. Pup-directed behavior (nursing, nonnutritive contact, licking and nets building) and self-directed behavior (self-grooming, resting, vertical activity and carrying) data was collected as previously described. However, since our understanding of rat maternal behavior may be biased by the available tools, and the fact that we might be losing important information regarding mother-pups interaction, the best way to completely exclude a maternal contribution for the development of the observed phenotype was to perform a cross-fostering experiment. For cross-fostering experiments, similar litters were exchanged between DEX and control

mothers at postnatal day 1 and progeny behavior was assessed in adulthood as previously described (Lavi-Avnon et al., 2005).

2.1.3 Structural analysis

Given the importance of the mesolimbic circuit for the development of addictive-like behaviors, we did an extensive characterization of the cell volumes and cell proliferation of NAcc and VTA, and neuronal morphology of these brain regions.

In order to investigate alterations in the general structure of NAcc and VTA, we used stereological tools. These tools refer to the group of techniques used to accurately estimate quantifiable characteristics of 3D objects, as volumes or number of cells and fibers. The use of these tools allows a precise and unbiased estimate of the numeric value of a parameter without actually having to measure it. This is done by taking, according to precise rules, only a systematic and random sample of the object, which is then measured and used to estimate the results of the whole (Gundersen et al., 1999).

For volume estimation, a grid of equally spaced points is superimposed to a sample of serial and equally spaced sections comprising the region of interest. The number of points falling inside the contour of the region of interest is counted and the result used according to the Cavalieri principle (Gundersen et al., 1988).

The optical fractionator (Gunderson et al., 1988; West et al., 1991) was employed to count cells in the studies presented in Chapter 3. Briefly, in this technique, a grid of virtual 3D-boxes, equally spaced sections, is superimposed within the region of interest, on a sample of serial and equally spaced sections and cells are counted whenever their nucleus came into focus. Besides its application to sections stained with common methods (Giemsa, hematoxylin-eosin), the optical fractionator method can also be used in immunocytochemically-stained sections, which allows, for instance, quantification of specific neuronal types, such a dopaminergic cells (TH-positive) or proliferating cells (Brdu-positive).

Stereological determinations of cell numbers and the volumes of a region of the brain provide an important perspective of its general structure. However,

we also wanted to examine the fine structural changes induced by pre-natal DEX in neurons of the NAcc and VTA. In order to achieve this goal, we analyzed 3D reconstructions of whole neurons stained by the Golgi-Cox method. This is one of several heavy metal impregnating methods (in this case mercury) derived from the original silver impregnation. The Golgi technique stains complete neurons, including its terminal dendritic branches and even spines; importantly, only a few (approximately 2-4%) are stained with this technique.

2.1.4. Neurochemical Determinations

Altered dopaminergic transmission may be the underlying cause of addiction and drug-seeking behaviors. In fact, while in the very first phases of acquisition, a hyperdopaminergic status seems to occur, in an addicted brain an hypodopaminergic status seems to exist (Melis et al., 2005). Thus, it is of extreme importance to determine dopamine levels in mesolimbic brain regions, although we cannot exclude the contribution of other neurotransmitters for the development of addiction. The levels of dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC), 4-hydroxy-3-methoxuphenylacetic acid (homovanillic acid, HVA), serotonin, HIAA, epinephrine and norepinephrine were assayed by high performance liquid chromatography combined with electrochemical detection (HPLC/EC) using a Glison instrument.

2.1.5 Molecular analysis

Early life stress can affect the dopaminergic circuits in specific brain regions by several ways, either by changing i) the neuronal cell fate, ii) neuronal morphology and functioning, iii) the production or turnover of DA or iv) the expression of DA receptors. Considering this, besides the structural analysis and neurochemical determinations, it was essential to measure dopamine receptors levels in the NAcc and VTA areas. We quantified mRNA levels of dopamine receptors using standard real-time PCR method using HPRT as housekeeping gene. Protein levels were determined using western blot technique with commercial antibodies against dopamine receptors and using alpha-tubulin as loading control. It is important to mention that several isoforms of dopamine receptor 1 and 2 exist (David et al., 1993; Sidhu, 1990); although no systematic

study has proven the biological relevance of such findings and which isoform is biologically active, we decided to quantify all isoforms identified so far.

It has been demonstrated that early life adversity can change the epigenetic status of several genes such as the GR and AVP and affect their expression in specific brain regions leading to altered behavior (Murgatroyd et al., 2009; Weaver et al., 2004). The long-term changes in Drd2 expression in DEX animals raised the hypothesis that this gene could also undergo epigenetic regulation. In fact, this theory seems plausible since in humans it was shown that Drd2 promoter region is hypermethylated in the blood cells of anorexia nervosa patients (Frieling et al., 2009). Using bioinformatic tools, we analyzed the promoter region of Drd2 gene and found a conserved CpG island in mouse, rat and human. DNA from the NAcc of control and DEX animals was isolated and converted using a standard bisulfite conversion kit. After this, ten clones per animal were sequenced and the levels of methylated cytosines were calculated. We analyzed the animals in a basal situation and after drug exposure (morphine plus ethanol) in order to assess if drugs of abuse can alter the epigenetic status of this gene.

2.1.6 Treatments

In an attempt to restore the dopamine levels in the NAcc, we decided to administer L-dopa to the animals. L-dopa is the natural form of dihydroxyphenylalanine and the immediate precursor of dopamine; it is widely used to treat Parkinson´s disease patients. L-dopa may be administered peripherally as it is known that it crosses the blood-brain barrier; is quickly picked up by dopaminergic neurons and converted to dopamine. We used a combination of L-dopa/carbidopa, as carbidopa inhibits L-dopa degradation in the bloodstream.

L-dopa/Carbidopa tablets (Sinemet, Merck) were crushed and dissolved in water. The solution was administered daily at a dose of 36.0/9.0 mg/kg L-dopa/carbidopa by oral gavage, starting three weeks before any type of evaluation. This concentration has been shown to have effects in the behavior of wistar rats but minor side effects such as dyskinesias (which are observed above 50mg/kg of L-dopa) (Lindner et al., 1997). L-dopa/carbidopa was administered

during all the behavioral procedures but with 8h interval before the last administration and the beginning of the test so it would not directly interfere with the test.

Chapter 3

Experimental Work

Chapter 3.1

"Programming effects of antenatal dexamethasone in developing mesolimbic pathway"

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Synapse (2007)

Programming Effects of Antenatal Dexamethasone in the Developing Mesolimbic Pathways

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KEY WORDS nucleus accumbens; ventral tegmental area; neurogenesis; dopamine (DA); stereology

ABSTRACT Elevated glucocorticoids, during pregnancy, alter emotionality and increase propensity to drug abuse later in life, albeit through substrates and mechanisms are largely unknown. In this study, we examined whether antenatal glucocorticoid exposure induces enduring structural changes in the nucleus accumbens (NAcc), an important relay point in the reward limbic circuitry. To this end, rat dams were exposed to the synthetic glucocorticoid dexamethasone (DEX) on days 18 and 19 of gestation, and stereological tools were used to assess the total volume of, and neuronal numbers in, the NAcc, as well as the density of mesencephalic dopaminergic inputs from the ventral tegmental area (VTA) to the NAcc in their adult offspring. Further, we used measures of bromodeoxyuridine incorporation into NAcc cells to examine whether DEX-induced effects on cell proliferation represent another mechanism through which glucocorticoids alter the structure of mesolimbic pathways and might influence addictive behavior. Our studies show that exposure to DEX during late gestation results in significantly reduced volumes and cell numbers in the NAcc. The latter measure correlated strongly with a reduced rate of cell proliferation in DEX-exposed animals. Moreover, the treatment resulted in a decreased number of cells expressing tyrosine hydroxylase in the VTA and an impoverished dopaminergic innervation of the NAcc. These observations, which identify glucocorticoid-sensitive structures and neurochemical targets within the developing ''reward pathway,'' pave way for future studies designed to understand how early life events can predispose individuals for developing drug dependence in adolescent and adult life. **Synapse 61:40–49, 2007.**

INTRODUCTION

The fetus is highly sensitive to perturbations of its chemical environment during critical developmental windows. However, the fetal brain is not only sensitive to teratogenic agents; for example, exposure to high levels of adrenal corticosteroids (CSs) can set long-term programs in behavior by altering brain chemistry and morphology. As we recently demonstrated in rats, pharmacologically induced hypercorticalism during late pregnancy leads to increased emotionality, a feature which persists throughout life (Oliveira et al., 2006). Moreover, exposure to excess levels of CSs during late fetal stages are thought to lead to increased susceptibility to a variety of neurological and psychiatric disorders during childhood and adult life (Boska and El-Khodor, 2003; Lewis and Levitt, 2002; Weinstock et al., 1988), including increased susceptibility to drug abuse (Meaney et al., 2002; Thadani et al., 2004). The latter is thought to be associated to disturbances in either emotional or mood states, or both (Regier et al., 1990).

The nucleus accumbens (NAcc) is a heterogeneous structure belonging to the ventral division of the striatum. Histological and neurochemical studies reveal two anatomically distinct regions: a core and a shell (Zahm and Brog, 1992). Both core and shell

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receive inputs from the amygdala, globus pallidus, and ventral pallidum. However, they differ in the density of their cortical afferents; the core receives projections predominantly from the prelimbic, anterior cingulate, and dorsal agranular insular cortex, whereas the shell receives projections predominantly from the infralimbic, ventral agranular insular, and piriform cortex (Zahm, 2000). Furthermore, and perhaps more importantly, these divisions innervate distinct areas; the core projects to the conventional basal ganglia circuitry including the ventral pallidum, globus pallidus, and the substantia nigra, whereas the shell projects to subcortical limbic structures such as the lateral hypothalamus and the ventral tegmental area (Zahm and Brog, 1992). These patterns of connectivities indicate that the two NAcc divisions may be involved in different aspects of the motivation-reward processes (Ikemoto and Panksepp, 1999) and the timing of the initiation of response patterns originating in the frontal corticostriatal loop systems (Groenewegen et al., 1999; Kelley et al., 2005).

The NAcc receives major dopaminergic inputs from the VTA. Importantly, the central dopaminergic systems, especially those in the NAcc that are crucial to the regulation of reward behavior, appear to be particularly vulnerable to perinatal insults. For example, maternal stress in late gestation (Berger et al., 2002; Diaz et al., 1998; Henry et al., 1995) or repeated periods of maternal separation during early postnatal development (Meaney et al., 2002) alter the pattern of DA receptor expression and decrease the dopamine transporter availability in the NAcc, later in life. Interestingly, Meaney and collaborators (2002) showed that postnatal maternal separation produces increased behavioral responses to stress and cocaine. In addition, there is evidence that prenatal and early postnatal stress results in decreased DA drive to the medial prefrontal cortex (Brake et al., 2000), a feature commonly associated with psychiatric disorders thought to have a neurodevelopmental basis (Lewis and Levitt, 2002).

The mechanisms through which stress alters NAcc function have not been fully investigated. On the premise that elevation of glucocorticoids is one of the most overt physiological manifestations of stress and that the brain is more sensitive to organization by glucocorticoids during early development, this initial study evaluated the potential impact of antenatal glucocorticoid administration upon NAcc structure and its dopaminergic innervation. Our results show that exposure of the fetus to dexamethasone (DEX) during late gestation leads to a reduction in both the volume and the number of cells in the $NAcc_{core}$ and $NAcc_{shell}$ of males, and volumes and number of neurons in the $NAcc_{shell}$ in females. These changes could be partly attributed to antenatal DEX-induced inhibition of cell proliferation in NAcc. Further, we report an association between fetal exposure to DEX and reduced numbers of tyrosine hydroxylase (TH)-positive cells in the adult VTA, paralleled by a markedly reduced density of dopaminergic inputs to the NAcc.

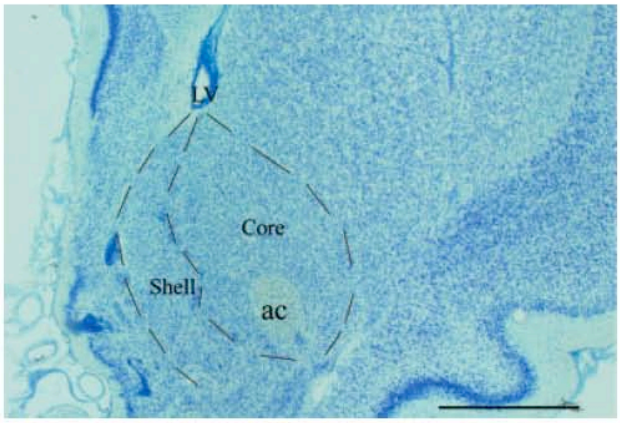

Fig. 1. Photomicrographs of Giemsa-stained glycolmethacrylate-embedded coronal sections of the NAcc shell and core divisions used for the cell counting with the SteroInvestigator software. LV, lateral ventricle; ac, anterior commissure. Scale bar = 200 μm.

MATERIALS AND METHODS

Animals and treatments

Adult pregnant Wistar rats (Charles River Laboratories, Barcelona, Spain) were individually housed under standard laboratory conditions (light/dark cycle of 12/12 h with lights on at 08:00; 22°C); food and water were provided ad libitum. Subcutaneous injections of dexamethasone (DEX, 0.1 mg/kg; $n = 4$) or saline (CONT, 1 ml/kg; $n = 4$) were administered on E18 and E19 days of pregnancy. Other groups of CONT- and DEX-treated mothers ($n = 3$) were given a single injection of bromodeoxyuridine (BrdU, 50 mg/kg, i.p.) on E18/19, to birthmark neurons in the offspring. On postnatal day 21, progeny were separated according to antenatal treatment and gender. All manipulations were done in accordance with local regulations (European Union Directive 86/609/EEC) and NIH guidelines on animal care and experimentation.

Tissue preparation

Groups of male and female rats born to mothers exposed to either saline or DEX (derived from four different litters) on E18 and E19 of pregnancy were deeply anesthetized and transcardially perfused with 4% paraformaldehyde (PFA) when 60 days old. Brains were removed and the cerebral hemispheres were separated by a longitudinal cut in the midsagittal plane. Blocks containing the NAcc were washed in tap water and dehydrated through a graded series of ethanol solutions, before being embedded in glycolmethacrylate (Tecnovit 7100, Heraeus Kulzer, Wehrheim, Germany). Microtome sections (30 μm) were placed on slides

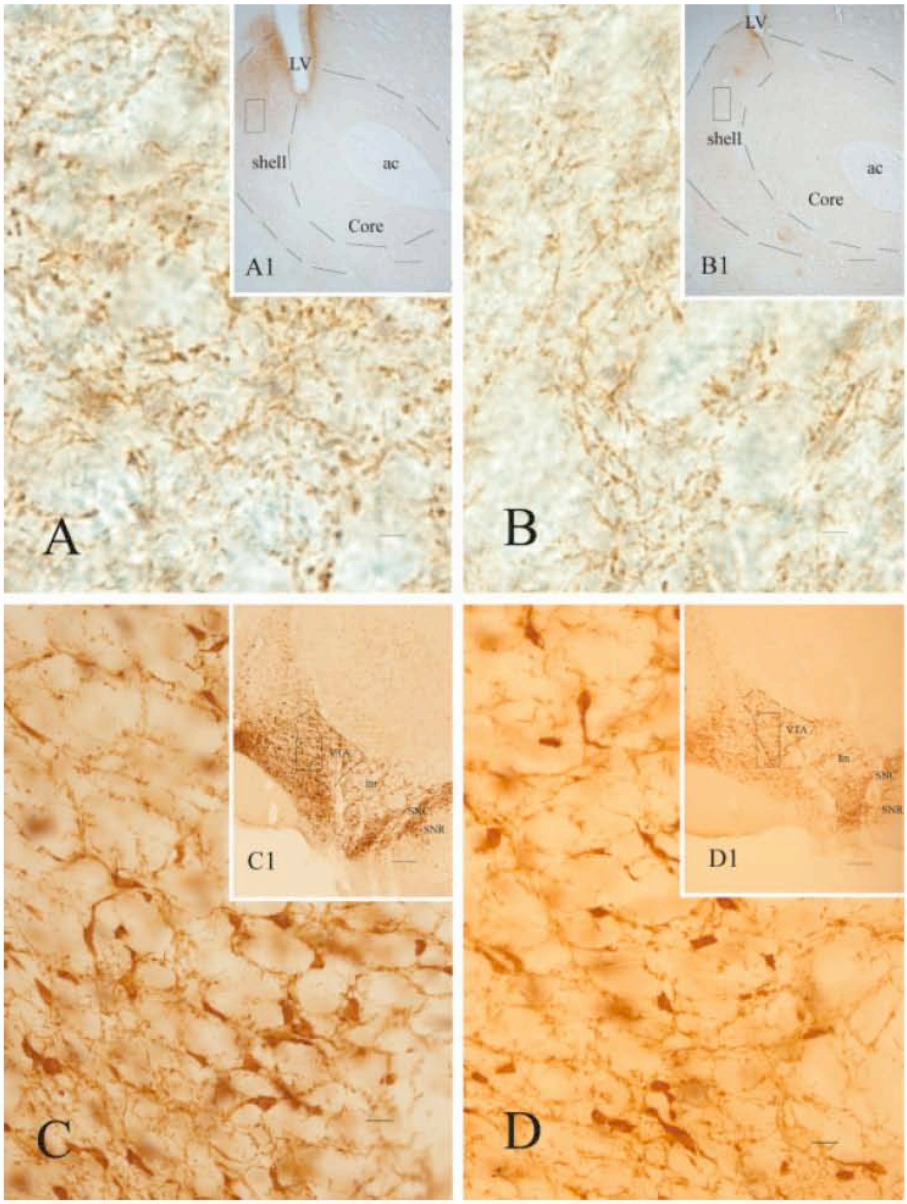

Fig. 2. Representative light microscopic photomicrographs of TH-positive immunocytochemistry in NAcc and VTA. Illustration of dopaminergic innervation of NAcc in control- (**A**) and DEX-treated (**B**) animals. Magnification of control- (**A1**) and DEX-treated (**B1**). Boxes represent the areas illustrated in (**A**) and (**B**), respectively. TH immunocytochemistry in control (**C,C1**) and DEX-treated (**D, D1**) in the VTA. LV, lateral ventricle; ac, anterior commissure. (A–D) – Scale bar = 200 μm and (A1–D1) – Scale bar = 5 μm.

coated with Entellan-new, before staining with Giemsa stain. The outline of the NAcc, including its core and shell areas (Zahm and Brog, 1992), was defined in each section using established landmarks (Fig. 1).

Another subset of male rats (derived from four different litters) was prepared for TH immunocytochemistry. Every 8th (30-μm thick) section, which included the NAcc and VTA, was treated with 3% H_2O_2 in PBS to eliminate endogenous peroxidase activity and blocked with 4% bovine serum albumin (BSA, Sigma) in PBS. Sections were then incubated overnight at 4°C in rabbit anti-TH serum (1:2000; Affinity Reagents, Exeter, UK), in blocking solution. Antigen visualization was carried out by sequentially incubating with biotinylated goat antirabbit antibody, ABC® (Vector, Burlingame, CA), and diaminobenzidine (DAB, Sigma) (Fig. 2).

Serial coronal cryosections, covering the entire length of the NAcc and VTA, were prepared from 3-day old pups (derived from three different litters) whose mothers had been treated with BrdU (Fig. 3). Every 2nd section from this series was mounted on poly-L-lysine-coated slides and stained for BrdU after fixation in 4% PFA (30 min), permeabilization (0.2% Triton X-100 in Tris buffer saline, 10 min), microwave treatment for antigen retrieval (20 min in 0.1 M citrate buffer), acidification with 2 M HCl, peroxidase and nonspecific blocking (3% H_2O_2 and 4% BSA, respectively). The BrdU antibody was a mouse monoclonal from Dako,

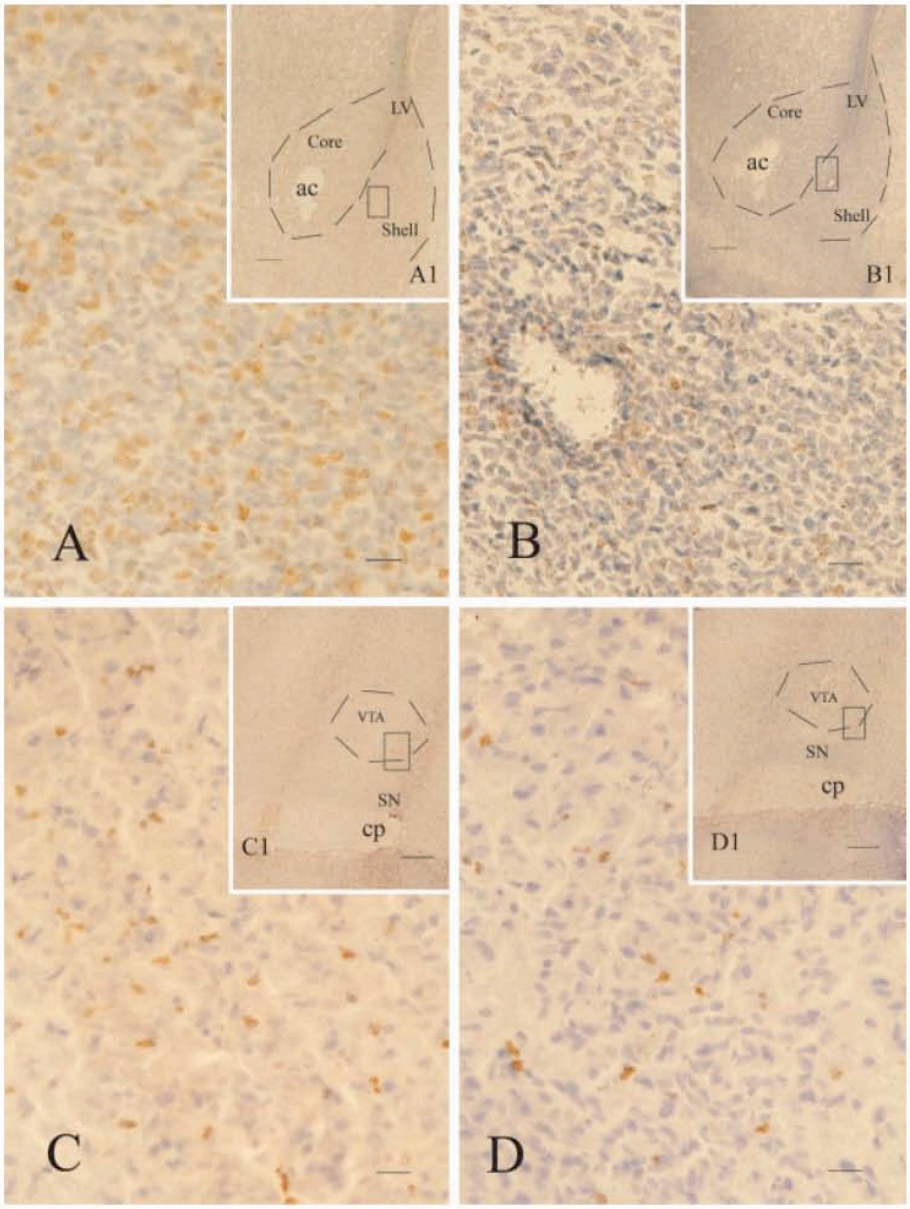

Fig. 3. Photomicrographs demonstrating BrdU immunoreactive cells in NAcc of controls (**A,A1**), VTA control (**C,C1**), DEX-treated (**B,B1**), and VTA DEX-treated (**D,D1**) in rats of PND3. (A–D) – Scale bar = 200 μm; (A1–D1) – Scale bar = 5 μm

(Glostrup, Denmark) (1:50). Antigen visualization was carried out using a universal detection system (BioGenex, San Ramon, CA) and DAB. Specimens were lightly counterstained with hematoxylin.

Stereology

Estimates of $NAcc_{core}$ and $NAcc_{shell}$ region volumes and cell numbers were obtained using StereoInvestigator® software (MicroBrightfield, VT) and a camera (DXC-390, Sony, Japan) attached to a motorized microscope (Axioplan 2, Carl Zeiss, Germany). Cavalieri's principle was used to assess the volume of each region. Every 8th section was used, and the cross-sectional area was estimated by point counting (final magnification ×112). We used a test point system in which the interpoint distance, at the tissue level, was 75 μm. The volume of the region of interest (ROI) was then calculated from the number of points that fell within its boundaries and the distance between the systematically sampled sections. Average cell numbers were estimated using the optical fractionator method as described elsewhere (West et al., 1991). Briefly, a grid of virtual 3D-boxes (30 μm × 30 μm × 15 μm) was superimposed on every 8th section of the ROI, spaced as for the volumetric estimation. An estimate of total number of cells was then derived from the number of cells falling inside the boxes, the volume of each box,

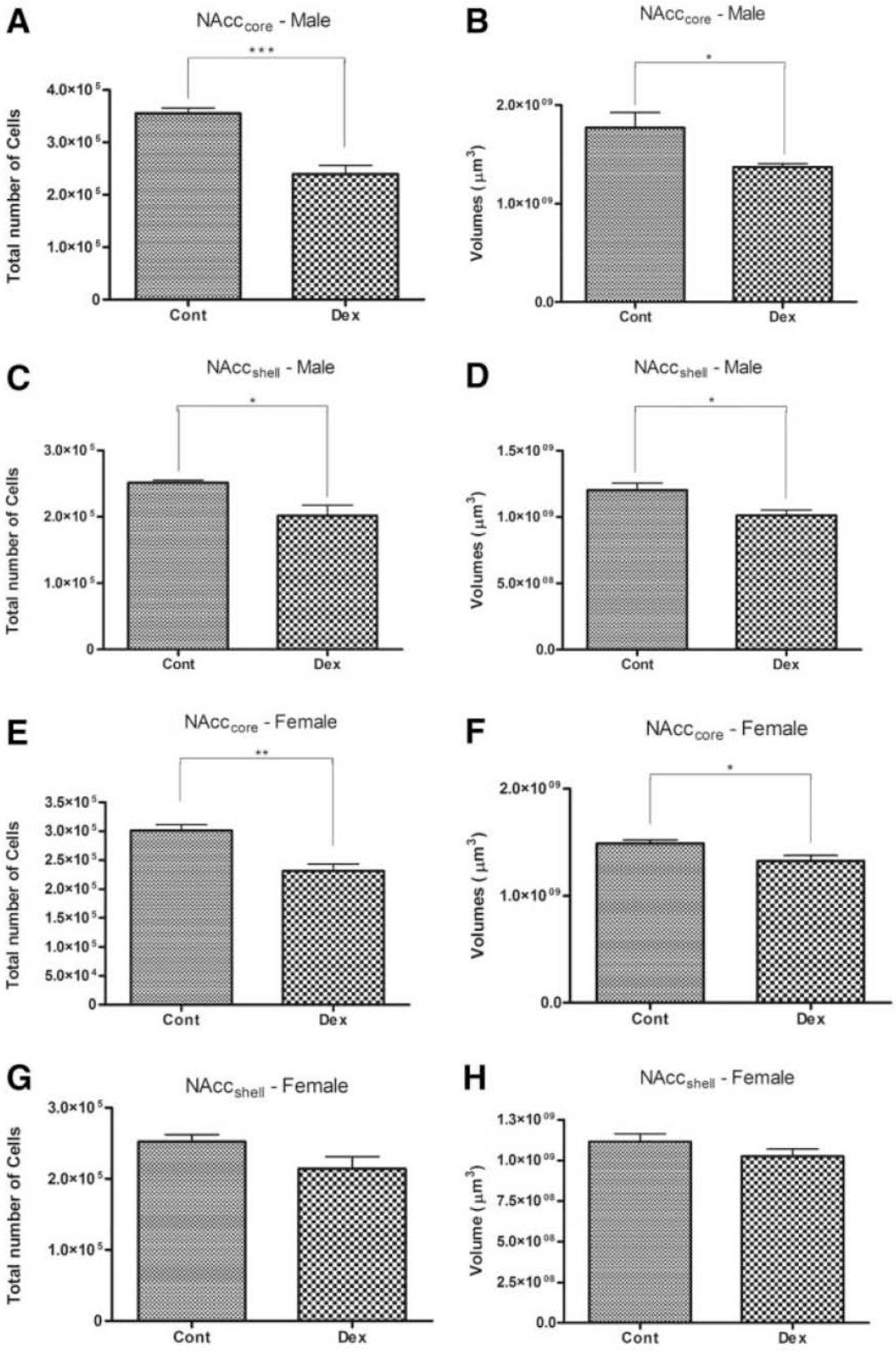

Fig. 4. Stereological data. Average volumes of NAcc regions in males (**A,C**) and females (**E,G**). Total number of neurons in NAcc divisons in males (**B,D**) and females (**F,H**). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

box spacing, and total number of boxes. Coefficients of error were automatically computed according to the formulas of Gundersen et al. (1999) for cell numbers and Gundersen et al. (1987) for volume estimations. Glial cells were not included in the estimations, and the discrimination between neuronal and glial cell body profiles was based on the criteria described by Ling et al. (1973) and Peinado et al. (1997).

The density of TH-positive fibers impinging upon the NAcc was estimated using a "staggered" cycloid test system (Baddeley et al., 1986; Emre et al., 1993; Issacs et al., 1993). The total number of intersections of the cycloid arcs with the stained fibers was obtained on randomly selected sections containing the core and shell regions. In addition, the number of TH-positive (VTA) and of BrdU-positive (NAcc and VTA) cells per unit area was estimated using StereoInvestigator software. To complement these measurements, a densitometric analysis of TH immunoreactivity in the NAcc (core and shell) was performed using NIH Image 1.52 software; the unit sampling area for these measurements was 1.0 mm^2, and measurements were made in three randomly-selected regions within core and shell of the NAcc.

Statistical analysis

Differences between groups were determined by a nonparametric procedure (Mann–Whitney) that does not assume normality or equal variance. For statistical analysis, the "n" of each experiment group is the number of litters from which individuals were derived. The results are expressed as group means ± standard error. Differences were considered to be statistically significant when $P < 0.05$.

RESULTS

Sustained effects of antenatal DEX on NAcc cytoarchitecture

The male progeny of DEX-treated dams displayed reduced volume ($P = 0.03$) and cell number ($P = 0.001$) in the $NAcc_{core}$ (Figs. 4A and 4B). $NAcc_{core}$ male controls: total number of cells = 352,000 (SEM = 8155, $n = 4$), volume = 1.75 mm^3 (SEM = 0.13, $n = 4$); male DEX: total number of cells = 235,600 (SEM = 19,050, $n = 4$), volume = 1.35 mm^3 (SEM = 0.03, $n = 4$). Similar observations, albeit of smaller magnitude, were made in their female counterparts (volume: $P = 0.001$; cell number: $P = 0.004$; Figs. 4E and 4F). $NAcc_{core}$ female controls: total number of cells = 306,400 (SEM = 11,720, $n = 4$), volume = 1.49 mm^3 (SEM = 0.03, $n = 4$); female DEX: total number of cells = 231,500 (SEM = 12.160, $n = 4$), volume = 1.27 mm^3 (SEM = 0.048, $n = 4$).

Prenatal DEX also significantly reduced the volume ($P = 0.04$) and number of cells ($P = 0.02$) in the $NAcc_{shell}$ of males (Figs. 4C and 4D). $NAcc_{shell}$ male controls: total number of cells = 252,000 (SEM = 4121, $n = 4$), volume = 0.12 mm^3 (SEM = 0.055, $n = 4$); male DEX: total number of cells = 199,600 (SEM = 17,290, $n = 4$), volume = 0.9 mm^3 (SEM = 0.03, $n = 4$). In contrast, there was no significant effect of antenatal DEX treatment in the shell region of the NAcc in females (volume: $P = 0.7$; cell number: $P = 0.4$) (Figs. 4G and 4H). $NAcc_{shell}$ female controls: total number of cells = 234,700 (SEM = 1649, $n = 4$), volume = 1.1 mm^3 (SEM = 0.03, $n = 4$); female

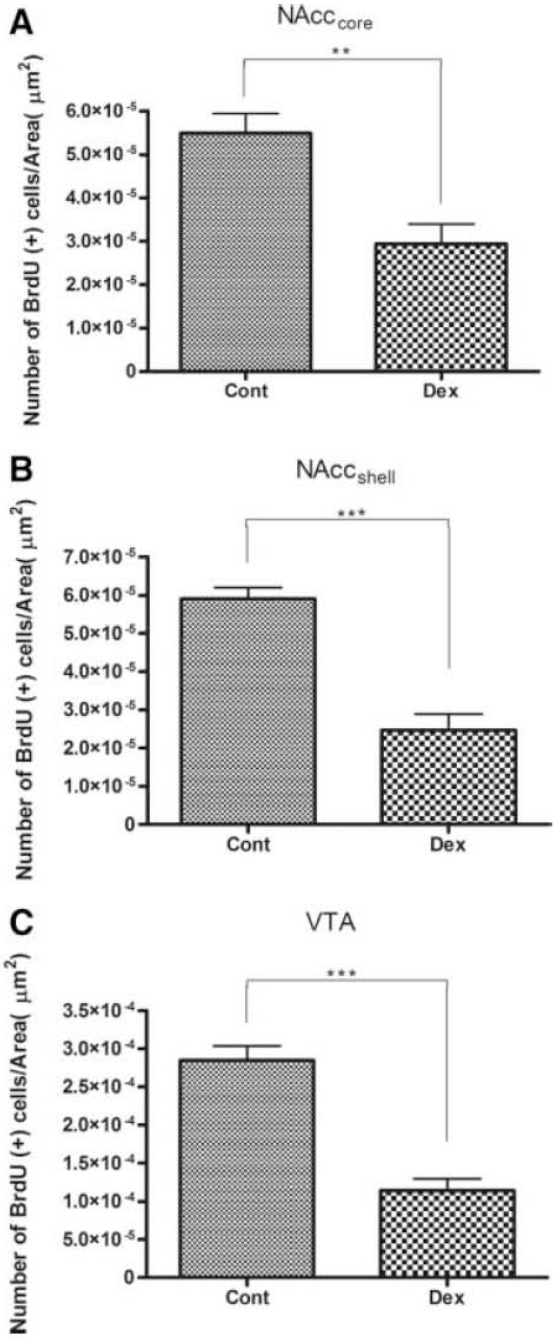

Fig. 5. Number of BrdU-positive cells in NAcc regions (**A,B**) and VTAs (**C**) in PND3. $^{**}P < 0.01$; $^{***}P < 0.001$.

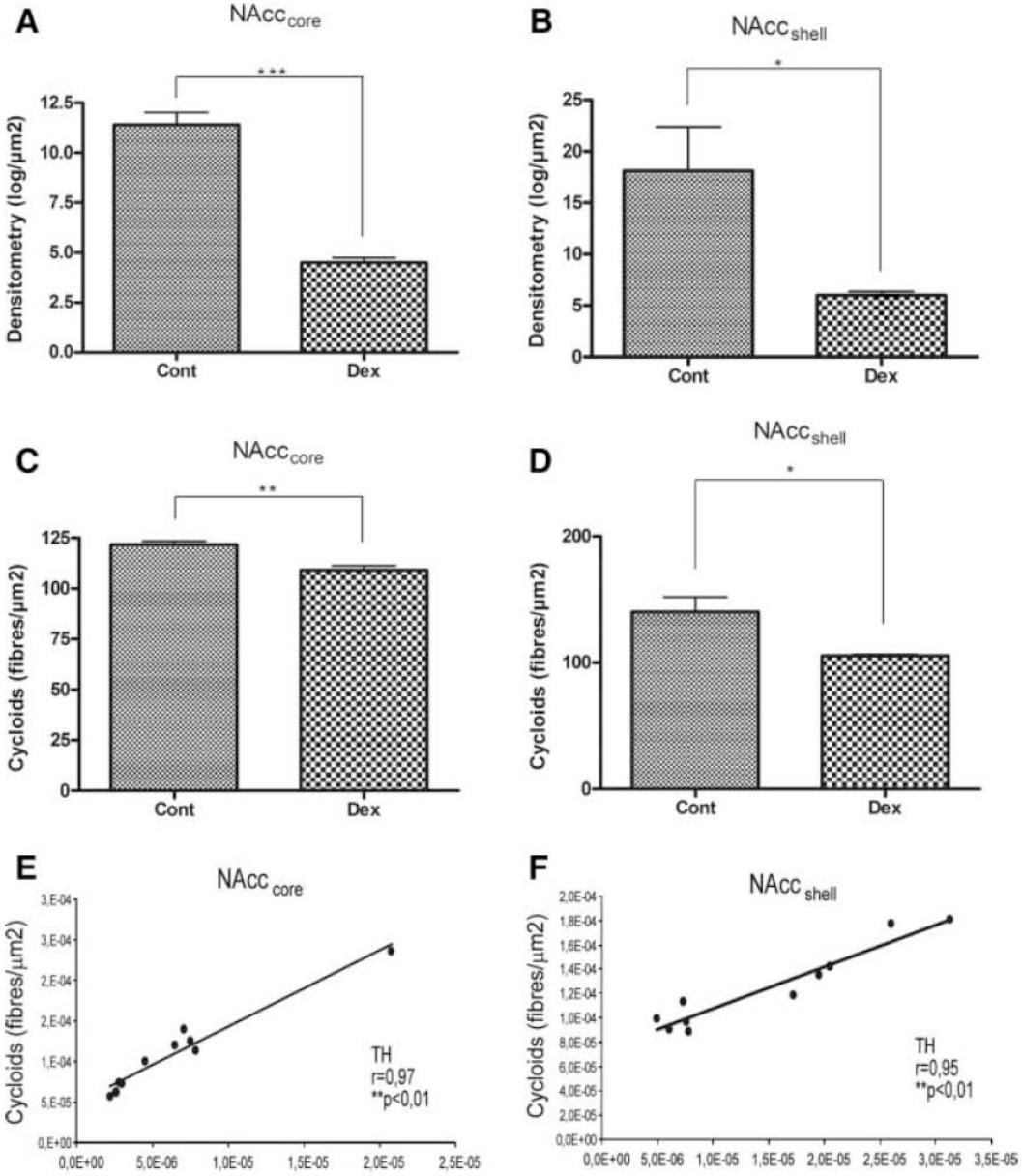

Fig. 6. Decreased TH content in the NAcc divisions of the progeny, after treatment with prenatal DEX. Densitometric (**A,B**) and stereological (**C,D**) data in core and shell, respectively. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Pearson correlation of both densitometric and stereologic quantifications of TH immunoreactive fibers in NAcc core (**E**) and shell (**F**).

DEX: total number of cells = 214,200 (SEM = 16,780, $n = 4$), volume = 1.02 mm^3 (SEM = 0.04, $n = 4$).

Inhibition of cell proliferation in the NAcc and VTA

The total number of cells in a given tissue is determined by the dynamic balance in cell proliferation and cell death (cell turnover). In view of the reduced number of cells in the NAcc core and shell of male progeny of DEX-treated mothers, as well as the known inhibitory effects of stress and glucocorticoids on neurogenesis, in this first study, we examined the possible contribution of altered cell proliferation on total NAcc cell numbers in male animals. As shown in Figures 5A and 5B, exposure to antenatal DEX resulted in a significant inhibition of BrdU incorporation (assessed at postnatal day 3) in the NAcc core ($P = 0.004$) and shell ($P = 0.0003$) regions, as well as in the VTA ($P = 0.0001$) (Fig. 5C).

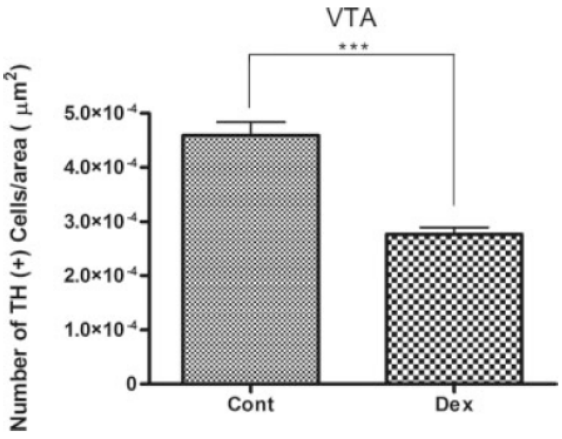

Fig. 7. TH-positive cells per surface unit of the VTA. ***$P < 0.0001$.

Reduced dopaminergic innervation of the NAcc after prenatal exposure to DEX

Densitometric and stereological measurements in the two divisions of the NAcc revealed significantly reduced densities of TH-positive fibers in the progeny of DEX-treated dams (by densitometry: core, $P = 0.04$ (Fig. 6A), and shell, $P = 0.001$ (Fig. 6B); by stereology: core, $P = 0.03$, (Fig. 6C) and shell, $P = 0.003$ (Fig. 6D). The correlation coefficient (Pearson analysis) between the two methods of estimation for core was $r = 0.97$ and $P < 0.01$ (Fig. 6E); shell: $r = 0.95$ and $P < 0.01$ (Fig. 6F).

Supplementing the earlier findings, we observed that the number of TH-positive cells per surface unit of the VTA was significantly reduced in animals that had received DEX during prenatal life ($P = 0.0003$) (Fig. 7).

DISCUSSION

Stressful experience during uterine or early postnatal life is thought to predispose individuals to addictive habits, as well as to increased risk for developing mental disorders such as schizophrenia and major depression (Hougaard et al., 2005; McClure et al., 2004). Motivation and the ability to respond to rewarding stimuli are key elements in all of these conditions. Work over the last two decades has identified the mesolimbic "reward pathway," of which the NAcc and VTA are crucial components (Wise, 2004a,b). Importantly, the VTA projects dopaminergic terminals to the NAcc (Jonjen-Relo et al., 1994). Since the NAcc plays a significant role in the timing of the initiation of response patterns originating in the frontal corticostriatal loop systems (Groenewegen et al., 1999), its implication in drug addiction and anhedonia (Di Chiara, 2002; Salamone et al., 2003) is not surprising.

Increased CS secretion is a key feature of the response to stress. CSs are small, lipid-soluble molecules; these characteristics allow them easy access to the brain, where they elicit both rapid and long-lasting changes in neural activity and behavior. Numerous studies from our own and other laboratories have demonstrated that GC can induce structural changes in various brain regions including the prefrontal cortex (Brown et al., 2005; Cerqueira et al., 2005), hippocampus (Donohue et al., 2006; Sousa et al., 1999, 2000), and striatum (Copeland et al., 2005; Haynes et al., 2001).

Recently, McClure et al. (2004) reported a reduction in the volume and number of neurons in the NAcc of rats born to mothers that had received an injection of saline during late pregnancy; they putatively attributed these structural changes to injection stress-induced CS secretion. The results of the present study largely confirm the supposition by McClure et al. (2004). We show that the adult offspring of rat dams that had been exposed to the synthetic CS DEX during the late stage of pregnancy have significantly decreased $NAcc_{core}$ volumes and total neuronal numbers. Similar observations were made in the $NAcc_{shell}$ of the male, but not female, progeny of DEX-treated mothers. While the significance of this sex difference is not known at present, it is interesting to note that males show greater susceptibility to early life events (McClure et al., 2004; Simon and Volicer, 1976) and that, in humans and animals, the two sexes display differences in their propensity to develop addictive behavior, and mood and other affective disorders (Simon and Volicer, 1976). Further, the two sexes differ significantly in their basal CS secretory profiles and endocrine responses to stress (Patchev et al., 1997), as well as in their hormonal and behavioral responses to prenatal stress (Bowman et al., 2004). Our finding that CS influences the structure of the $NAcc_{shell}$ (but not $NAcc_{core}$) in a sex-specific fashion calls for the establishment of the relationship between sex, stress, and the regulation of specific components of the motivation-reward circuit. Current views hold that the $NAcc_{shell}$ is responsible for anticipatory responses (Chang et al., 1994), while the $NAcc_{core}$ participates in the timing of the initiation of response patterns originating in the frontal corticostriatal loop systems; it will be interesting to know whether these processes are differentially regulated between the sexes and to what extent their different susceptibilities to antenatal CS (and presumably stress) translates into sex differences in behavior.

The changes in NAcc volume and neuronal numbers in animals exposed to antenatal CS were accompanied by significant reductions in the dopaminergic innervation of the NAcc by the VTA (confirming a recent report by McArthur et al., 2005), as well as by reduced numbers of dopaminergic cell bodies in the VTA (not observed by McArthur et al., 2005). Interestingly, these

changes appear to result from antenatal DEX-induced alterations in cell proliferation: our results record, for the first time, that CS can significantly reduce postnatal neurogenesis in both, the VTA and NAcc. The inhibitory actions of CS on neuronal proliferation in the postnatal brain, especially in the hippocampus (Gould et al., 1991), have been extensively described, but their ability to interfere with neurogenesis during prenatal life have been recognized only recently (Lemaire et al., 2000). CS receptors are expressed in many areas of the rat brain, including the basal ganglia striatum and brainstem nuclei, from embryonic day 15.5 (Diaz et al., 1998); since the NAcc and VTA undergo considerable structural organization during late gestation (Hynes and Rosenthal, 1999), our antenatal CS paradigm on E18/19 is likely to have had a major impact on this process.

The NAcc seems to be one of the most important brain centers in determining drug-addiction behavior. When stimulated by DA, neurons in NAcc produce feelings of pleasure and satisfaction; in normal conditions, such response keeps the subject focused on basic biological goals. However, genetic as well as natural and pharmacological interventions can modulate dopaminergic drive upon the NAcc. The decrease in basal dopaminergic in the NAcc after prenatal DEX treatment (associated with an impoverishment structural complexity in this nucleus) may determine an increase susceptibility to drug-seeking behavior. Thus, the results of the present study lend support to the view that early exposure to stress or CS might "program" the mechanisms and neural substrates governing reward-seeking behavior throughout the lifespan (Marinelli and Piazza, 2002; Piazza et al., 1998).

Importantly, the implications of the present results are plausibly also relevant to our understanding of the biological basis of schizophrenia, since there are reports that cell losses contribute to volumetric reductions in the NAcc of schizophrenic patients (Pakkenberg, 1990). Moreover, CS released during stress might contribute to the neurodevelopmental anomalies that underlie schizophrenia (Boksa and El-Khodor, 2003); in support to this hypothesis, Koenig et al. (2005) have shown that two parameters used for the validation of animal models of schizophrenia—diminution of the prepulse inhibition of the acoustic startle response and disruption of auditory sensory gating—are modulated by prenatal stress. In addition, our findings that antenatal CS exposure leads to decreased DA input to the NAcc and reduced numbers of TH-positive cells in the adult VTA show that CS may be important determinants of mesolimbic function in the adult. Lastly, our observations that antenatal exposure to CS can have enduring effects on the cytoarchitecture and neurochemistry of the VTA and NAcc are important in the light of the common use of CS in the prevention of preterm birth in women.

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Chapter 3.2

"Dopamine reverts vulnerability to drug abuse"

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Dopamine reverts vulnerability to drug abuse

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Running title: Dopamine alters drug-seeking behavior

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Supplementary Material: 1 + 1 (Figures)

Abstract

Prescription of glucocorticoids, such as dexamethasone, is common practice during late gestation in pregnancies at risk of premature delivery, to promote lung development. This practice is expected to increase given the advices of the World Health Organization on maternity care. Recent studies have shown that early life events trigger programming effects on brain structure and function that alter susceptibility to disease later in life, including addictive behaviors. Herein, we show that adult offspring of dexamethasone (DEX)-injected dams display increased sensitivity to drug abuse in both contingent and non-contingent paradigms. This behavioral phenotype is associated with structural changes at the synaptic level and diminished levels of dopamine in the nucleus accumbens (NAcc). In addition, DEX-treated animals present an over-expression of the dopamine receptor 2 (Drd2) in a basal situation in this region of the mesolimbic reward circuit. Interestingly, after chronic morphine/alcohol exposure, DRD2 was significantly down-regulated and this was correlated with increased methylation of the promoter region of Drd2 in the NAcc. Importantly, administration of a dopaminergic agonist rescues this behavior phenotype. Altogether we unravel a mechanism that may underlie the increased vulnerability to addiction triggered by early life events but also its pharmacological modulation, thus opening new perspectives in the prevention and treatment of addictive disorders.

High levels of corticosteroids during critical developmental periods can set long-term programming effects in behavior by altering brain chemistry and morphology[1-3]. Recent studies demonstrated that administration of corticosteroids during late pregnancy in equipotent doses to those used for prevention of pre-term delivery, leads to increased emotionality throughout life[4]. These findings might underpin the reported increased susceptibility to a variety of neurological and psychiatric disorders observed in subjects exposed to excess levels of corticosteroids during late fetal stages[5-7], including increased susceptibility to drug abuse[8,9].

The mesolimbic dopaminergic system, of which the projection of the ventral tegmental area (VTA) to the nucleus accumbens (NAcc) is a central component, is implicated in the regulation of reward behaviors[10-12], which underlie addiction. Importantly, there is evidence that this dopaminergic pathway is particularly vulnerable to prenatal insults[13]. Previous studies showed that maternal stress in late gestation[14,15] or repeated periods of maternal separation during early postnatal development[8] alter the pattern of dopamine receptor (DR) expression and decrease dopamine transporter availability in the NAcc later in life; interestingly, these changes in the dopaminergic system are associated with increased behavioral responses to stress and cocaine[8,12]. These studies suggested that a differential sensitivity to drugs of abuse may occur as the result of structural and/or functional changes in the brain reward system during gestation.

To test this hypothesis we performed a behavioral assessment of addictive behavior in adult male rats previously exposed to dexamethasone (DEX) during foetal development (E18-E19). The conditioned-place paradigm (CPP) revealed a significant difference among control and DEX groups (Fig. 1); while both controls and DEX-treated animals show CPP formation for morphine, DEX-treated animals spend significantly more time in the conditioned place than controls.

To further test for the development of addictive behavior, we measured, in another set of animals, voluntary ethanol consumption (EC) in a two-bottle free-choice paradigm. The daily and total intake of ethanol by male progeny of DEX-exposed rats was significantly higher than that of controls (Fig. 1b,c), albeit

no differences were found in sucrose or food consumption (Supplementary Material).

Enhancement of locomotor activity after drug administration is modulated by dopamine release in the NAcc, being an important indicator of altered susceptibility to drugs of abuse[8]. We assessed locomotor activity after morphine administration in an open-field (OF) arena; as expected, controls increased their global activity after morphine administration (Fig. 1d). Despite a decreased locomotor activity in basal conditions, after morphine administration the offspring of DEX-treated dams travelled longer distances on the arena than control animals; the enhancement of the locomotor activity due to morphine of DEX animals was 65% compared with only 25% in controls (Fig. 1d).

A growing body of evidence shows that exposure to inadequate maternal care is predictive of numerous behavioral and endocrine alterations relevant to psychiatric diseases[16]. Interestingly, on a background of increased HPA axis responsiveness in DEX-progeny[4], we did not observe any effects in self-directed and pup-directed behavior as a function of maternal strain and pup type as well as interactions between these variables (Supplementary Material). Likewise, and to completely exclude the possibility that DEX effects on offspring development were related to maternal care, we studied cross-fostered animals. Of notice, Dex animals displayed identical addictive behavior when crossed fostered (Fig. 1e-h).

Considering the role of NAcc and VTA in the development of addictive behaviors[12], and our previous observations that DEX-exposed animals display volumetric atrophy and reduced neuronal number these two brain regions[17] (herein confirmed, data not shown), we performed a 3D morphological analysis of dendrites and spines of NAcc and VTA neurons to explore the long-term response to prenatal DEX at the level of dendritic/synaptic plasticity. In this analysis, the core and shell of NAcc displayed a different response to prenatal DEX exposure; signs of dendritic/synaptic plasticity were only found in the shell division (Fig. 2a-h). Specifically, these neurons displayed increased number of total spines (Fig 2a, d), as well as an increased percentage of both ramified and thin spines (Fig. 2b) that partially compensate for the loss of neurons in this brain region. In contrast, no signs of plasticity was detected in the core division, which reveals that this division of the NAcc is, at the long-term, more strongly

affected by DEX-treatment. The VTA also displayed a significant increment in the total number of spines (Fig. 2i, l) but maintained the proportion of different spine types (Fig. 2j) and showed no changes in total dendritic length.

We next evaluated the levels of cathecolamines in control and DEX-treated animals in the NAcc and VTA, since dopamine release has been suggested to modulate behavior response in addiction[8,12]. DEX-progeny displayed significantly decreased dopamine levels and increased dopamine turnover ratio in the NAcc (Fig. 3a, b). In contrast, in the VTA, dopamine levels were higher and DOPAC levels were significantly lower in DEX animals (Fig. 3c) and a trend for diminished dopamine turnover was observed (Fig. 3d). No changes were seen in other catecholamines. Overall, these data suggest an association between the behavioral phenotype of the DEX animals with an hypodopaminergic status in the NAcc; this view is further supported by a reduced number of tyrosine hydroxylase positive fibers, both in the core and shell divisions of the NAcc (Fig. 3e-h). The NAcc seems particularly sensitive to the effects of glucocorticoids (GCs) administration early in life since no differences were found in the dopamine levels in other brain areas such as the prefrontal cortex and the hippocampus (data not shown).

To further dissect the molecular mechanisms underlying these behavioral, morphological, and neurochemical changes, we analyzed the expression of candidate genes by qRT-PCR. No significant differences were found in the expression levels of genes encoding for recognized modulators of neuronal function and synapse formation such as BDNF, synapsin, CdK5, CREB, NCAM between control and DEX animals. Additionally, no differences were found in the expression of several encoding for molecules involved in the stress response such as the GR, Chr1, and Chr2 (Supplementary Material). On the contrary, DEX-exposed animals presented an overexpression of the dopamine receptor 2 (Drd2) mRNA in the NAcc; no changes in the expression of Drd1, Drd3-5 were found (Fig. 3i). In the VTA, dopamine receptors expression was unchanged, with the exception of Drd5 down-regulation (Fig. 3j). Importantly, this transcriptional differences were reflected at the protein level: DEX-treated animals presented increased levels of both the 35kDa precursor, the 47kDa isoform and the 72kDa glycosylated Drd2 receptor in the NAcc (Fig. 3k, 3m); no significant changes were

found in the 50kDa isoform or in glycosylated Drd1 (74kDa) (Fig. 3k, 3l). No changes were observed in Drd1 and Drd2 protein levels in the VTA (data not shown). Interestingly, while in a basal situation DEX progeny displayed higher levels of Drd2 in the NAcc, after repeated morphine exposure, these levels were significantly reduced when compared to controls (Fig. 3n-o); a similar trend was observed after ethanol consumption. This altered Drd2 response pattern suggested changes at the transcriptional level. To further explore this possibility, and considering the latest findings that suggest that psycostimulants can induce significant epigenetic changes in specific genes in the NAcc[31-33], we analyzed the methylation pattern of a conserved human-rodent CpG island in the Drd2 gene promoter (Fig. 3p). In a basal situation, no differences in the methylation status of DEX animals when compared to controls were found (Fig. 3q). Surprisingly, after repeated drug exposure, DEX animals present a significant increase in Drd2 methylation (Fig. 3q), consistent with the expression levels of Drd2 after stimulus.

Altogether, DEX-exposed animals display high vulnerability to drug-seeking behavior, in parallel with a "hypodopaminergic status" in the NAcc. Noticeable, daily treatment with L-dopa/carbidopa (36.0/9.0 mg/kg daily) for three weeks [which restored the dopamine levels both the NAcc and in the VTA (Fig 4a, b)] completely reverted the vulnerability to drug-seeking in both contingency and non-contigency paradigms (Fig. 4c, d). Moreover, L-dopa in DEX-exposed animals also rescued the hyperlocomotor phenotype in the open-field after morphine (Fig. 4e). To determine whether the behavior reversal was sustained in time, in another set of animals we interposed a three week interval between the end of L-dopa treatment and the behavior assessement (chronic-interval group); noticeably, the behavioral rescue by L-dopa was still fully present (Fig. 4c-e). Finally, an additional group of animals was studied for the therapeutic effects of a shorter treatment (3 days) with L-dopa (acute group). While the reversal in behavior was also observed shortly after treatment (Fig. 4f-h), it was not sufficient for a not sustained maintenance in time (i.e. after an interval of three weeks; acute-interval group) in the contigent and non-contigent paradigms (Fig. 4f-h).

The present study confirms that early life exposure to DEX triggers an increased vulnerability to drugs (morphine and ethanol)[18-23].This behavioral phenotype is linked to structural changes at the synaptic level, particularly in the NAcc shell, that most likely compensate for the loss of neurons triggered by exposure to DEX early in life[16]. These findings corroborate the implications of the NAcc shell in stress–induced sensitization of motor activity but also in drug rewarding effects[24]. This behavioral/structural correlate is further strengthen by the observation of an hypodopaminergic status in the NAcc of DEX-exposed rats. Work over the last two decades has highlighted the role of dopamine in the NAcc in the timing of the initiation of response patterns originating in the frontal cortico-striatal loop systems[25], which are now known to be vulnerable to stress[26]. When stimulated by dopamine, neurons in the NAcc produce feelings of pleasure and satisfaction[24,27]. In cases of altered structure of the mesolimbic circuit or altered release of dopamine this reward circuit may predispose individuals to addictive behavior.

One hallmark of the "addicted brain" seems to be an hypdomaminergic status [12,28], which fits the current observation in DEX-exposed rats. It is important to mention that this reduction in dopamine levels in the NAcc may not be attributed to major changes in dopamine in the production site (VTA), given the fact that DEX animals have even slightly higher levels of dopamine in the VTA. The NAcc hypodopaminergic condition might implicate an increased drug-seeking behaviour to obtain similar rewarding effects. Alternatively, but not mutually exclusive, DEX-exposed rats might display an altered threshold to dopamine release in the NAcc and/or altered responsiveness of dopamine receptors. In fact, the present study shows that the increased vulnerability to drugs of abuse displayed by DEX-exposed rats is associated to an impaired expression of Drd2 in response to morphine or ethanol, which are stimuli that release dopamine in the NAcc. In accordance, others have suggested that de-regulation of dopamine receptors could modulate vulnerability to addictive behaviors[28]. Interestingly, such blunted Drd2 response to drug-exposure in the NAcc seems to be linked to an increased pattern of methylation of the CpG islands in this gene, most likely triggered by the exposure to high levels of GCs early in life. It is known that a window of susceptibility to such changes occurs in

this period of life, in order to imprint dynamic environmental experiences on the unchanging genome, resulting in stable and adaptive alterations in the phenotype. For example, *bad* maternal behaviour leads to alterations in the methylation status of the GR promoter in the progeny, accompanied with impaired HPA axis and behavioral deficits[29]. Interestingly, the Drd2 promoter is also hypermethylated in anorexia nervosa[30], suggesting that such Drd2 epigenetic changes may have behavioural implications. Recently it has been shown that drugs of abuse but also chronic stress can induce long-lasting changes in Dnmt3a (DNA methyltransferase) and in MeCP2 expression (methyl DNA binding protein) levels in the NAcc [31-33].

Noticeably, the drug-seeking behavior of DEX-exposed animals was reverted by restoring the levels of dopamine by acute or chronic administration of L-dopa. This fits previous observations that pre-treatment with dopamine D1 or D2 agonists causes marked reductions in voluntary ethanol intake and in cocaine self-administration[34-36]. Interestingly, when prolonged, the response to treatment was sustainable in time (at least for three weeks). If translable to humans, these findings are of major clinical relevance, as they demonstrate that modulation of central dopamine may be a target in the treatment and, equally importantly, in the prevention of addictive-like behaviors.

Methods summary

Animals and behavioral tests

Adult pregnant Wistar rats were individually housed under standard laboratory conditions (light/dark cycle of 12/12 hours with lights on at 08:00; 22ºC); food and water were provided *ad libitum*. Subcutaneous (sc) injections of DEX (0.1mg/kg) or saline (control) were administered on E18 and E19 days of pregnancy. On postnatal day 21, progeny were separated according to antenatal treatment and gender. All manipulations were done in accordance with local regulations (European Union Directive 86/609/EEC) and NIH guidelines on animal care and experimentation.

Behavioral evaluation was performed with at least 8 male rats derived from 4 different litters when animals were 3-4 months of age. All tests were

conducted during the daily light phase. More details about behavioral tests in Supplementary Material.

Drugs

Morphine hydrochloride (Labesfal Pharmaceutical) at a dosage of 10 mg/kg or saline was administered sc. L-dopa/Carbidopa (Sinemet, Merck) was administered daily at a dose of 36.0/9.0 mg/kg L-dopa/carbidopa by oral gavage in water.

Tyrosine hidroxylase immunohistochemistry

Immunofluorescence and quantification of TH-positive fibers was performed as described[17].

Structural analysis

Analysis of brains stained with Golgi-Cox solution and for TH immunohistochemistry were done with morphological tools using the Neurolucida/Stereoinvestigator software (Supplementary Material).

Macrodissection

Animals were anaesthetized, decapitated and heads were immediately snap-cooled in liquid nitrogen. Brain areas were rapidly dissected in an ice cold petri dish, using a stereomicroscope and following anatomical landmarks. Samples were snap-frozen in dry ice and kept at -80ºC until use.

Neurochemical Determinations

Levels of cathecolamines and its metabolites were assayed by HPLC combined with electrochemical detection using a Gilson instrument (Gilson), fitted with an analytical column (Supleco Supelcosil LC-18 3 µM).

Molecular analysis

Western blotting was done using standard protocols.

Real-time PCR was performed using Quantitec SyberGreen (Qiagen) and the Biorad q-PCR CFX96 apparatus, using HPRT as the housekeeping gene and the DDCT method for relative. Primer list and detailed protocols in Supplementary material. Epigenetic analysis was performed as previously described[29] (cf in Supplementary Material).

Figure legends

Figure 1. Prenatal DEX exposure enhances addictive-like behaviors. (a) DEX animals spend significantly more time in the morphine-associated compartment than controls. Daily (b) and total (c) ethanol consumption is higher in prenatal DEX animals than in controls. (d) DEX progeny present reduced locomotor activity in the open field in a basal situation but morphine administration (Mor) triggers increased locomotor activity in DEX-exposed rats. Cross-fostered DEX animals also display enhanced CPP formation (e) increased daily (f) and total (g) ethanol consumption. (h) Cross fostered DEX animals no longer display basal hypolocomotor phenotype, but after morphine administration they present increased activity when compared to controls. Data is presented as mean ± SEM. DEX: dexamethasone-exposed, CONT: controls, Mor: morphine (10 mg/kg) injection. *** $p<0.001$; ** $p<0.01$; * $p<0.05$.

Figure 2. Neuronal remodelling in the NAcc and VTA areas following prenatal DEX exposure. DEX animals present increased total number of spines in the NAcc shell when compared to controls (a). These changes are a result of increased numbers of both ramified and thin spines (b). Dendrite length is increased in the shell of DEX-exposed animals (d) Representative neuron from NAcc shell in DEX and controls. No differences were observed regarding total number of spines (e), spine type (f) or dendrite length (g) in NAcc core. (h) Representative neuron from NAcc core in DEX and control animals. DEX-exposed animals display increased number of spines (i), although the ratio between the different types of spines is maintained in the VTA (j). No differences were observed in the length of dendrites (k) in the VTA area in DEX animals. (l) Representative neurons from VTA in DEX and control animals. Data is presented as mean ± SEM. DEX: dexamethasone-exposed, CONT: controls; ** $p<0.01$; * $p<0.05$.

Figure 3. Impaired dopaminergic circuitry in DEX progeny. (a) Prenatal exposed DEX animals display reduced levels of dopamine (DOPA), DOPAC and HVA in the NAcc in comparison to control animals as measured by HPLC. (b) DEX-animals present enhanced dopamine turnover, which was assessed by the ratio between dopamine metabolites (DOPAC + HVA) versus dopamine levels. (c) Dopamine was increased in VTA of DEX animals while DOPAC was diminished and no significant changes were found in dopamine metabolism (d). Reduced TH positive fibers in both NAcc (e, f) and VTA (g, h) areas. (i) Expression levels of dopamine receptors in the NAcc. D2 was augmented in DEX animals when compared to controls and no changes were found in the other receptors expression. (j) No changes were found in the expression levels of all dopamine receptors in VTA except for Drd5 which was downregulated in DEX animals. (k) Representative western blot of Drd1 and Drd2 in 5 control and 5 DEX animals. No major differences were found in Drd1 levels of glycosylated (74kDa) or non-glycosylated form (~50 kDa) in NAcc (l). However, the levels of the Drd2 percursor (35kDa), the non-glycosylated form (~50kDa) and the glycosylated receptor (74kDa) were higher in DEX animals (m). (n) Drd1 levels display a trend to be increased in DEX animals but after a morphine injection 10 mg/kg (1x

morph), they are significantly downregulated. No differences were found in Drd1 levels in NAcc between controls and DEX animals with 4 injections of morphine (in 4 different days at the same dosage; 4x morph) or after ethanol consumption (EC). (o) While in a basal situation Drd2 is upregulated, after 1 or 4 injections of morphine the levels of this receptor are significantly lower in DEX animals when compared to controls. After ethanol consumption there is a trend for diminished Drd2 expression, although not statistically significant. (p) Scheme of the CpG region of Drd2 promoter. (q) Percentage of total methylation of each CpG nucleotide in the Drd2 promoter region in the NAcc of control and DEX-exposed animals in a basal situation and after morphine and alcohol consumption. After drug exposure, DEX animals present and increase in the methylation status of several CpG nucleotides. Data is presented as mean ± SD. DEX: dexamethasone-exposed, CONT: controls; ** $p<0.01$; * $p<0.05$.

Figure 4. Enhanced addictive-like behavior of DEX animals is reverted by chronic L-dopa administration. (a) Schematic design of the experimental groups used for L-dopa treatment. (b) Prenatal exposed DEX animals fed with water display reduced levels of dopamine (DOPA) in the NAcc. Chronic treatment with L-dopa increase the levels of dopamine in the NAcc of the two groups, although DEX animals still exhibit less dopamine than controls. (c) In the VTA, dopamine levels are higher in both groups subjected to L-dopa treatment when compared to water treated animals. No statistical differences exist between the two L-dopa groups regarding dopamine levels. (d) L-dopa supplementation impairs the morphine-induced CPP in both control and DEX animals from chronic group, although the effect is significantly more pronounced in DEX animals. Controls from chronic-interval group still display CPP formation while DEX animals do not. (e) Ethanol consumption is significantly reduced in DEX animals given L-dopa in the two experimental groups (chronic and chronic-interval) when compared to DEX animals given water. (f) DEX progeny have reduced locomotor activity in the OF when compared to controls (given water). After morphine administration (Mor), both groups display enhanced locomotor activity, although more pronounced in DEX animals. Chronic L-dopa administration reverts the enhanced locomotor activity in both groups. Control and DEX animals from chronic-interval group behave similarly in response to morphine as animals given water. (g) Acute L-dopa treatment reverts the enhanced conditioning of DEX animals in the CPP. Animals from acute-interval group still maintain increased conditioning. (h) Ethanol consumption is significantly reduced in DEX animals given L-dopa in the two experimental groups (chronic and chronic-interval) when compared to DEX animals given water. Nevertheless, DEX animals from acute-interval group consume more ethanol than paired controls. (i) DEX progeny have reduced locomotor activity in the OF when compared to controls (given water). After morphine administration (Mor), both groups display enhanced locomotor activity. Acute L-dopa administration reverts the enhanced locomotor activity in DEX animals. Control and DEX animals from acute-interval group behave similarly in response to morphine as animals given water. Data is presented as mean ± SEM. DEX: dexamethasone-exposed, CONT: controls, Mor: after morphine injection 10 mg/kg. *** $p<0.001$; ** $p<0.01$; * $p<0.05$.

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Figures

Figure 1.

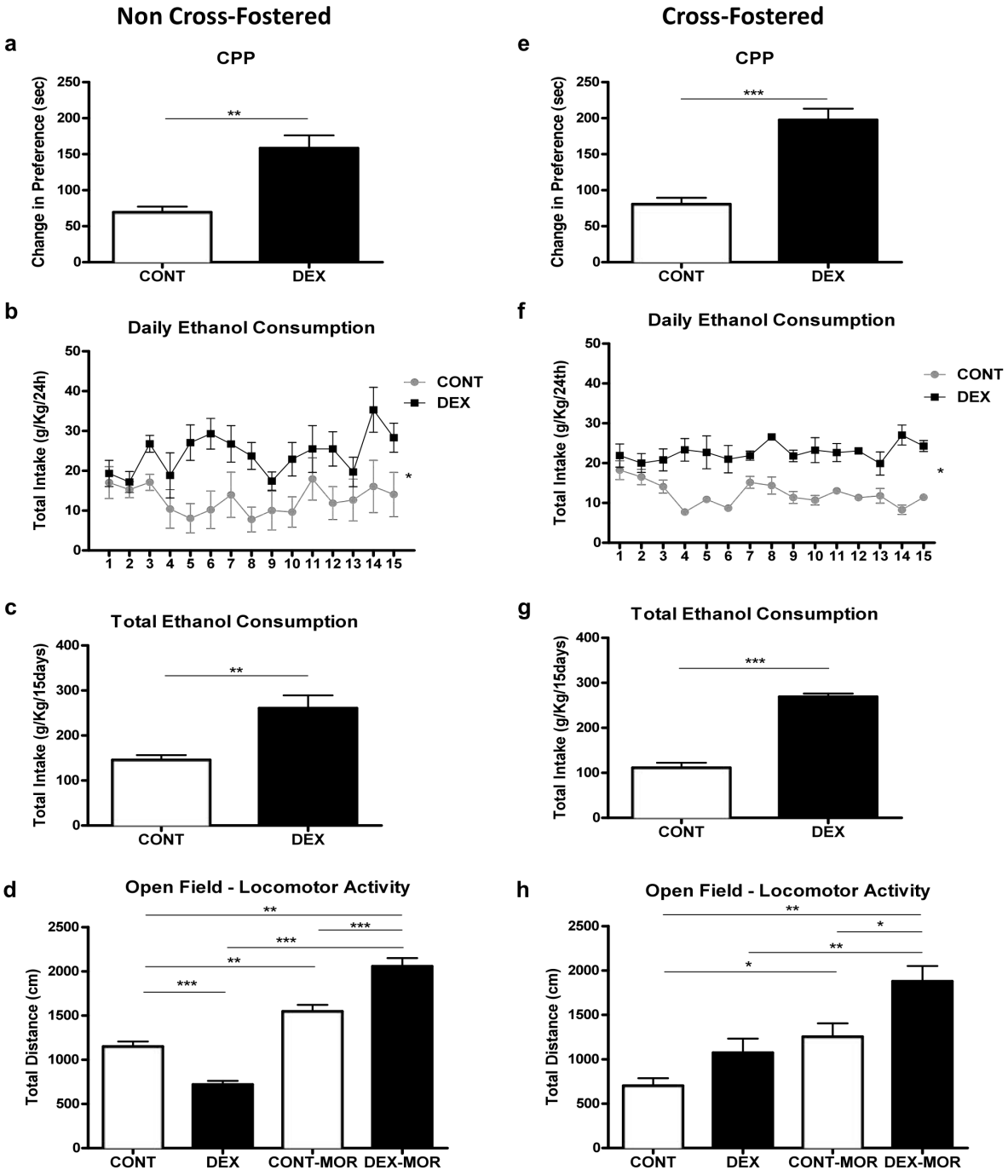

Figure 2.

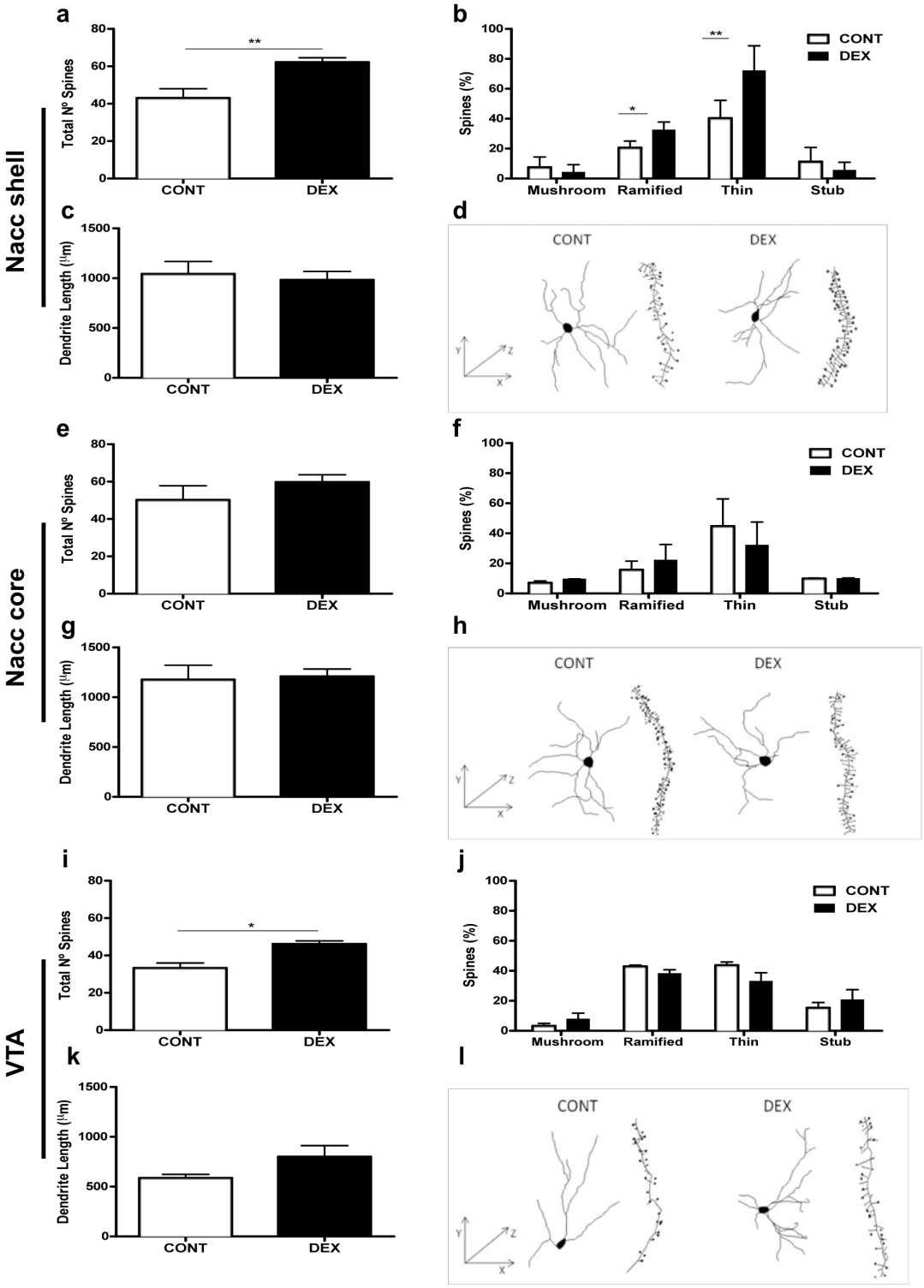
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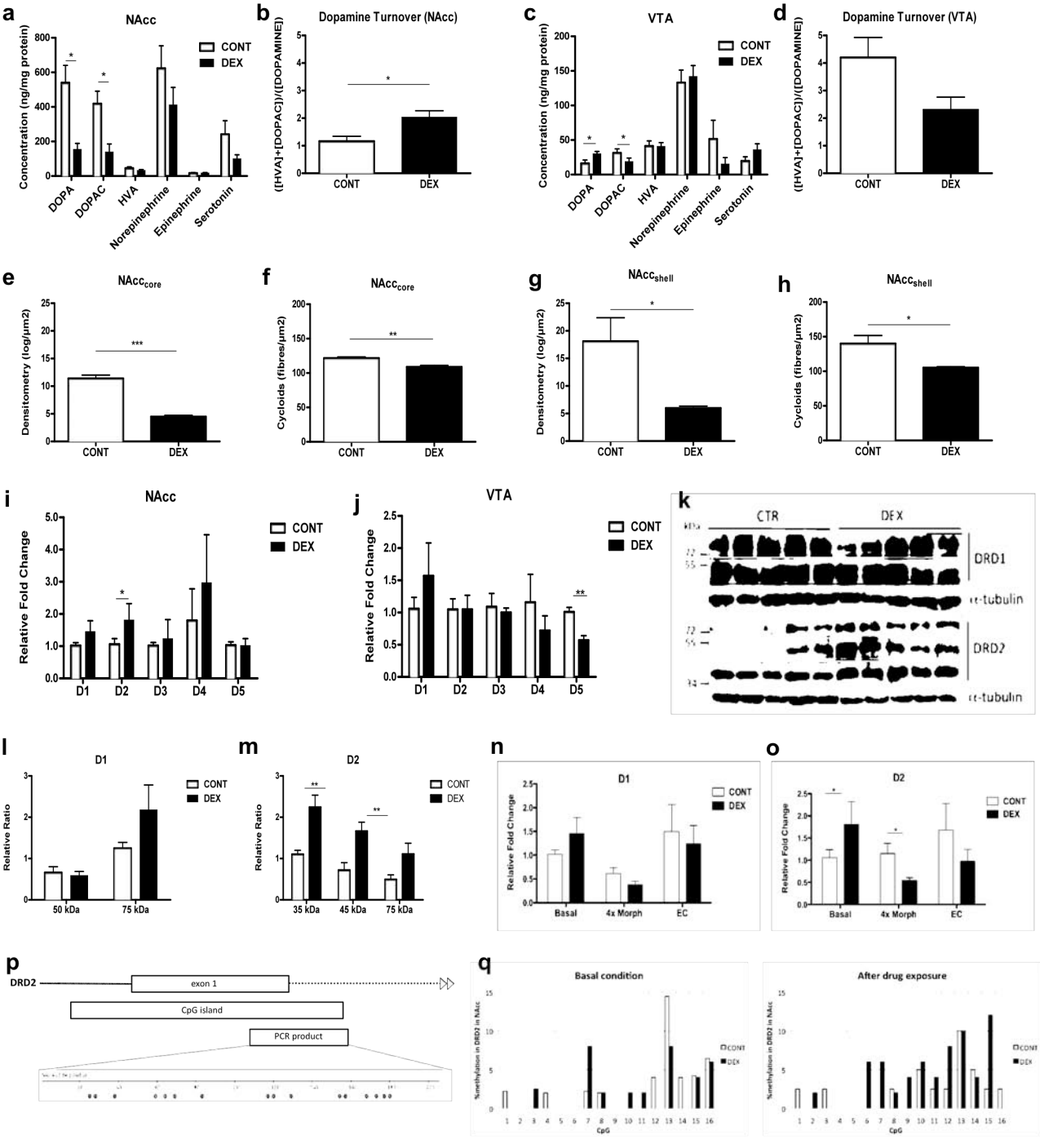
Figure 3.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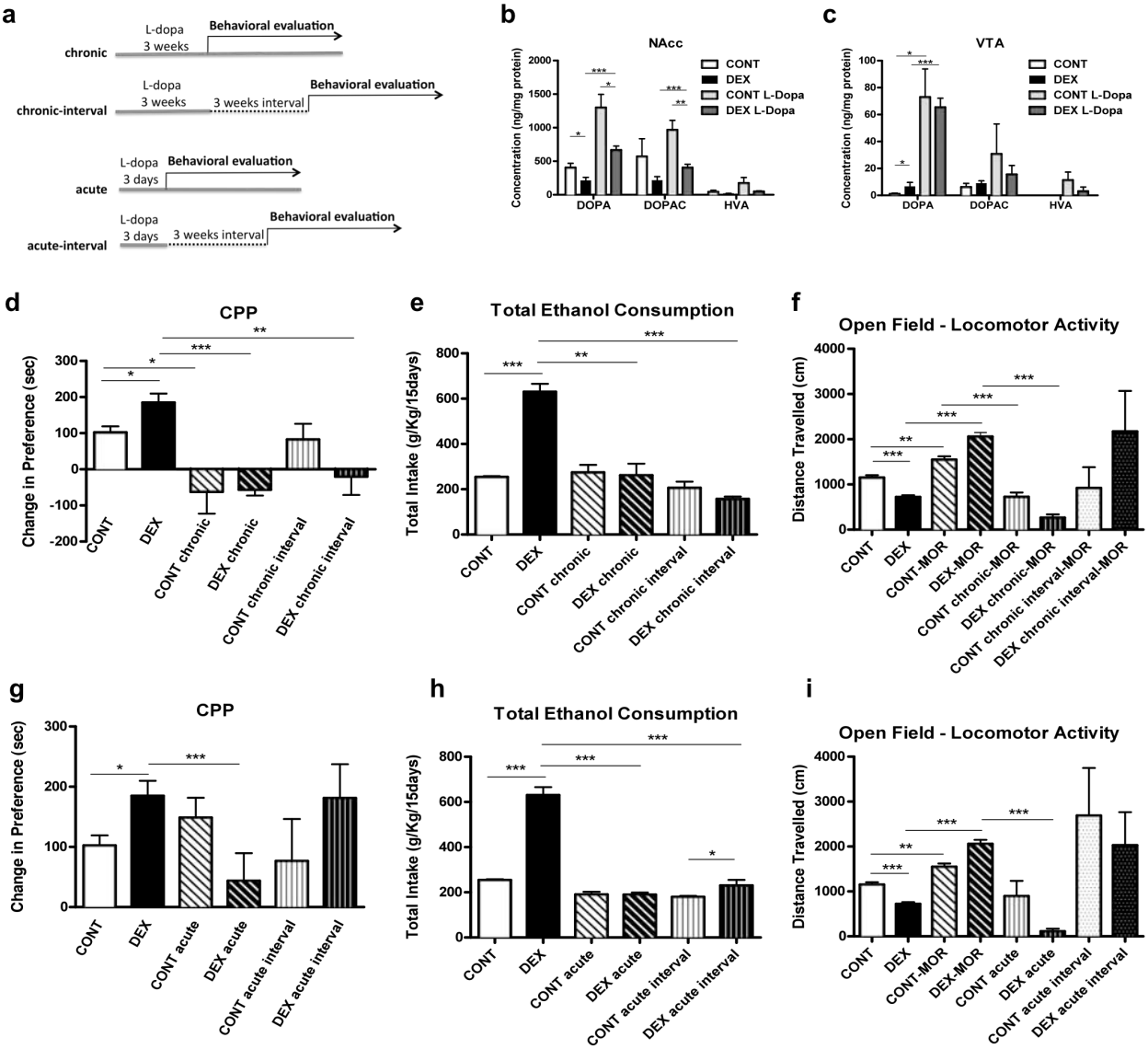
CpG

Figure 4.


a
chronic
L-dopa
3 weeks
Behavioral evaluation
chronic-interval
L-dopa
3 weeks
3 weeks interval
Behavioral evaluation
acute
L-dopa
3 days
Behavioral evaluation
acute-interval
L-dopa
3 days
3 weeks interval
Behavioral evaluation
b
NAcc
Concentration (ng/mg protein)
CONT
DEX
CONT L-Dopa
DEX L-Dopa
DOPA
DOPAC
HVA
c
VTA
Concentration (ng/mg protein)
CONT
DEX
CONT L-Dopa
DEX L-Dopa
DOPA
DOPAC
HVA
d
CPP
Change in Preference (sec)
CONT
DEX
CONT chronic
DEX chronic
CONT chronic interval
DEX chronic interval
e
Total Ethanol Consumption
Total Intake (g/Kg/15days)
CONT
DEX
CONT chronic
DEX chronic
CONT chronic interval
DEX chronic interval
f
Open Field - Locomotor Activity
Distance Travelled (cm)
CONT
DEX
CONT-MOR
DEX-MOR
CONT chronic-MOR
DEX chronic-MOR
CONT chronic interval-MOR
DEX chronic interval-MOR
g
CPP
Change in Preference (sec)
CONT
DEX
CONT acute
DEX acute
CONT acute interval
DEX acute interval
h
Total Ethanol Consumption
Total Intake (g/Kg/15days)
CONT
DEX
CONT acute
DEX acute
CONT acute interval
DEX acute interval
i
Open Field - Locomotor Activity
Distance Travelled (cm)
CONT
DEX
CONT-MOR
DEX-MOR
CONT acute
DEX acute
CONT acute interval
DEX acute interval

Supplementary Material

Detailed Methods

Open Field (OF)

Locomotor behaviour was assessed using the open field test in a brightly illuminated (white light) room. Briefly, rats were placed in the center of an arena (43.2 x 43.2cm; transparent acrylic walls and white floor) (MedAssociates Inc.) and position was monitored online over a period of 15min. Total distances travelled were used as indicators of locomotor activity. Animals were injected with saline or morphine and tested 30 min after injection.

Conditioned place preference (CPP)

The place preference apparatus consisted of two compartments with different patterns on floors and walls, separated by a neutral area (MedAssociates Inc). Animals were placed in the central neutral area and allowed to explore both compartments, allowing definition of the preferable compartment (day 1). During the conditioning phase (Day 2–4), rats were confined to the pre-test preferred compartment for 20 min after saline injection (1 ml/kg, sc) and, after a 6h gap, to the opposite compartment for 20min, 30 min after injection of morphine (10 mg/kg, sc). CPP was assessed on day 5 (20min) with all compartments accessible as described for the preconditioning phase. Results were expressed as the difference of time spent in drug-paired and saline-paired side during test.

Ethanol Consumption (EC)

The two-bottle choice protocol was carried out for 15 days as described previously[1]. After three days of taste habituation (ethanol 10% and sucrose 5%) animals were offered 10% ethanol in one bottle, while the other contained sucrose 5%. These two drinking bottles were continuously available to each rat, and each bottle was weighted daily. Bottle positions were changed every day to control for position preference. Quantity of ethanol consumed (g/Kg/day) was calculated for each animal and these values were averaged for ethanol consumption. Throughout the experiment, evaporation/spillage estimates were calculated every day from bottles (one containing sucrose and the other containing ethanol solution) placed on an empty cage.

Cross-fostering and maternal behaviour

For cross-fostering experiments, similar litters from 5 CONT and 5 DEX mothers were exchanged at postnatal day 1. To assess maternal behavior, records were made every two days during 30min per mother until weaning. Pup-directed behavior (nursing, nonnutritive contact, licking and nets building) and self-directed behavior (self-grooming, resting, vertical activity and carrying) data was collected.

Structural analysis

Rats were transcardially perfused with 0.9% saline under deep pentobarbital anesthesia and processed according to the protocol described by Gibb and Kolb[2]. Briefly, brains were removed and immersed in Golgi–Cox solution (a 1:1 solution of 5% potassium dichromate and 5% mercuric chloride diluted 4:10 with 5% potassium chromate[3]) for 14 days; brains were then transferred to a 30% sucrose solution (minimum 3 days), before being cut on a vibratome. Coronal sections (200 μm thick) were collected in 6% sucrose and blotted dry onto cleaned, gelatin-coated microscope slides. They were subsequently alkalinized in 18.7% ammonia, developed in Dektol (Kodak), fixed in Kodak Rapid Fix (prepared as per package instructions with solution B omitted), dehydrated through a graded series of ethanols, cleared in xylene, mounted, and coverslipped. For each selected neuron (NAcc and VTA), all branches of the dendritic tree were reconstructed at 600x magnification using a motorized microscope (Axioplan 2, Carl Zeiss, Germany), with oil objectives, and attached to a camera (DXC-390, Sony Corporation) and Neurolucida software (Microbrightfield, VT). A 3D analysis of the reconstructed neurons was performed using NeuroExplorer software (Microbrightfield). 20 neurons were studied for each animal of the 2 experimental groups, and neurons from the same animal were averaged. Several aspects of dendritic morphology were examined. To assess differences in the arrangement of dendritic material, a 3D version of a Sholl analysis[4,5] was performed. For this, we counted the number of intersections of dendrites with concentric spheres positioned at radial intervals of 20 μm; in addition, we also measured the length of the dendritic tree located between 2 consecutive spheres. The method for sampling dendritic branches for spine density (i.e., spines per micron dendritic length) was designed as follows: only branches that 1) were either parallel or at acute angles to the coronal surface of the section and 2) did not show overlap with other branches that would obscure visualization of spines were considered. In Golgi-impregnated material, the spines emerging toward the surface or directly into the section are not well visualized. Furthermore, an attempt to correct for hidden spines[6] was not made because the use of visible spine counts for comparison between different experimental conditions had been validated previously[7]. To assess treatment-induced changes in spine morphology, spines in the selected segments were classified according to Harris[8] in mushroom, thin, wide, and ramified categories.

Neurochemical Determinations

Levels of dopamine (DOPA), 3,4-dihydroxyphenylacetic acid (DOPAC), 4-hydroxy-3-methoxuphenylacetic acid (homovanillic acid, HVA) were assayed by high performance liquid chromatography, combined with electrochemical detection (HPLC/EC) using a Glison instrument (Glinson, Inc., Middleton, WI, USA), fitted with an analytical column (Supleco Supelcosil LC-18 3 μM; 7,5cm x

4,6mm flow rate: 1.0-1.5mL/min; Supelco, Bellefonte, PA, USA). Adequate volume of 0.2N of perchloric acid was added to each sample and samples were kept overnight at -20ºC. On the following day, samples were sonicated on ice for 5 min, centrifuged at 5000 rpm and supernatant collected. Supernatant was then filtered through a Spin-X HPLC column (Costar) to remove debris and 150 ml aliquots were injected into the HPLC system, using a mobile phase of 0.7 M aqueous potassium phosphate (monobasic) (pH 3,0) in 10% methanol, 1-heptanesulfonic acid (222mg/l) and Na EDTA (40mg/l). A standard curve using known concentrations of all cathecolamines was run each day.

Treatments

Animals were subjected to one injection of morphine 10mg/kg and sacrificed 1 hour after (morph 1x) or subjected to four injections at distinct days and sacrificed on the 4th day, 1 hour after the last injection (morph 4x). Another set of animals was allowed to have access to ethanol for 15 days (EC) and sacrificed one week after the consumption. Brains were snap-frozen in liquid nitrogen and brain areas were macrodissected to be used for RNA isolation.

Molecular analysis

For western blotting procedures, ice-cold lysis buffer (50 mM Tris-HCl pH 7.4, 50 mM NaCl, 1 mM PMSF, complete protease inhibitors (Roche)) was added to each frozen area. After disruption of the tissue using a 23G needle, SDS (Cf=0.1%) and Triton X-100 (Cf=1%) was added to each sample to solubilize proteins. After incubation on ice for 1h, samples were centrifuged at 13,000 rpm 10 minutes at 4ºC, the supernatant was collected and proteins were quantified using the Bradford method. Forty mg of total protein was loaded into SDS-PAGE gels and then transferred to nitrocellulose membranes. After incubation with the primary antibodies: rabbit anti-Dopamine receptor D1 (1:2500, ab20066, Abcam), rabbit anti-Dopamine receptor D2 (1:2000, ab21218, Abcam), mouse anti-alpha-tubulin (1:200, DSHB); the secondary antibodies were incubated at a 1: 10,000 dilution (SantaCruz Biotechnologies). Detection was done using ECL kit (Pierce). Band quantification was performed using ImageJ (http://rsbweb.nih.gov/ij/) as advised by the software manufacturers using alpha-tubulin as the loading control.

Data analysis

One-way ANOVA followed by Tukey post-hoc comparisons, was performed to analyse data from CPP, open-field tests, neurochemical as well as cell densities and molecular determinations. Repeated measures ANOVA were used to analyze daily ethanol and sucrose consumption. The results are expressed as group means ± standard error. Differences were considered to be statistically significant when $p<0.05$.

Table 1. List of primers used in this study.

Primer Name	Sequence
Hprt_F	GCAGACTTTGCTTTCCTTGG
Hprt_R	TCCACTTTCGCTGATGACAC
GR_F	AGGCCGGTCAGTGTTTTCT
GR_R	CAATCGTTTCTTCCAGCACA
Crhr1_F	CCTTTCAGGGCTTCTTTGTG
Crhr1_R	GGACTGCTTGATGCTGTGAA
Crhr2_F	TTTTCCTAGTGCTGCGGAGT
Crhr2_R	AGCCTTCCACAAACATCCAG
NCAM_F	AAAGGATGGGGAACCCATAG
NCAM_R	TAGGTGATTTTGGGCTTTGC
Synapsin_F	CACCGACTGGGCAAAATACT
Synapsin_R	TCCGAAGAACTTCCATGTCC
Bdnf_F	GCGGCAGATAAAAAGACTGC
Bdnf_R	GCAGCCTTCCTTCGTGTAAC
Cdk5_F	ATTGTGGCTCTGAAGCGAGT
Cdk5_R	CACAATCTCAGGGTCCAGGT
Creb1_F	TCAGCCGGGTACTACCATTC
Creb1_R	CCTCTCTCTTTCGTGCTGCT
DRD1_F	TCCTTCAAGAGGGAGACGAA
DRD1_R	CCACACAAACACATCGAAGG
DRD2_F	CATTGTCTGGGTCCTGTCCT
DRD2_R	GACCAGCAGAGTGACGATGA
DRD3_F	GGGGTGACTGTCCTGGTCTA
DRD3_R	TGGCCCTTATTGAAAACTGC
DRD4_F	GTGCTGGTGTTGCCTCTCTT
DRD4_R	ACAAACCTGTCCACGCTGAT
DRD5_F	ACCAAGACACGGTCTTCCAC
DRD5_R	CACAGTCAAGCTCCCAGACA

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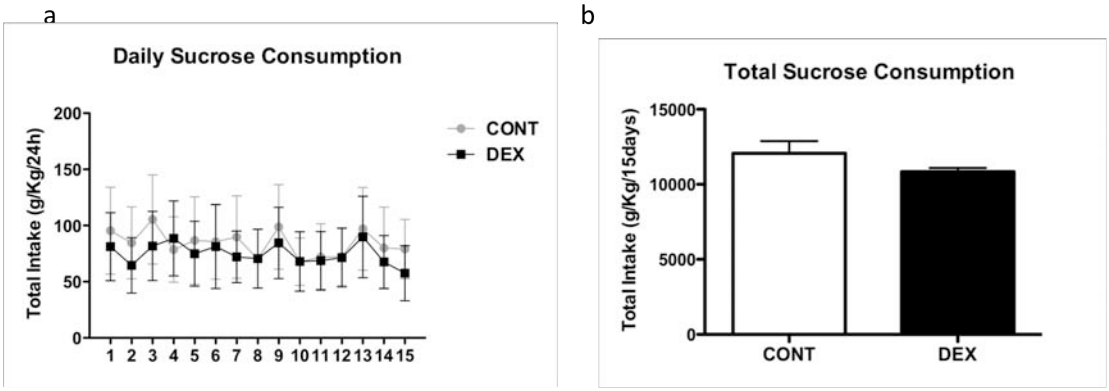
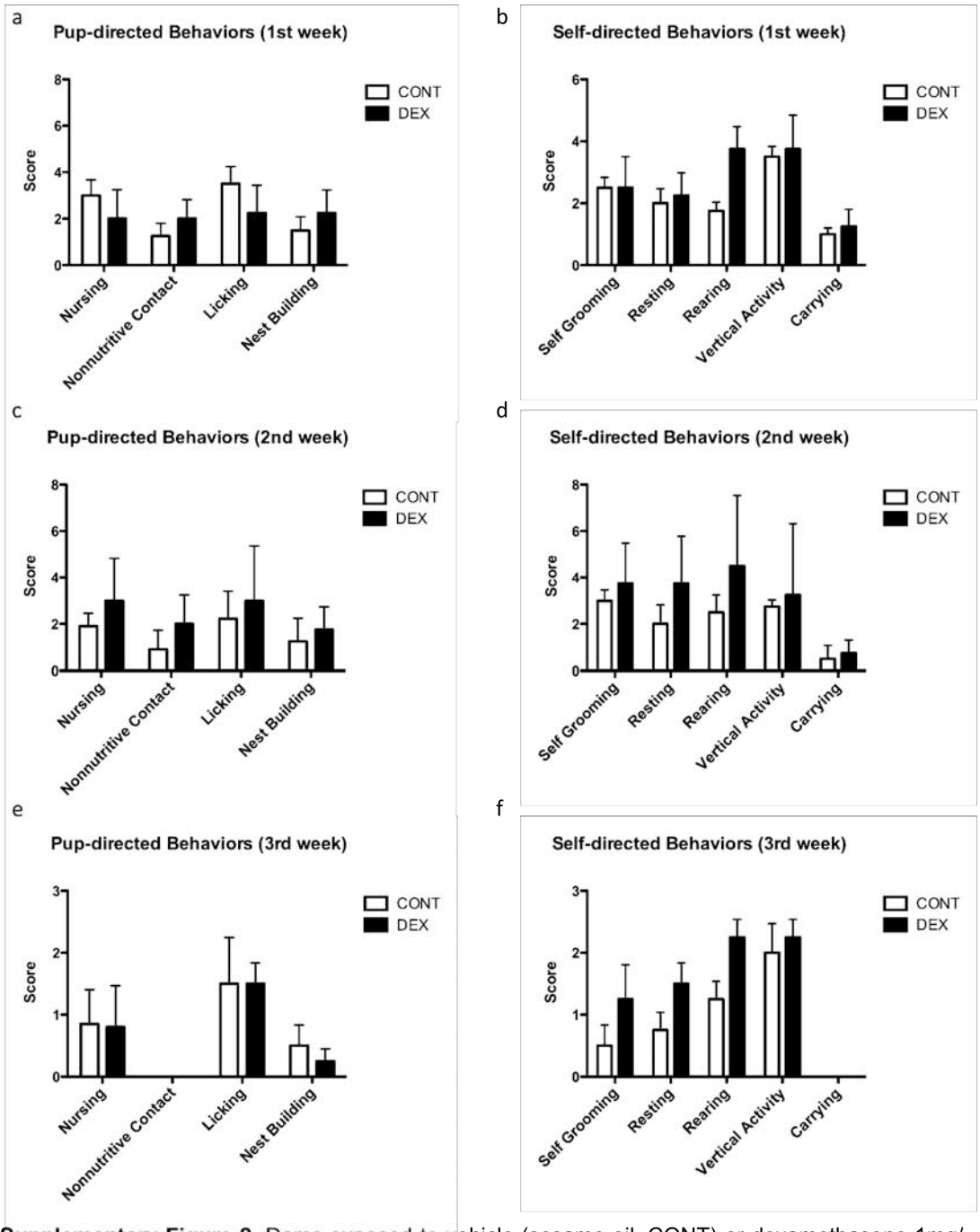
Supplementary Figures

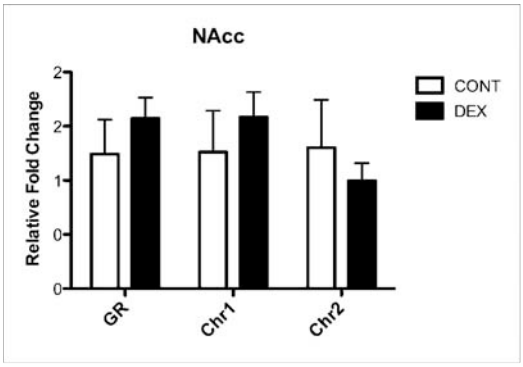


Supplementary Figure 1. Control and DEX animals have no differences in daily (a) or total (b) sucrose consumption. DEX, dexamethasone-exposed, CONT, controls.

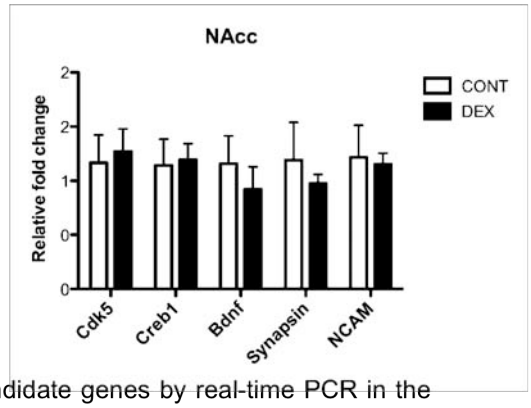


Supplementary Figure 2. Dams exposed to vehicle (sesame oil, CONT) or dexamethasone 1mg/kg (DEX) do not display major changes in pup-directed and self-directed behaviors in the three weeks post-delivery.

a



b



Supplementary Figure 3. Measurement of candidate genes by real-time PCR in the Nacc of control and DEX-exposed animals. No significantsignificant changes in the mRNA expression levels of glucocorticoid receptor (GR) or Corticotropin releasing hormone receptor 1 or 2 (Crhr1 and Crhr2) (a) nor in molecules important for synaptic function/plasticity/activation (b) was found.

Chapter 4

General Discussion

4.1 Discussion

4.1.1 Early life events alter adult behavior

Prenatal stress and/or GCs administration are known to produce numerous neurobiological programming alterations within the brain (Mesquita et al., 2009; Appendix 1), that are associated with increased risk for depression, schizophrenia, anxiety and substance abuse (Agid et al., 1999; Bernet and Stein, 1999; Chapman et al., 2004; Dube et al., 2003; Heim and Nemeroff, 2001; Kendler et al., 2004; Weiss et al., 1999; Young et al., 1997). If we consider that around 80% of adults who experienced early life stress (ELS) are predicted to suffer at least one episode of a psychiatric disorder such as depression and anxiety or from a behavioral disorder such as addiction (Edwards et al., 2003; Espejo et al., 2007; Gutman and Nemeroff, 2003; Heim and Nemeroff, 2001; McFarlane et al., 2005), it becomes obvious the clinical and social relevance of the study of the neurobiological consequences of ELS.

In this work, we analyzed the effects of acute prenatal (E18-19) DEX exposure at the behavioral, neurochemical, and molecular level in the adult progeny. The results presented herein suggest that prenatal DEX administration induces a behavioral sensitization to drugs of abuse both in contigent and non-contingent paradigms by changing the response of the mesolimbic dopaminergic circuit. To understand if DEX administration could increase the vulnerability to develop addictive-like behaviors, we used the CPP paradigm, a gold standard test in the field of addictive behavior, widely used to measure the rewarding properties of drugs of abuse. We found that adult male rats prenatally exposed to DEX at E18-19 are more vulnerable to the conditioning effects of morphine in the CPP, clearly demonstrating a different behavioral response than controls to the reinforcing effects of morphine. Some previous studies also showed that prenatal stress increases locomotor activity, motivational and neurochemical responses to cocaine in the CPP test (Kippin, 2008).

Interestingly, besides the proneness to addiction in a non-contigent paradigm, DEX-animals also displayed increased alcohol consumption in a two-bottle paradigm, as previously described by others (Darnaudery et al., 2007). Other types of ELS, such as prolonged maternal separation, also trigger increase

in alcohol intake during adulthood (Gustafsson and Nylander, 2006; Gustafsson et al., 2005; Huot et al., 2001; Jaworski et al., 2005; Ploj et al., 2003; Roman et al., 2003), whereas intake is decreased after brief episodes of neonatal separation in some (Jaworski et al., 2005; Ploj et al., 2003), but not all studies (Lancaster, 1998; Weinberg, 1987). The existing evidence suggests that antenatal or maternal separation procedures produce increased vulnerability to increased alcohol consumption supporting the view that adverse early life events are linked to a lack of resilience and that to later alcoholism (Enoch, 2006; Tiet et al., 1998; Zimmermann et al., 2007; Zuker et al., 2008). However, it remains unclear if this propensity toward "alcoholism" reflects a specific or a general vulnerability to neuropsychiatric disease, as reflected by high comorbidity with other disorders (Cornelius et al., 2003; Krystal et al., 2006; Kushner et al., 2000; Schuckit, 2006), for which stress is a contributing factor. As an example, a recent study conducted by Darnaudery et al. (2007) examined the long-term effects of prenatal stress and its interaction with stressors on voluntary alcohol intake. Under basal conditions, prenatal stress rats did not differ from controls in terms of free access alcohol intake, but a subgroup of prenatal stress females that displayed initial high preference for alcohol further increased their intake when subjected to intense stressors as adults, whereas stress-induced changes in intake were not observed in prenatal stress females exhibiting an initial low preference for alcohol or in prenatal males, regardless of their initial alcohol preference (Darnaudery et al., 2007). Interestingly, prenatal stress produces enduring neurochemical abnormalities within brain regions analogous to those exhibiting pathologies in alcoholic individuals (Moselhy et al., 2001; Volkowet al., 1990) which raises the distinct possibility that the enhanced motivation of prenatal stress to self-administer alcohol (Campbell et al., 2009) may be related to enduring effects of this early developmental manipulation on the dopaminergic limbic system.

In further support of the hypothesis that DEX-animals have a different behavioral response to drugs of abuse was the fact that in the OF test, these animals displayed a hyperlocomotory profile when compared to control animals when injected with morphine 10 mg/kg, although in a basal situation these animals have decreased locomotor activity. The OF test is a simple assessment

used to determine general activity levels, gross locomotor activity, and exploration habits in rodents. However, the specific phenotype in the OF we measured is known to be dependent on the activation of the mesolimbic dopaminergic transmission (Piazza et al., 1991b). Indeed, the locomotor activity can be enhanced by the injection of dopamine or psychostimulants in the accumbens (Delfs et al., 1990; Kelly and Iversen, 1976; Pijnenburg and Van Rossum, 1973; Robbins and Everitt, 1982) or of opioids in the VTA (Joyce and Iversen, 1979; Kalivas et al., 1983; Vezina and Stewart, 1984). Furthermore, previous studies demonstrated that the rewarding effect of drugs of abuse can be predicted by the locomotor activity in a novel environment (Piazza et al., 1991b; Saigusa et al., 1999). Animals displaying hyperlocomotion (high responders- HR) i) release more corticosteroids in response to stressors (Piazza et al., 1991; Rots et al., 1995), ii) display significant conditioning by drugs of abuse and iii) are more prone to display addictive-like behaviors than low responder animals (LR) (Piazza et al., 1991b). Interestingly, the locomotor activity is positively correlated with DA levels in the NAcc both in a basal situation but also in environmental - tail pinch (Rougé-Pont et al., 1993) or pharmacological – cocaine (Hooks et al., 1992) challenges.

Such as HR animals, prenatal DEX-exposed animals present increased release of corticosteroids in response to chronic stress when compared to controls (Oliveira et al. 2007). On the other hand, DEX progeny present lower levels of DA in the NAcc in a basal situation, which is in agreement with their decreased locomotor activity in the OF but might seem in contrast to the enhanced addictive-like behavior observed in these animals. However, it is likely that the mesolimbic system of DEX animals is somehow more sensitive to the rewarding effects of drugs of abuse, which is further supported by the observed hyperlocomotor profile of DEX animals when compared to controls after morphine administration, suggesting that they display a different dopaminergic tone after this stimulus (see section “De-regulation of dopaminergic mesolimbic circuit in DEX- animals”).

The contribution of the maternal behavior for the phenotype of DEX progeny

GCs administration during pregnancy leads to a hyperanxious phenotype in the dams and it is known that exposure to inadequate maternal care is predictive of numerous behavioural and endocrine alterations relevant to psychiatric diseases including addiction (Agid et al., 1999; Aisa et al., 2007; Brake et al., 2004; Caldji et al., 1998; Edwards et al., 2003; Espejo et al., 2007; Heim and Nemeroff, 2001; Matthews et al., 1996; Moffett et al., 2006; Moffett et al., 2007; Weaver et al., 2004). To exclude a maternal contribution for the observed phenotype of DEX progeny, we analyzed the maternal behavior of DEX dams and performed a cross-fostering experiment. We did not observe any differences in the maternal behavior of DEX dams, although some previous studies have proven so (Brabham et al., 2000). To further complement our analysis, we showed that cross-fostered animals still display enhanced addictive behavior in the CPP and AC paradigms, suggesting a direct effect of DEX rather than altered maternal behavior. Noteworthy to mention that DEX animals reared by a control mother no longer displayed the basal hypolocomotor phenotype in the OF, suggesting at least a partial contribution of the maternal behavior for this behavior. Nevertheless, after morphine stimulus, fostered DEX animals display similar behavior response as non-cross-fostered animals, proving that the behavioral response to morphine is maintained. Altogether, these results suggest a direct effect of DEX *in utero* rather than a significant maternal contribution for the observed phenotype and provide extra evidence of the importance of studying both the immediate and long-term changes of GCs administration during pregnancy.

Stress and addiction

Drugs of abuse exert their reinforcement effects largely through the mesolimbic dopaminergic system, also known as “reward pathway”, that, as previously said, encompasses two main areas: the VTA and the NAcc, but also includes modulating effects from the PFC, the amygdala and hippocampus. Drugs, and natural rewards such as food and sex, induce release of dopamine in the NAcc, providing a “pleasurable experience” to the individual (Piazza and Le Moal, 1996). Importantly, the mesolimbic circuit is also highly responsive to

stress since increased GC levels and stress exposure enhance dopamine release in the NAcc (Kalivas and Duffy, 1995; Rouge-Pont et al., 1998; Takahashi et al., 1998; Thierry et al., 1976). In fact, there is evidence of a peculiar analogy between the effects of acute stress and drugs of addiction in the mesolimbic system, in as much as exposure to either of these seems to produce a similar pattern of changes in the brain at synaptic and dendritic levels (Liston et al., 2006; Robinson et al., 2001; Robinson and Kolb, 1999; Saal et al., 2003).

Unequivocally, there is a close link between stress and addiction, with dopamine certainly playing a central role in this interplay. Stress and GCs levels are intrinsically related to drug seeking, intake and addiction; repeated exposure to stress enhances behavioral responses not only to subsequent stress but also to drugs of abuse (Robinson and Becker, 1986; Sorg and Kalivas, 1991; Stewart and Badiani, 1993), and this sensitization process has been associated with augmented dopamine release in the NAcc (Doherty and Gratton, 1992; Kalivas and Stewart, 1991). GCs also facilitate the psychomotor stimulant effects of cocaine, amphetamine and morphine (Cools, 1991; Marinelli et al., 1994) and adrenolectomy decreases to a great extent alcohol consumption and drug intake in rats (Fahlke et al., 1994; Marinelli and Piazza, 2002; Marinelli et al., 1997a; Marinelli et al., 1997b). Corticosterone levels before drug self-administration are positively correlated with the extent of self-administration of cocaine at low doses in both stressed and unstressed animals (Goeders and Guerin, 1994; Piazza et al., 1991a). Even more interesting, is the fact that corticosterone has been suggested to act as a positive reinforcer since naive rats will self-administer corticosterone in a dose-response curve similar to other reinforcing drugs (Piazza et al., 1993). Our results further support these findings since DEX animals, which also present high corticosterone levels (Oliveira et al., 2007), also display enhanced conditioning in the CPP and enhanced locomotory activity in the OF in response to morphine.

But what are the causes (neurobiological? neurochemical?) of such altered response to morphine stimulus? Considering the fact that in rodents the dopaminergic circuitry is only fully maturated three weeks after birth, and that the developing dopaminergic systems are highly sensitive to perturbations, including stress and high levels of GCs, it is plausible to assume that synthetic

GCs administration during late gestation (E18-19) can significantly impact their correct and balanced development. In fact, mesolimbic dopaminergic cells possess glucocorticoid receptors (Hlrfstrand et al., 1986) and there is evidence that corticosterone can modify metabolism (Ho-Van-Hap et al., 1967; Rothschild et al., 1985), extracellular concentrations (Imperato et al., 1989; Mittleman et al., 1992) and reuptake of dopamine (Gilad et al., 1987) in these neurons.

Despite the direct role of GCs on dopaminergic neurons, stress-induced corticosterone secretion can also modulate dopamine-dependent responses to drugs by acting on other neuronal systems. The opioid system is one of the most probable targets of corticosterone. First, opioid afferents to dopaminergic neurons modulate stimulant effects of drugs (Kalivas, 1985). Second, corticosterone potentiates both biochemical and certain behavioral effects of morphine (Deroche et al., 1993). Third, corticosterone has been found to increase the effects of enkephalin on hippocampal slices (Vidal et al., 1986), whereas adrenalectomy decreases preproenkephalin mRNA in the striatum (Chao and McEwen, 1990). Corticosterone may also control stress-induced sensitization of dopamine- dependent effects of drugs also by acting on GABA, serotonin, and excitatory amino acid transmission. GCs have been reported to modulate the binding capacity of serotonin receptors (Biegon et al., 1985) and GABA receptors (Majewska et al., 1986; Majewska et al.,1987; Sutanto et al., 1989) and to potentiate glutamatergic transmission (Tischler et al., 1988; Sapolsky, 1990), that, in turn, can modulate the dopaminergic transmission in the brain. Besides this, stress can increase endocannabinoids receptors (Choukèr et al., 2010); and endocannabinoids control the activation of various neuronal circuits including those involved in neuroendocrine stress processing (Steiner and Wotjak, 2008). This seems to occur by the inhibition of glutamate and GABA release, which in turn can modulate dopaminergic transmission, thus contributing to addictive-like behaviors.

4.1.2 Prenatal stress modifies NAcc and VTA structural organization

Considering: i) the late maturation of the mesolimbic dopaminergic system, ii) the fact that dopaminergic cells possess GC receptors, and iii) the effects of GCs on dopamine metabolism and release, it is not surprising that high GCs

during pregnancy have long-term effects on the neuronal populations of this circuit. Indeed, treatment with DEX at E18-E19 caused significant neuroanatomic changes in the NAcc and VTA areas of adult progeny (Chapter 3.1). A remarkable decrease in BrdU-positive cells was found in 3 days old animals in the NAcc and VTA areas. However, it is important to mention, that in despite of metholological differences, others have described quite contrasting effects of prenatal dexamethasone in the survival and phenotypic expression of dopamine neurons in the NAcc and VTA (McArthur et al., 2005). Besides the immediate consequences of DEX exposure found at 3 days of postnatal life, we also found long-term changes in neurons of the mesolimbic system in the progeny when adults. In fact, administration of DEX leads to a decreased volume and number of cells in the VTA and both the core and shell areas of the NAcc similar to what has been found in other models of early life stress (McLure et al., 2004).

The significant reduction of BrdU-positive cells in the NAcc and VTA suggests that cell proliferation and/or survival can be down regulated by exposure to this prenatal insult. It should be noted that because the BrdU marker was injected during the late gestational period, the results observed in neonatal rats could mainly reflect the changes in cell proliferation during the late embryonic rather than early postnatal period. In this regard, our results suggest that prenatal DEX-induced impairment in brain development is evident already during the embryonic period. In agreement with our data, Kawamura showed that prenatal restraint stress (PRS) for E13-17 also lead to a significant decrease in cell proliferation in the NAcc (Kawamura et al., 2006), suggesting that the mesolimbic pathway is extremely sensitive in terms of stress/GCs levels during development. It is also interesting to note the finding of reduced levels of BDNF in the offspring of prenatal stressed rats (Burton et al., 2007), which might potentially contribute to explain the reduction in neuronal proliferation/survival. One alternative mechanism is the hyperactivation of the HPA axis that is observed in DEX-treated animals (Oliveira et al., 2006). It has been demonstrated that excessive maternal stress hormones, may be able to reach the fetal brain, and high levels of GCs are known to have inhibitory effects on the cell proliferation of neonatal rats (Tanapat et al., 1998) and adult neurogenesis in the dentate gyrus (Gould et al., 1992; Fuchs and Flugge, 1998).

The aforementioned elevation of GCs could also underlie the aversive effects of prenatal stress on early NAcc development, culminating in a reduction of accumbal cell proliferation and morphological changes, as revealed in this study.

Besides these effects on neuronal cell fate and volumes of the NAcc, early life adverse exposure also triggers relevant changes in neuronal morphology in the NAcc. Overall, there is a volumetric reduction, most likely due to a decrease in neuronal numbers (that is probably a consequence of decreased proliferation in the embryonic period). However, it is remarkable that our work shows the existence of compensatory mechanisms in adult animals, particularly in the NAcc shell region; neurons in this division of the NAcc present an increase in the number and density of spines, mainly due to an increase of thin subtype spines (Chapter 3.2). These results may suggest a compensatory phenomenon and may also imply that the synaptic transmission in this area is partially restored, especially because augmented density of dendritic spines seems to be a consequence of a change in the number of synaptic inputs onto dendrites (Peters and Feldman, 1976; Wilson et al., 1983) and results in increased synaptic efficacy (Luscher et al., 2000; Malinow et al., 2000; Scannevin and Huganir, 2000). The NAcc shell is mainly involved in mediating the rewarding effects of psychostimulants (Ito et al., 2004; Parkinson et al., 1999; Rodd-Henricks et al., 2002). Thus, the increase in spine density and potentially the increased synaptic efficacy in the NAcc shell could even contribute to explain the enhanced addictive-like behavior of DEX animals.

In contrast, and although we cannot fully exclude that some subtle differences may occur, there seems to be no compensatory synaptic response in NAcc core after prenatal DEX exposure, even though this subdivision also presents reduced volumes and neuronal numbers. The NAcc core is mainly implicated in the psychomotor activating effects of drugs of abuse (Boye et al., 2001; Sellings and Clarke, 2003; Weiner et al., 1996; West et al., 1999), although the shell (Heidbreder and Feldon, 1998; Ito et al., 2004; Parkinson et al., 1999) or both divisions together may also play a role (Ikemoto, 2002; Pierce and Kalivas, 1995). The lack of apparent compensatory mechanisms in the core may indicate that this area is less plastic, and, thus, more vulnerable to the detrimental effects of prenatal DEX, which is likely to be of relevance to the behavioral phenotype

herein described.

In summary, our morphological data demonstrate that mesencephalic dopaminergic neurons are exquisitely sensitive to relatively mild, transient perturbations in circulating GC levels during development and, moreover, the responses are population-specific. The mechanisms leading to this impairment are currently unknown but seem to be largely dependent on changes in proliferation/survival of neurons during brain development.

4.1.3 De-regulation of dopaminergic mesolimbic circuit in DEX- animals

In this work, we observed not only a reduced number of cells in the NAcc and VTA along with changes in the spine number and densities, but we also found that DEX-animals display a sustainable reduction in the number of TH-positive fibers in these two areas. Although the influence of early stressors on the mesolimbic dopamine system is known, our demonstration of a clear effect of prenatal DEX exposure on TH+ cell numbers in the NAcc and VTA further confirms the particular susceptibility of this pathway to this prenatal insult. Because GCs can influence neurogenesis and neuronal migration in the CNS (Gould and Cameron, 1996), our prenatal treatment regimen (E16–E19) may disrupt the normal course of NAcc and VTA neuronal development/migration/maturation. After neuronal migration, significant neuronal apoptosis occurs in the mesolimbic pathway from at least E20 until P8 (Tepper et al., 1994), raising the hypothesis that excessive prenatal GCs may have a significant pro-apoptotic role on mesencephalic dopamine neurons in the newborn rat, besides the decrease in their proliferation.

The decrease of TH-positive fibers was obviously matched by a significant reduction in dopamine content in the NAcc of adult DEX- exposed animals when compared to controls. One important remark is that for the neurochemical determinations, we did not individualize the NAcc shell and core, which could be biasing our results, given the differential functional roles of each area. One should investigate each of these subdivisions in more detail in order to assess if the neurochemical differences observed are area-specific, specially if we

consider the fact that core and shell regions have different basal DA levels (Deutch and Cameron, 1992; Hedou et al., 1999; King and Finlay, 1997).

It is important to mention that this reduction in dopamine levels cannot be attributed to major changes in dopamine in the production site (VTA), given the fact that DEX animals have even slightly higher levels of dopamine in the VTA. One possibility is that dopamine transport along the axons is somehow disrupted as it has been shown in animals subjected to chronic drug administration for example (Beitner-Johnson et al., 1992; Beitner-Johnson and Nestler, 1993).

Hypofunctioning of the mesolimbic circuit as the underlying cause of drug-seeking behavior

This "hypodopaminergic" status of the NAcc is particularly interesting since in the addicted brain, and during withdrawal, a hypofunctioning of dopaminergic system occurs (Melis et al., 2005). In fact, withdrawal of rats from chronic ethanol, morphine, cocaine and amphetamine resulted in a sustained reduction in extracellular dopamine concentration in the ventral striatum (Rossetti et al., 1992). In addition, it has been shown that the extracellular levels of dopamine in the NAcc of cocaine-, cannabinoid- or alcohol-treated animals were decreased during withdrawal (Diana et al., 1998; Diana et al., 1993; Parsons et al., 1991). Thus, conceptually, either too much, or too little, dopamine can induce drug-seeking behaviors: while hyperdopaminergic activity may increase the motivational impact of drug reward, low dopaminergic such as the one observed in our DEX-exposed animals can diminish the motivational impact of drugs or 'natural' rewards (such as food or sex) and enhance the seeking behavior of the drug/rewards, similar to a withdrawal condition (Appendix 2). This low dopaminergic content in the NAcc of DEX animals is further supported by the basal hypolocomotory profile in the OF arena, as it is known that exploratory activity is directly correlated to dopamine release in the mesolimbic circuit as previously mentioned. One apparently contradictory result to this "hypodopaminergic theory" seems to be the enhanced lomocotor activity in response to morphine of DEX animals when compared to controls. This observation could suggest that DEX animals present i) a different neurochemical

(dopaminergic) response and/or ii) differential abundance/sensibility of dopamine receptors to morphine. Interestingly, and in agreement with the second hypothesis, we found that DEX animals presented an up-regulation of Drd2 both at mRNA and protein levels in a basal situation in the NAcc. It is important to mention that dopamine receptors expression varies in the core and shell regions, which may also explain their different behavioral involvement. Drd2 are quite sparse in the NAcc shell while Drd1 are abundant, and the opposite is seen in the core region (Bardo and Hammer, 1991). Hence, it would be important to distinguish between core and shell regions in order to identify what is the area more affected due to GCs exposure in terms of DA receptors expression, and understand its contribution for the observed phenotype.

The Drd2 increase in the NAcc might occur as a compensatory mechanism due to the hypodopaminergic status observed in this brain region. Indeed, some studies show that if a neurotransmitter is downregulated, receptor up-regulation is usually seen as a compensatory mechanism. In agreement with our data was the fact that D2 expression/binding in the NAcc seems to be increased in other models of prenatal stress (Berger et al., 2002; Barros et al., 2004; Henry et al., 1995) or in postnatal stress (Moffett, 2007), albeit some other studies have found contradictory results (Meaney et al., 2002). These differences may be explained by changes in the type, duration and time of the stressor; nevertheless, it strongly suggests that dopamine receptors expression may be modulated by early life events.

A remarkable finding of this work was the fact that DEX-exposed rats have an impaired expression of Drd2 in response to morphine or ethanol. While in a basal situation DEX-exposed animals have higher levels of Drd2, after morphine exposure the expression of this receptor is significantly down-regulated when compared to control animals. This may indicate that the system is hypersensitive to dopamine release. It can also indicate that the available Drd2 receptors have enhanced binding capacity, although this hypothesis needs further validation. Importantly, such blunted Drd2 response to drug-exposure in the NAcc seems to be linked to the pattern of methylation of the CpG island in this gene, most likely triggered by the exposure to high levels of GCs early in life. While in a basal situation, DEX animals display a methylation pattern similar to

that of controls, after stimulus, they present increased methylation in the Drd2 promoter region. Interestingly, it has been previously shown that D2 promoter is hypermethylated in anorexia nervosa (Frieling et al., 2009) and it was suggested that methylation differences in the promoter region of D2 can be responsible for monozygotic twin discordance in schizophrenia (Petronis et al., 2003), which suggest that the epigenetic status of the Drd2 promoter can be correlated with altered behaviors in humans as well. In concordance with our observations is the fact that other models of early life stress also show significant differences in the epigenetic status of GR and AVP gene promoters (Murgatroyd et al., 2009; Weaver et al., 2004). Importantly, recent data shows that drugs of abuse and chronic stress can modulate the complex epigenetic regulation of gene expression. Cocaine can induce long-lasting changes in histone acetylation in several genes important for neuronal function and for the response to psychostimulants such as Bdnf, c-fos, cdk5 in the NAcc (Renthal and Nestler, 2008). In addition, drugs of abuse can exert a modulatory effect in enzymes responsible for the epigenetic control. For example, cocaine exposure and chronic social defeat stress can increase the levels of the DNA methylating enzyme Dmnt3a in the NAcc; interestingly, such Dmnt3a expression change significantly alters cocaine-induced conditioned place preference (LaPlant et al., 2010). Other studies showed that manipulation of MeCP2, a methyl DNA-binding transcriptional regulator, in the NAcc, bidirectly modulates amphetamine-conditioned place preference (Deng et al., 2010; Im et al., 2010). Altogether, these findings show that early life stress can induce permanent changes in the expression of target genes in specific brain regions due to epigenetic mechanisms with concomitant effects on behavior. A critical issue to be raised is that epigenetic changes (methylation, acetylation) are potentially reversible. For example, it has been shown that memory disturbances in aging are associated with altered hippocampal acetylation and that restoration of acetylation levels can recover the cognitive impairment (Peleg et al., 2010). “Bad maternal behavior” leads to increased methylation of the GR promoter, translated into an hyperanxious progeny; this phenotype can be rescued by the use of TSA (histone deacetylase inhibitor) (Weaver et al., 2004). Importantly, recent data suggests that administration of TSA can potentiate the rewarding effects of cocaine

(Kumar et al., 2005). Now the question is whether these epigenetic alterations that we found in the NAcc of DEX animals can be modified by using drugs with the potential to reverse DNA methylation for example (e.g. 5-aza-2'-deoxycytidine, already approved for use in cancer) and if this rescues the addictive-like behavior of these animals.

But what are the behavioral correlates of altered Drd2 expression? An inverse correlation between D2 receptors expression and vulnerability to the reinforcing effects of cocaine in primates seems to exist (Nader and Czoty, 2005). In humans, the expression levels of D2 receptors can predict the individuals experience to methylphenidate (a stimulant drug similar to cocaine) (Volkow et al., 2004); while higher levels of D2 receptors lead to an "unpleasant feeling", lower D2 receptor levels were associated with a "pleasant feeling" after taking the drug (Volkow et al., 2004), suggesting that this receptor plays a central role in the development and/or maintenance of addiction. These studies are in agreement with our results as we also observed lower Drd2 expression after stimulus, suggesting that DEX animals may have a "more pleasant feeling" than controls in response to morphine due to lower Drd2 expression.

4.1.4 Restoring dopamine levels as a possible treatment for addiction

As described above, this work shows that rats treated with DEX in the prenatal period are most susceptible to addictive behaviors and further pinpoints changes in the mesolimbic dopaminergic system as the putative underlying cause. More specifically, these animals present a hypodopaminergic status in the NAcc, which could potentially be relevant for the acquisition of the addictive behaviors, if one considers the important role of this neurotransmitter in addiction.

At this point it is interesting to establish a parallel with other conditions in which there is a decrease in dopaminergic transmission such as in Parkinson's disease (PD). These patients present striatal DA depletion and subsequently motor and cognitive deficits from the earliest disease stages (Owen et al., 1992). In contrast to our model, in PD, the DA depletion progresses from the dorsal to the ventral striatum, so that, in early PD, the dorsal striatum is severely depleted,

but the ventral striatum is relatively intact (Farley et al., 1977; Kish et al., 1988). Hence, mild PD provides a unique model for assessing dopaminergic drug effects on neural systems with differential baseline DA levels but with contrasting topography to our model. Current treatment for PD includes the administration of specific doses of L-dopa, a dopamine precursor. L-dopa administration significantly improved, not only the motor symptoms, but also PD non-motor symptoms such as anhedonia (Maricle et al., 1995). Antidepressant drugs that increase dopaminergic transmission (inhibitors of monoamine oxidase inhibitors, catechol-O-methyltransferase, DA reuptake, and DA receptor agonists) have mood-improving effects in these patients (Papakostas, 2006). These findings are quite interesting in the sense that indicate that boosting DA levels can also affect individuals "pleasure/mood", suggesting that it may also have an effect in modulating the reward properties of drugs of abuse.

Considering the low DA levels in the NAcc of our DEX-exposed rats, our strategy also involved the supplementation of these animals with L-dopa/carbidopa at a dosage previously shown to have therapeutic effects in rodent models (Lindner et al., 1997). Side effects usually associated with L-dopa administration such as diskinesia and involuntary movements were not observed in our animals. Moreover, we administered L-dopa daily by oral gavage, to mimic a putative human therapeutic trial, in different treatment regimens prior to the behavioral, neurochemical and molecular analysis. As expected, we were able to restore dopamine levels in the NAcc of DEX-animals and, noticeably, we observed a complete reversal of the addictive phenotype in both contingency and non-contingency tests. Worthy to mention that although acute treatment with L-dopa was able to revert the addictive behavior in both paradigms, it was not sustained in time, while the effects of chronic (3 weeks) L-dopa administration were. These findings are quite interesting in the sense that they confirm that dopamine levels in the NAcc are critical for the development of addictive-like behaviors and open the possibility of new therapeutic approaches for the treatment and/or prevention of addiction.

4.2 Conclusions

In summary, we proved herein that prenatal exposure to DEX leads to enhanced vulnerability to addictive-like behaviors. Elevated GCs during early life seems to have repercussions on the mesolimbic circuit, especially in the NAcc (but also in VTA), where cell loss occurs as a consequence of decreased cell proliferation. Both the shell (“emotive area" of the NAcc) and the core ("motor area" of the Nacc), were structurally affected but only in the shell we could observe compensatory synaptic mechanisms in adulthood. Accompanying these morphological changes, there was a reduced dopaminergic innervation and increased Drd2 in the NAcc of DEX animals. Importantly, DEX exposed animals presented a downregulation of Drd2 gene in response to morphine, and these Drd2 gene expression changes were correlated with the methylation status of the promoter region of this gene. Interestingly, supplementation with L-dopa and the consequent restoration of dopamine levels in the NAcc reverts the addictive-like phenotype. These findings are of clinical and social relevance as they bring a new perspective for the prevention and treatment of addictive behaviors.

Chapter 5

Future Perspectives

Future perspectives

Although we were able to fully revert the addictive phenotype in both contingent and non-contigent paradigms by L-dopa administration, several questions arise: Besides the neurochemical and behavioral changes, are we also able to revert the morphological changes in the mesolimbic system? Are the NAcc core and shell areas equally affected? A systematic and longitudinal morphological study will be crucial to answer this question. Are there any side effects of chronic L-dopa administration? In fact, as previously mentioned, either hyper- or hypo-activity of dopaminergic systems may lead to pathological conditions. While increased dopaminergic transmission in the mesocortical system and amygdala (an important modulator of the mesolimbic system) may result in schizophrenia and increased fear, respectively, reduced DA activity in mesocorticolimbic circuits may lead to memory (hippocampus and frontal cortex) and mood (frontal cortex) deficits and addiction, as demonstrated in this study. Considering the importance of a well-balanced dopamine circuit in the brain, one important question is the identification of the implications of long-term L-dopa administration. For example, in PD patients, it was hypothesized that the beneficial effect of L-dopa on task-switching reflects a remediation of dopamine levels in severely depleted dorsal fronto-striatal circuitry, while, conversely, the impairing effect of L-dopa on reversal learning reflects a detrimental 'over-dosing' of intact ventral fronto-striatal circuitry (Cools et al., 2001; Gotham et al., 1988; Swainson et al., 2000). Furthermore, there is ample evidence that a subset of PD patients, after chronic L-dopa treatment, develop pathological gambling, shopping, sexual and eating behaviors (Merims and Giladi, 2008; Weintraub, 2009). Although it has been suggested that genetic factors may account for the gambling behavior (Eisenegger et al., 2010), other causes remain obscure. We may raise the hypothesis that continuous L-dopa administration resets the dopaminergic systems and make them more prone to addictive-like behaviors. Indeed, L-dopa may be seen as a drug *per se*, given that some PD patients start to continuously increase L-DOPA doses and develop withdrawal symptoms (Merims and Giladi, 2008).

If a specific neurotransmitter is continuously up-regulated, quite often there is a downregulation of its receptor(s) in order to maintain the homeostasis. In this sense, if dopamine is continuously reaching the system in excess due to L-dopa supplementation, this may cause downregulation of D2 receptors for example, which can vulnerabilize the individual for addictive-like behaviors. Indeed, D2 receptor downregulation is a hallmark of addicted brain (Melis et al., 2005; Volkow et al., 2004). According to Volkow and colleagues, this could reflect a decreased sensitivity of reward circuits to concomitant natural rewards in these individuals which will, as a consequence, enhance the need to "seek for pleasure" to activate this circuit (Volkow et al., 2004). It remains to be answered, however, if these animals will display such changes in D2 receptor and also if they will develop addictive-like behaviors with the long-term administration of L-dopa. One should also consider the potential reversion of the addictive phenotype of DEX animals by using drugs with the potential to rescue the methylation status of Drd2 promoter region.

Other pertinent question is the involvement of other dopaminergic systems in the development and maintenance of addiction. Whereas the mesolimbic pathway (especially the NAcc core) is responsible for the rewarding effects of drugs during the initial phases of addiction, the nigrostriatal system assumes an increasingly important role at later stages as drug consumption increases (Everitt et al., 2008; Everitt and Robbins, 2005; Wise, 2009). The NAcc core is important not only for the rewarding effect of drugs of abuse (Wise, 2004) but also mediates the motivational drive or "wanting of a reward" that underlies drug-craving (Berridge, 2007), and assures efficiency of response-outcome associative learning (pavlovian conditioning) (Yin and Knowlton, 2006). However, second-order protocols of drug reinforcement and pharmacological experiments revealed that the dorsal striatum, rather than the NAcc, is essential for drug-seeking behaviour after repetitive drug exposure (Ito et al., 2000). Importantly, lesions in the nigrostriatal dopamine system disrupt stimulus-habit formation (Faure et al., 2005).

Our hypothesis is that addiction occurs in "two-waves": while the initial proneness for addiction is mediated by the dopaminergic mesolimbic circuit,

once the individual has experienced the drug pleasure, it becomes “habituated” and the nigrostriatal dopaminergic pathaway gets involved. Thus, it is of extreme importance to analyze the effects of prenatal DEX exposure in the nigrostriatal pathway, in order to assess if this circuit is also being affected, making these animals more prone to habit formation. Therefore, analysis of the operant behavior to quantify the behavior towards habit would be of relevance, in particular if combined with a morphological, neurochemical and molecular analysis of the brain regions involved in this pathway.

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Appendix 1

"Glucocorticoids and neuro- and behavioural development".

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Glucocorticoids and neuro- and behavioural development

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SUMMARY

Epidemiological evidence links exposure to stress hormones during fetal or early postnatal development with lifetime prevalence of cardiac, metabolic, auto-immune, neurological and psychiatric disorders. This has led to the concept of 'developmental programming through stress'. Importantly, these effects (specifically, hypertension, hyperglycaemia and neurodevelopmental and behavioural abnormalities) can be reproduced by exposure to high glucocorticoid levels, indicating a crucial role of glucocorticoids in their causation. However, there can be important differences in outcome, depending on the exact time of exposure, as well as duration and receptor selectivity of the glucocorticoid applied. The mechanisms underlying programming by stress are still unclear but it appears that these environmental perturbations exploit epigenetic modifications of DNA and/or histones to induce stable modifications of gene expression. Programming of neuro- and behavioural development by glucocorticoids and stress are important determinants of lifetime health and should be a consideration when choosing treatments in obstetric and neonatal medicine.

1. Introduction

Endogenous corticosteroids have a wide spectrum of physiological functions throughout early development, including the regulation of surfactant production by the lungs, salt-water homeostasis, lipid and carbohydrate metabolism, muscle growth and development, immune responses[1] and, as will be discussed, neuro- and behavioural development. Exogenous corticosteroids are indicated in obstetric and pediatric care and are widely used in childhood to treat allergic and/or dermatological symptoms. Corticosteroids are classified as mineralocorticoids or glucocorticoids (GCs), given their respective contributions to ion and water transport in the kidney or in the mobilisation of glucose, respectively. Aldosterone is the prototypic endogenous mineralocorticoid, while cortisol and corticosterone represent the major circulating GCs in humans and rodents, respectively.

Adrenal production of GCs and mineralocorticoids is governed by the secretion of pituitary adrenocorticotropin, controlled by corticotropin-releasing hormone and arginine vasopressin neurons within the paraventricular nucleus (PVN) of the hypothalamus. This top-down sequential activation of the adrenal cortex is tightly regulated through the negative feedback actions of GCs at the pituitary and hypothalamus,[2,3] as well as in higher brain centres, such as the hippocampus and prefrontal cortex (PFC).[4] The hypothalamic–pituitary–adrenal (HPA) axis, like most physiological processes, displays a robust circadian rhythm of activity, but its activation upon perception of a threatening physical, physiological or psychological stimulus (stress) plays a particularly important role in survival. Noxious stimuli occur unpredictably, often at times of the day when the HPA axis is at 'rest'. Mounting an adequate and balanced behavioural and physiological response to adverse stimuli demands finely tuned homeostatic mechanisms that can go awry (transiently or in a protracted fashion) and lead to various brain and other pathologies.

While recent evidence suggests that corticosteroids may produce rapid non-genomic cellular effects[5] via putative membrane-bound receptors,[6] corticosteroids are classically considered to exert their biological action through two ligand-activated transcription factors, mineralocorticoid receptors (MRs) and glucocorticoid receptors (GRs). Ligand binding results in the translocation of the receptor–ligand complexes to the nucleus, where they bind to glucocorticoid response elements (GREs) in the promoter region of target genes to influence gene transcription. Mapping studies have revealed that whereas GRs are ubiquitously distributed, MRs have a more discrete distribution; for example, in the brain MR expression is mainly restricted to one hypothalamic nucleus (the PVN), certain hippocampal subfields and the septum. These patterns of distribution, as well as the pharmacological

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profiles of each receptor, to some extent explain the wide spectrum of physiological and behavioural homeostatic functions that are subject to regulation by corticosteroids. Nevertheless, certain enigmas still surround how specificity of actions may be attained given that hippocampal neurons, for example, co-localise both receptors, share presumably identical GREs, and bind the same ligand (cortisol/corticosterone) in the brain; moreover, MRs and GRs differ in affinity by only some 10-fold.[7] Recent attempts to examine whether nuclear co-regulatory proteins may be differentially recruited by the two receptors provided some promise for resolving this important issue.[8,9]

There are convincing links between exposure to GC during early life and the subsequent development of metabolic syndrome (a cluster of cardiovascular risk factors including hypertension, insulin resistance and hyperlipidaemia)[10,11] as well as susceptibility to auto-immune[12] and neurological[13] and psychiatric disorders.[14] According to the increasingly popular concept of developmental programming through stress and GCs, stimuli or insults that occur during critical periods of development can permanently alter tissue structure and function, producing effects that may persist throughout life. The shaping of brain and other physiological functions through endogenous and exogenous signals (stress, GC) at critical periods of development may be compared to the well-known 'organisational' effects of sex steroids during sexual differentiation of the brain.[15,16]

2. Programming of neuro- and behavioural development by glucocorticoids during early life

2.1. Antenatal period

The placenta and most fetal tissues express GRs from mid-gestation onwards.[17] The expression of MRs is more restricted and, at least in rodents, is only detectable during late gestation.[18] In the fetus, steroid hormones are typically involved in organ development and maturation (e.g. lungs, heart, liver, gut and kidneys) and their action during this time has long-term (organisational) effects on the function of the organ in later life. As exogenous GCs can accelerate these processes, they are used therapeutically in preterm labour as well as in the perinatal period, in particular to help lung maturation.[19] Given their small size and lipophilicity, GCs can access the brain; the magnitude of their damaging effects in the brain depends largely on the particular stage of development when exposure occurs,[20] indicating that there are 'critical windows' of sensitivity to the detrimental effects of GCs on brain tissue; again, these windows of sensitivity may be analogous to those described for sexual differentiation of the brain through the actions of sex steroids.[15]

Spatio-temporal fine-tuning of GC levels during ontogeny is mandatory for appropriate organ maturation while avoiding undesirable side-effects. The fetus has only limited capacity to degrade steroids, especially synthetic (xenobiotic) GCs, which means that even low levels of GCs can have prolonged access to sensitive tissues. This can have detrimental effects especially in tissues undergoing rapid proliferation and differentiation, e.g. the brain. Here, it should be noted that although endogenous GC can bind both GRs and MRs, their effects (at least in the brain) may be regulated in a rheostatic manner, depending on their relative occupation of each receptor[21,22]; on the other hand, the typical GCs used in obstetrics and neonatology (e.g. dexamethasone, betamethasone) tend to be have a greater affinity for GRs which, as will be discussed later, appear to mediate many of the undesired actions of GCs in the brain. In this context, it should be mentioned that fetal GC levels are much lower than those found in the maternal circulation, as a consequence of the action of the enzyme 11β-hydroxylase 2 (11β-HSD2), which regulates the conversion of active GCs (cortisol and corticosterone) into their inactive 11-keto metabolites (cortisone and 11-dehydrocorticosterone, respectively).[23,24] 11β-HSD2 is present in the syncytiotrophoblast, the site of maternal–fetal exchange, where it is well-positioned to serve as the barrier for GC transfer and, consequently, to protect the fetus from the adverse effects of excessive maternal GC. However, this mechanism does not provide complete protection; studies in rats and humans indicate considerable variations in placental 11β-HSD2 activity towards the end of pregnancy,[25] possibly explaining why stress-induced increases in GC secretion in the mother may surmount the placental barrier and produce deleterious effects in the developing fetus. The fetal brain transiently expresses high levels of 11β-HSD2; levels decline after mid-gestation in a manner that varies from one brain area/neuronal population to another.[26]

Experimentally, the effects of increased GC load on the fetal brain have been studied by exposing pregnant rodent dams either to exogenous natural or synthetic GCs or to various stressors (e.g. repeated immobilisation) from the last week of gestation (typically, from embryonic day 14 [E14], with delivery at E21/22, corresponding, approximately, to the second trimester of human pregnancy).[27] It should be noted however, that differences in maternal care[28] may be an important confounding factor in the interpretation of results involving such paradigms, especially those pertaining to brain maturation and eventual psychopathology.

Synthetic GC (e.g. dexamethasone) administration to pregnant rat or mouse dams is frequently used to experimentally increase the foetus' exposure to GC. If at all, exogenous GCs are poor substrates for 11β-HSD2, and they therefore readily cross the placenta.[29] Rats receiving acute dexamethasone (selective GR agonist) during the last third of pregnancy display an anxious phenotype and signs of impaired GC negative feedback in adulthood; such signs are not seen in animals receiving corticosterone (binds to MR and GR) at an equivalent dosage, indicating the differential effects of GR vs MR occupation in the programming of behavioural functions.[30] Interestingly, not all behavioural domains are differently affected by corticosterone and dexamethasone, suggesting that the neurochemical and anatomical substrates which mediate anxiety behaviour might be differentially sensitive to corticosteroids at different developmental stages due to the relative temporal availability of GR and MR in a specific brain area.[31] Prenatal stress affects mood and emotional behaviour in a manner similar to that observed with exogenous GC: as adults, animals that experienced stress in utero display depression-like[32] and hyper-emotional (anxious) phenotypes.[33] The underlying anatomical and neurochemical correlates of these altered behaviours remain ambiguous, but a variety of neurotransmitter systems and signalling cascades can be expected to be affected by antenatal GC exposure. Interestingly, some of the stress-induced behavioural symptoms can be normalised with antidepressants,[34] a finding that matches reports that antenatal GC exposure reduces 5-HT1A receptor expression,[35] a phenomenon causally related to depression-like behaviour.

Apart from these well-known behavioural effects of inappropriate antenatal GC exposure and their clinical projections for the pathogenesis of mood and anxiety disorders, accumulating evidence suggests that other behavioural functions are also subject to GC-mediated programming. Prenatal stress reportedly also results in reduced exploratory behaviour, presumably a reflection of their reduced motivational drive.[36] Locomotor behaviour has been frequently assessed in animals that were exposed to prenatal stress or exogenous GC, but the findings for this behavioural endpoint have been somewhat controversial. Locomotor activity after acute adult pharmacological or environmental challenges in prenatally stressed or GC-treated animals reflects the malprogramming effects of stress/GCs on motivational and appetitive functions. For instance, amphetamine-stimulated locomotion was

found to be accentuated in adult rats that had been stressed during prenatal life, but interestingly, these effects were only manifest when these animals were exposed to an acute stressor in adulthood.[37] These patterns have been attributed to stress-induced alterations in catecholaminergic systems.[38] The behavioural patterns observed in animals are similar to those seen after acute amphetamine application in unmedicated schizophrenic patients; striatal dopaminergic transmission is disturbed in such patients who are characterised by their impaired ability to discriminate between relevant (signal) and irrelevant (noise) signals in the prepulse inhibition (PPI) test.[39] Importantly, there are strong links between maternal stress in human pregnancy and the incidence of schizophrenia in the offspring.[40]

Addictive behaviour represents another behavioural domain that seems to be susceptible to GC programming during early life. An increasing number of studies indicate that the experience of high GC levels during late gestation can predispose individuals for developing drug dependence in adolescent and adult life. In trying to identify the neuromorphological correlates of these behavioural changes, we recently evaluated the impact of prenatal GC exposure on the structure and function of the nucleus accumbens (NAcc), a crucial relay point in the limbic circuitry of reward. We observed that the volume and number of cells in the NAcc were significantly reduced in the adult progeny of dams treated with dexamethasone during their last trimester of pregnancy; these findings were paralleled by reduced rates of cell proliferation in the ventral tegmental area (VTA, an area that sends dopaminergic projections to the NAcc) as well as in the shell and core of the NAcc. In addition, prenatal GC treatment resulted in reduced dopaminergic innervation of the NAcc.[41] Previously, major structural reorganisation of the NAcc and VTA were described during late gestation[42]; the data from Leão et al.[41] therefore indicate that GC may interfere with this process, producing structural and neurochemical alterations that predispose to drug-seeking behaviour.

Most psychiatric disorders (depression, anxiety and schizophrenia) associated with gestational stress are characterised by cognitive impairments. The hippocampus, which is critical for learning and memory, has an abundant population of corticosteroid receptors.[21] A very robust finding in the literature is that hippocampal volumes and numbers of neurons and synaptic contacts are reduced in prenatally stressed rats.[43] Moreover, the adult offspring of mothers subjected to stress during gestation show significant impairments in spatial learning[44] and long-term potentiation, the electrophysiological correlate of memory[45]; these physiological and behavioural impairments most likely occur secondarily to the above-mentioned morphological changes. Nevertheless, and despite many published studies on the association between antenatal stress and reduced cognitive performance in animals, we and others failed to observe deficits in learning ability and memory in the offspring of rats born to mothers that were either stressed or exposed to exogenous corticosteroids during pregnancy.[30,46] Such discrepancies could arise from the quality of the postnatal environment (including health status of the mother); for example, rats born to dexamethasone-treated mothers, but reared by vehicle-treated mothers, were found to display normal spatial learning and enhanced sensitivity to GC negative feedback, compared with animals born to, and raised by, dams that were given dexamethasone during pregnancy.[47] Thus, the early postnatal environment seems to play a critical role in the determination of behavioural and neuroendocrine outcomes.[48] Importantly, these findings also point to the reprogrammability of events initiated during prenatal life by postnatal experience; this reprogramming will, presumably, be compromised by any stressful experience after birth, and it is likely to be limited to periods after birth when neuronal plasticity (within and between brain regions) is still dynamic.

2.2. Neonatal period

Behaviour is critically dependent on brain development, including appropriate neuron numbers and synapses, as well as connectivity between different brain areas. Despite this, there is a notable paucity of information available about how stress and GC experience during early postnatal life affect behaviourally relevant brain structures. Early studies described hippocampal atrophy following exposure to stress or exogenous corticosteroids during early life. This atrophy can be accounted for by dendritic/axonal changes,[49] and a decrease in neuronal number[50]; the latter could be a consequence of changes in neuronal survival and plasticity as a result of reduced neurogenesis and increased apoptosis[51] and altered neurotrophin expression.[52] Since the hippocampus plays a critical role in learning and memory and connects to other brain regions that are involved in the regulation of mood and emotionality, as well as neuroendocrine regulation, these permanent changes can be expected to have a major impact on behaviour and physiology. One such region is the PFC which is implicated in the regulation of cognition, mood, emotion and motivation. Braun and collaborators found that early life stress results in a decrease in dendritic spine density and number of pyramidal neurons in the anterior cingulate cortex, and an increase in these measures in the dorsal part of the PFC,[53] although the functional significance of these findings remains to be elucidated.

The programming effects of stress in early postnatal life (first week of life in rodents corresponds, roughly, to late pregnancy in primates) have been extensively assessed. Most studies employed the so-called maternal separation (MS) stress paradigm, which is based on interference with the mother–infant interaction. Even though phenotypic outcomes are not always easy to compare between studies, possibly due to subtle differences in experimental protocols (e.g. frequency and duration of MS, age at which the paradigm is first applied, level of social deprivation, as well as the quality of the post-separation and post-weaning environments), most studies report behavioural aberrations that may be related to many aspects of mental illness in humans, such as mood and anxiety disorders as well as schizophrenia. For instance, Ellenbroek and colleagues[54] showed that separation of rat pups from their mothers for a single 24 h session results in attention deficits and in a disruption of PPI which, as previously mentioned, suggests schizophrenia-like dysfunction. Interestingly, this behavioural phenotype was only expressed after puberty,[54] thus resembling the temporal profile observed for the onset of schizophrenic symptomatology in patients and in line with the concept that the impact of early-life stress on sensorimotor gating depends on the age at which the stress is experienced.[54,55] The mechanisms through which early-life stress induces these behavioural abnormalities remain unclear at present; however, their responsiveness to antipsychotic drugs[54] indicates hypersensitivity in the dopaminergic system. In addition, MS-stressed rats (A.R. Mesquita, unpublished data) and mice[56] display, as adults, signs of increased learned helplessness, a sign of depressive-like behaviour that can be prevented by antidepressant administration at the end of the MS paradigm.[57] In these types of experiments too, narrow periods of sensitivity to the deleterious effects of GCs or MS have been observed. However, these 'windows of vulnerability' may differ from one behavioural domain to another. For example, prenatal manipulations of the GC milieu do not influence learning and memory[30]; nevertheless, significant impairments of these measures of cognition have been observed in MS-stressed rats (unpublished data).

Variations in maternal care may serve an important role in determining the behavioural outcome of early-life stress. For instance, Liu et al.[58] found that the learning impairments induced by MS were greater in the adult offspring of animals reared by

poor-caring vs those fostered by good-caring mothers. Similar reports exist with respect to quality of maternal care and MS-induced deficits in sensorimotor gating, mood and anxiety.[59,60]

Besides the behavioural programming described above, early-life events are known to affect somatic well-being on measures of body mass and growth. However, their impact on the timely reaching of critical milestones has not been studied extensively. Recent studies in our laboratory[61] have shown that MS on postnatal days 2–15 delays the acquisition of neurological reflexes that are dependent on vestibular and cerebellar function. On the other hand, MS-treated animals showed earlier eye and ear opening, indicating advanced physical maturation. Similarly, Neal[62] reported that dexamethasone administration during the first postnatal week leads to retardation of acquisition of the above-mentioned neurological reflexes. While some authors have suggested that delayed myelination can account for these neurological deficits,[63–65] we recently found that the serotoninergic system might also be involved[61]; specifically, MS-experienced rats showed increased serotonin turnover in the dorsal raphe nucleus.

It is important to note that the neurochemical bases of MS are poorly understood. Importantly, GCs do not seem to be directly responsible for any of the above-mentioned effects since GCs are not secreted in response to stress in the rodent neonate[66]; this so-called stress hyporesponsive period is confined to about the first two weeks of the rodent's postnatal life. The fact that MS and postnatally administered GCs may lead to similar behavioural and neuroendocrine outcomes, suggests possible redundancy of signals that converge on stress-sensitive neural substrates. Resolution of this issue remains a challenge for future research.

3. Are animal studies translatable into clinical practice?

In its 2001 Mental Health Report, the World Health Organization observed that, 'Contrary to popular belief, mental and behavioural disorders are common during childhood and adolescence. Inadequate attention is paid to this area of mental health'. The Report went on to point out that, 'Mental and behavioral disorders of childhood and adolescence are very costly to society in both human and financial terms. The aggregate disease burden of these disorders has not been estimated, and it would be complex to calculate because many of these disorders can be precursors to much more disabling disorders during later life'. Happily, these concerns are currently being addressed,[67] and some of the results discussed above indicate that the various experimental paradigms and animal models may be relevant to human health.

Glucocorticoids are indicated in obstetric and paediatric conditions, such as infection, connective tissue and allergic disorders, and are commonly used in obstetric practice to accelerate lung maturation in cases of threatened preterm labour; the latter affects up to 10% of pregnancies. While GC therapy makes a significant contribution to the reduction of infant mortality,[68] there is considerable debate about which GC should be used, and according to what regimen, for a particular underlying condition, so as to induce the minimum degree of damage. Only a few studies have assessed the impact of perinatal GC exposure in humans and there is usually sparse follow-up information available. The significant findings that have so far emerged may be summarised as follows: (i) children exposed to dexamethasone and who were born at term, had increased emotionality, unsociability/social withdrawal and general behavioural problems[69] as well as persistent impairments in tests of verbal working memory[70]; (ii) the offspring of women given multiple doses of antenatal GCs because of risk of preterm delivery have reduced head circumferences, and show significantly increased aggressive/violent behaviour and attention deficits.[71] Likewise, maternal stress has been shown to induce long-term programmes in the fetus that prevail at least until middle childhood[72] and, as shown by recent work from Rachel Yehuda's laboratory, children of mothers with post-traumatic stress disorder display altered cortisol levels that are accompanied by signs of behavioural distress during the first nine months of life.[73] In addition, the stress-induced release of striatal dopamine is reduced in subjects that had experienced poor parental bonding, indicating dysregulation of neurotransmitter systems by stressful experiences during early life.[74]

These clinical reports reveal a pattern similar to that observed in experimental animal models, but which itself is insufficient to allow immediate extrapolation to humans. Taken together, they do, however, point to the long-term negative impact that high GC levels can have on the development of the brain and behaviour. However, one should consider that risk–benefit judgements are confounded by the fact that subjects born preterm are already at risk for delayed neurodevelopment. A recent systematic review concluded '… prenatal steroids reduce the occurrence and severity of … health problems in the first few weeks of life …. However, these benefits are associated with a reduction in some measures of weight and head circumference at birth, and there is still insufficient evidence on the longer-term benefits and risks'.[75]

4. Mechanisms of programming by stress and glucocorticoids

Stable alterations of gene expression that are independent of changes of the DNA sequence can be caused by epigenetic mechanisms. Covalent modifications, e.g. through methylation of DNA at cytosine residues of CpG dinucleotides or through various histone modifications, are among the best known of these. Work by Avishai-Eliner et al.[76] and Liu et al.[58] showed that social environmental cues, such as postnatal handling, promote maternal care, resulting in long-lasting increases in GR expression in the hippocampus of rat pups raised by high-caring mothers. Recently, pioneering work in the laboratories of Michael Meaney and Moshe Szyf demonstrated that demethylation at the nerve growth factor1A (NGFI-A, also known as Egr1, zif268 or Krox 24) binding site may underlie the increased expression of GRs; NGFI-A is a key transcription factor in the GR promoter, activating transcription at the GR 1_7 promoter.[77,78] Studies in vitro and in vivo demonstrated that demethylation at the NGFI-A binding site in the rat GR 1_7 promoter is initiated by enhanced serotonin input and activation of 5-HT_7 receptors which, in turn, activate the cAMP/protein kinase A pathway, increasing NGFI-A expression; NGFI-A binding to its recognition element in the 1_7 GR promoter initiates cytosine demethylation and leads to the recruitment of a histone acetylase (cyclic AMP response element-binding protein), an event that could potentially explain enhanced transcription of the GR.[78] Interestingly, cross-fostering studies revealed a direct effect of maternal care, rather than genetic inheritance, suggesting trans-generational transmission of these epigenetically programmed traits.[79] While pointing to mediation through serotonin, it should be mentioned that GCs themselves have been shown to have the potential to induce epigenetic changes in non-neural tissues; for example, chronic treatment of rat hepatoma cells with GC induces fast chromatin remodelling in an enhancer region of the GR target gene tyrosine aminotransferase, an event followed by recruitment of the transcription factor hepatic nuclear factor-3 (HNF3) and slow, but persistent DNA demethylation at GC-responsive units within the enhancer.[80,81]

5. Conclusions

An abundance of data in rodents and humans indicates that exposure to GC at specific time-points during ante- and postnatal development can induce undesired cardiovascular, metabolic, neuroendocrine and behavioural phenotypes in adulthood. The

concept that stress and GCs act by reprogramming the genetically determined phenotype through epigenetic mechanisms is becoming increasingly popular; more intriguing is the evidence that some of these acquired changes may be transmitted across generations. Improvements in our understanding of these latter two mechanisms are a major challenge for researchers in this field; in particular, it will be of interest to discover ways in which human health can be improved by over-writing these changes.

Conflict of interest statement

None declared.

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Appendix 2

"Potential programming of dopaminergic circuits by early life stress".

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Psycopharmacology, *accepted for publication*

Potential programming of dopaminergic circuits by early life stress

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Keywords

Programming, glucocorticoids, dopamine, mesolimbic, mesocortical, nigrostriatal, tuberoinfundibular, addiction, depression, anxiety, nucleus accumbens, ventral tegmental area.

Abbreviations

DA: dopamine; DAergic: dopaminergic; TH: tyrosine hydroxilase; L-DOPA: levodopa; early life stress (ELS); ADHD: attention deficit hyperactivity disorder; HPA: hypothalamus-pituitary-adrenal axis; GC: glucocorticoids; EPM: elevated plus maze; OF: open field; VTA: ventral tegmental area, NAcc: nucleus accumbens.

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Abstract

Stress and high levels of glucocorticoids during pre- and early postnatal life seem to alter developmental programs that assure dopaminergic transmission in the mesolimbic, mesocortical and nigrostriatal systems. The induced changes are likely to be determined by the ontogenetic state of development of these brain regions at the time of stress exposure and their stability is associated with increased lifetime susceptibility to psychiatric disorders, including drug addiction. This article is intended to serve as a starting point for future studies aimed at the attenuation or reversal of the effects of adverse early life events on dopamine-regulated behaviors.

Introduction

The catecholaminergic neurotransmitter dopamine (DA; 4-[2-aminoethyl]benzene-1,2-diol) is prominently involved in a number of brain functions such as cognition, emotion, reward and motor control (Nieoullon and Coquerel, 2003; Wise, 2008), as well as neuropsychiatric disorders such as schizophrenia, drug addiction, attention deficit hyperactivity disorder (ADHD), and Parkinson's disease (Genro et al., 2010; Howes and Kapur, 2009; Melis et al., 2005; Oades et al., 2005; Piazza and Le Moal, 1996; Weiner, 2002). DA is also implicated in the regulation of depression, social behaviour and pain processing (Kapur and Mann, 1992; Wood, 2008). DAergic activity changes in a graded fashion over the lifespan, resulting in the manifestation of age-related behavioral profiles and neurological conditions. In rodents, DA-producing neurons begin to form during early mid-gestation (E10.5); at E12.5, these neurons start to express tyrosine hydroxylase, the rate limiting enzyme in the conversion of L-tyrosine into L-DOPA (3,4-dihydroxyphenylalanine) and subsequently, into DA. Thereafter, the generation of DAergic cells gradually declines, and importantly, DAergic neurons increasingly undergo two peaks of apoptosis: immediately after birth and again, during the second week of postnatal life (Burke, 2004; Oo and Burke, 1997). It is estimated that adult human and rat brains contain some 600,000 and 45,000 DAergic cells, respectively (German and Manaye, 1993) – a relatively small proportion of the total population of neurons in the brain.

Knowledge of the various transcription factors that contribute to the

ontogeny of DAergic neurons has grown considerably in the last decade (Prakash and Wurst, 2006). On the other hand, besides knowing that increased levels of reactive oxygen species derived from neurotoxins and that, perhaps, some therapeutic agents can compromise the viability of DA neurons, our understanding of other environmental and physiological factors that are responsible for the survival and demise of these neurons is surprisingly limited. In light of the narrow window within which DAergic cells are born, and the fact that the fate of the developing nervous system is particularly sensitive to environmental influences (Bjorklund and Dunnett, 2007), studying how early life events may sculpt DAergic circuits, and therefore predispose individuals, or indeed contribute to their resilience to DA-related disorders later in life, is particularly important.

This article focuses on how early life stress, implicated in a number of behavioral disorders associated with DAergic dysfunction, may exert its effects. Notably, a number of studies, mainly carried out in adult animals, have shown that glucocorticoids (GC), the primary humoral effectors of the physiological response to stress, can upregulate TH synthesis and therefore, DA production (Makino et al., 2002; Markey et al., 1982; Ortiz et al., 1996). While these effects are likely to reflect direct GC actions on TH neurons following their activation of glucocorticoid receptors (GR, which have transcriptional properties), indirect regulation of TH synthesis through intersecting pathways cannot be excluded (Otten and Thoenen, 1975).

Programming of behaviour by early life stress (ELS)

Adversity during early life, including physical and emotional neglect and traumatic experiences, can induce persistent effects on physical and mental health (Heim and Nemeroff, 2002; Teicher et al., 2003). Specifically, there is now well-documented evidence that adversity in childhood increases the risk for development of conduct disorders, personality disorders, ADHD, major depression, posttraumatic stress disorder, schizophrenia, anxiety and addictive disorders (Agid et al., 1999; Bernet and Stein, 1999; Chapman et al., 2004; Dube et al., 2003; Heim and Nemeroff, 2001; Kendler et al., 2004; Weiss et al., 1999; Young et al., 1997). The clinical importance of these findings can be better

appreciated when one considers that some 80% of adults who experienced abuse or neglect in early life are predicted to suffer at least one episode of a psychiatric disorder such as depression and anxiety or a behavioral disorder such as addiction (Edwards et al., 2003; Espejo et al., 2007; Gutman and Nemeroff, 2003; Heim and Nemeroff, 2001; McFarlane et al., 2005). In contrast, the predicted incidence of such disturbances is much lower in women abused as adults (Brown and Moran, 1994; McCauley et al., 1997), a finding that points to the existence of critical time windows during which the organism is particularly sensitive to stress-induced pathology later in life.

Most of the above clinical conditions are linked to impaired DAergic transmission and are likely to be underpinned by structural alterations in the nervous tissue which, in turn, translate into a resetting of homeostatic mechanisms that promote either adaptation or pathology. Much attention has been recently focused on the ability of ELS to programme the hypothalamic-pituitary-adrenocortical (HPA) axis (Heim et al., 2008; Tarullo and Gunnar, 2006). Information about the physical and psychological environments converges on this axis, which, through its secretion of glucocorticoids (GCs), determines the organism's physiological and behavioral response. In a simplistic way, physical or physiological stress activates the production of corticotrophin-releasing factor (CRF) in the hypothalamus, which in turns binds to specific receptors in pituitary cells stimulating the production of adrenocorticotropic hormone (ACTH). ACTH is then transported to adrenal glands, culminating with the secretion of GCs (cortisol in humans and corticosterone in rodents). GCs have a series of metabolic effects for alleviating the harmful effects of stress an act through negative feedback to both the hypothalamus and the anterior pituitary, once the state of stress subsides. Yet, it should be noted that stress response involves far more than the elevation of GCs and, as a consequence, the stress effects cannot be confined to elevations of GCs. Indeed, it has been shown that severe forms of stress can also result in decreased levels of GCs release; as an example, insufficient GC signaling may lie beneath the pathophysiology of some stress-related disorders such as posttraumatic stress disorder (Raison and Miller, 2003).

Importantly, *in utero* exposure to GC/stress has also been found to be

associated with long-lasting deficits in cognitive, mood and affective, as well as addictive and affiliative behaviors in humans (French et al., 1999; Heim and Nemeroff, 2001; MacArthur et al., 1982; Malaspina et al., 2008; Sinha, 2001) and in animal models (Caldji et al., 1998; Liu et al., 1997; Oliveira et al., 2006; Rayburn et al., 1997). It is of interest to note that GC administration or separation of rodents from their mothers during the first week of postnatal life shifts the timing of a number of neurodevelopmental milestones. Such treatments delay the acquisition of neurological reflexes (e.g. righting and postural reflexes, negative geotaxis) that depend on vestibular and cerebellar function (Ellenbroek et al., 2005; Mesquita et al., 2007), while advancing eye and ear opening. On the other hand, prenatal stress advances the time of ear-flap and eye opening (Secoli and Teixeira, 1998). While these neurodevelopmental changes may reflect delayed myelination (Ferguson and Holson, 1999; Murphy et al., 2001; Valkama et al., 2000), there is strong evidence for a role of altered catecholaminergic transmission in the vestibular region, the ventral tegmental area (VTA) and raphe nuclei (Mesquita et al., 2007). Since these brainstem structures project to corticolimbic structures, it is plausible that their altered activity impacts on neuroendocrine (HPA axis activity) and behavioural functions.

In the majority of cases, the behavioral consequences of ELS are attributable to transient or persistent dysregulation of GC secretion which, in turn, is causally related to increased susceptibility to depression and anxiety disorders (Carroll et al., 1976; Heim et al., 2001; Heim et al., 2000; Holsboer, 2001; Yehuda et al., 1991), impaired social behaviors (Rinne et al., 2002), ADHD (Sullivan and Brake, 2003; Swanson et al., 2007) and drug abuse (Huizink et al., 2006; Prendergast and Little, 2007), all of which appear to involve an altered DAergic tone. Yet, whereas severe stress is usually associated with HPA-mediated pathology, mild stressful experiences may be linked to 'positive' effects and/or resilience in rodents (Catalani et al., 1993; Levine, 1957; Macri et al., 2009).

Pioneering work by Meaney and colleagues showed that the HPA axis can be epigenetically programmed (McGowan et al., 2009; Weaver et al., 2004) and further, that epigenetic (methylation) marks may be transmitted across

generations. Other studies have shown that ELS-induced alterations in the epigenetic control of the activity of the HPA axis are associated with enduring expression of impaired cognitive- and depressive-like behavior in rodents (Murgatroyd et al., 2009). It remains to be demonstrated whether drugs with the potential to reverse DNA methylation (e.g. 5-aza-2'-deoxycytidine, already approved for use in cancer chemotherapy), can reverse the central effects of ELS. It should be noted that stress also leads to transient epigenetic alterations by deacetylation of histones with concomitant changes in behavior; such changes are drug-reversible with inhibitors of histone decaetyltransferase (HDAC) which have also proved effective in reversing age-dependent cognitive decline in experimental animals (Peleg et al., 2010).

Linking ELS to DAergic activity

The developing, postnatal and adolescent brain is characterized by high levels of neuroplasticity and reorganization. Given the evidence that prenatal, perinatal and early postnatal life represent windows of susceptibility to the long-lasting effects of stress on brain pathologies related to DAergic dysfunction, it is reasonable to assume that DAergic circuits are direct or indirect targets of stress and stress hormones (GC). The clinical studies about ELS, DAergic transmission and psychiatric conditions are sparse. Nevertheless, it has been shown that low parental care is associated with higher cortisol and, consequently, dopamine levels in response to a psychosocial stress task (Pruessner et al., 2004). Moreover, it has been shown that a polymorphism in the DA enzyme COMT and childhood trauma may interact together to contribute to the risk of developing psycopathological personality traits (Savitz et al., 2010), suggesting a close link between DA, stress and mental illness. Stress may influence DAergic i) cell fate; ii) neuron metabolism (DA production and turnover); iii) neuron morphology; and/or iv) receptor expression and synaptic transmission. Its effects, whether transient or permanent, can thus be expected to have long-term consequences on the shaping and expression of DA-regulated behaviors. Notably, the consequences of ELS appear to be different upon the different DAergic circuits. Perinatal stress seems to decrease steady state levels of DA in the PFC and

increase it in both the NAcc and striatum (Boksa and El-Khodor, 2003), suggesting different vulnerabilities of the mesocortical, mesolimbic and nigrostriatal pathways to the deleterious effects of stress. A different timing of development and maturation of neurons of each circuit or different intrinsic sensibilities may explain this differences, although this needs to be further explored.

DAergic neurons show marked anatomical and functional heterogeneity. They are principally located in the diencephalon, mesencephalon and olfactory bulb (Bjorklund and Dunnett, 2007); the largest number (~ 90%) is found in the ventral part of the mesencephalon. These mesencephalic neurons are the origin of the so-called mesocortical, mesolimbic and nigrostriatal DAergic systems (Figure 1); a fourth set of DAergic neurons, less relevant to this article, follow the tuberoinfundibular pathway to terminate in the hypothalamo-pituitary unit. Both the mesolimbic and mesocortical systems arise from the ventral tegmental area (VTA). While the mesocortical pathway terminates in the cortex, where it is thought to control cognition and executive functioning, the mesolimbic projections innervate limbic areas such as the nucleus accumbens (NAcc), amygdala and hippocampus and serve in the regulation of memory, motivation, reward and addiction. Due to their common origins in the VTA, these two pathways are jointly referred to as the mesocorticolimbic system, although the activity of each is subject to regulation by distinct feedback loops. DAergic neurons that project from the substantia nigra to the striatum comprise the nigrostrial system; this pathway is mainly implicated in the initiation and maintenance of motor behavior. As already mentioned, these midbrain DAergic neurons are formed during early development, according to a rostrolateral to caudomedial gradient (Bayer et al., 1995) and their fibers project to terminal fields in the mesocortical and nigrostrial areas (Kawano et al., 1995). All these DAergic systems are thought to be fully mature and functional by the first few weeks of postnatal life in both rats (Voorn et al., 1988) and humans (Prakash and Wurst, 2006), although some others have suggested that this maturation can occur until early adulthood in the prefrontal cortex (PFC) for example (Benes et al., 2000).

Indicating that the developing and maturing DAergic systems are highly

sensitive to perturbations, including stress and high levels of GC, experiments from our laboratory found that GC administration during late gestation (E18-19) significantly increases the ratio of apoptotic to proliferative cells in the VTA, resulting in a sustained decrease in DAergic inputs to the NAcc (Leao et al., 2007). The same treatment altered a number of DA-regulated behaviors, including anxiety (Oliveira et al., 2006), prepulse inhibition and drug preference (Leão, Rodrigues et al., unpublished observations). Some of these behavioural changes might be additionally explained by prenatal stress-induced variations in DA turnover in the PFC (Fride and Weinstock, 1988) and NAcc (Alonso et al., 1994), reflected in altered sensitivity to certain drugs of abuse. Remarkably, ELS also adjusts DAergic tone in response to certain drugs of abuse and to stress. For example, progeny from stressed dams display higher DA output under basal conditions and in response to amphetamine or cocaine exposure (Kippin et al., 2008; Silvagni et al., 2008). Similarly, maternal separation (MS) enhances DA release in the NAcc following amphetamine administration (Hall et al., 1999; Moffett et al., 2006). Variations in MS and handling cause changes in ethanol and cocaine self-administration with concomitant changes in DA receptors in the NAcc (Moffett et al., 2007). A short-term insult such as perinatal anoxia results in long-term alterations in the DAergic response to tail-pinch (Brake et al., 1997). ELS also affects DA transporter (DAT) and DA receptor expression, function and sensitivity. The role of DAT1 which regulates DAergic tone by clearing DA in the synaptic cleft may be significant in this respect; this is exemplified by the fact that drugs such as cocaine induce pleasurable feelings by stimulating DAT1 activity. In this vein, it is interesting to note that MS decreases DAT levels in the NAcc (Brake et al., 2004; Meaney et al., 2002).

Besides their well-described ability to determine neuronal cell fate (Yu et al., 2010) and neuronal morphology in the hippocampus (Fujioka et al., 2006; Seidel et al., 2008; Sousa et al., 2000) and PFC (Bock et al., 2005; Cerqueira et al., 2007a; Cerqueira et al., 2007b; Michelsen et al., 2007; Murmu et al., 2006), stress (early or in adulthood) and GCs have been found to influence the morphology of neurons in the mesocorticolimbic circuitry. In the above-mentioned study by Leao et al. (2007), we observed that GC during late gestation results in a significant reduction in the volume of the NAcc with significant changes in spine

density and morphology (Leão, Rodrigues et al., unpublished observations). These findings were extended by recent work from Martinez-Tellez *et al*. (Martinez-Tellez et al., 2009) who demonstrated decreased spine densities in the NAcc and hippocampus of the progeny of rat dams subjected to restraint stress from mid-late gestation. Since spine density and morphology correlates with synaptic transmission and plasticity (Blanpied and Ehlers, 2004; Luscher et al., 2000; Murthy et al., 2001), these findings indicate that ELS interferes with transmission at neuronal networks. Interestingly, however, prenatal stress has been shown to alter the relative number of mushroom spines in the PFC (Michelsen et al., 2007); as compared to other spine types, mushroom spines are relatively stable, i.e. do not show spontaneous appearance and disappearance, suggesting a mechanism through which early life manipulations of the GC milieu might leave a permanent trace within mesocorticolimbic pathways.

As mentioned earlier, there is a convincing correlation between adverse experience during early life and depression (Edwards et al., 2003; Felitti et al., 1998; McCauley et al., 1997). Given that the therapeutic efficacy of the antidepressant tricyclic drugs was based on their ability to inhibit norepinephrine (NE) and serotonin (5-HT) transporters, the role of dopamine in depression was less explored over the years. Yet, ELS has long-term effects not only on noradrenergic and serotonergic but also on DAergic circuits (Schneider et al., 1998; Takahashi et al., 1992). Research, based on measurements of DA metabolites, suggests that a hypo-DAergic state may be causally related to the depressed state; for example, depressed patients display reduced cerebrospinal fluid levels of homovanillic acid (HVA) (Mendels et al., 1972) and levels of dihydroxyphenylacetic acid (DOPAC) are reduced in the caudate, putamen and NAcc of depressed suicide victims (Bowden et al., 1997). Hypofunction of the mesocorticolimbic DA system is thought to underlie anhedonia, a cardinal symptom in depression, as well as the loss of motivation experienced by subjects suffering from cognitive and mood disturbances. Interestingly, boosting DA levels through administration of L-DOPA to Parkinsonian patients improves their depressive symptoms (Maricle et al., 1995), and antidepressant drugs that increase DAergic transmission (inhibitors of monoamine oxidase inhibitors, catechol-O-methyltransferase, DA reuptake, and DA receptor agonists) have

mood-improving effects (Papakostas, 2006). It should be noted, however, that other authors failed to observe any anti-depressant actions of L-DOPA (Cools, 2006; Shaw et al., 1980). Again, it is important ho highlight that disruption of other monoamines transmission such as NE may underlie depression basic symptoms. In fact, drugs that act selectively to enhance either DA or NE transmission can produce a clear antidepressant action; moreover, DA is able to modulate noradrenergic transmission and vice-versa (El Mansari et al., 2010). Importantly, some strategies acting on both systems have been shown to be more effective, not only in drug naive patients, but also in treatment-resistant depression (El Mansari et al., 2010).

Schizophrenia, a neurodevelopmental disorder in which symptoms are first seen in teenagers and young adults, is clearly associated with disturbed DAergic tone. Childhood malnutrition and viral infection, as well as obstetric complications or genetic defects are thought to be triggers of the disease (Bayer et al., 1999; Cannon et al., 2003; Murray and Fearon, 1999), although in the more recent 'two-hit' hypothesis on the origins of schizophrenia, stress during young adulthood has been added to the list of aforementioned neurodevelopmental factors in disease causation (Bayer et al., 1999; Malaspina et al., 2008; Pantelis et al., 2003). Indeed, the role of stress in schizophrenia has recently received support from studies in humans (Weber et al., 2008) and animals (Choi et al., 2009). Currently, the leading hypothesis is that a deficit in DA activity at D1 receptors in the PFC is responsible for the cognitive impairment and negative symptoms of schizophrenia, while hyperstimulation of D2 receptors by subcortical (mesolimbic) DA is responsible for core ("positive") disease symptoms (hallucinations, delusions) (Toda and Abi-Dargham, 2007).

Early life adversity such as lead exposure, drug abuse (smoking, alcohol, cannabis), low birth weight or premature birth can increase the risk for developing ADHD, although genetic factors also play a substantial role on its etiology (Sullivan and Brake, 2003; Swanson et al., 2007). A dysfunction of DAergic mesocortical (but also mesolimbic (Russell et al., 1995)) transmission is thought to underlie ADHD, though the involvement of other neurotransmitters such as noradrenaline has to be considered (Oades et al., 2005). Briefly,

hypofunctioning (especially) of the DAergic transmission in the right PFC seems to occur in ADHD, and this is particularly interesting since ELS can induce lateralized changes on PFC DAergic function (Fride and Weinstock, 1988). Other findings support the involvement of DA in ADHD: i) changes in DAT expression were found in ADHD patients compared to controls (Dougherty et al., 1999); ii) genetic analysis identified an association between specific alleles of D4 receptor (Faraone et al., 2001; Rowe et al., 1998) and of DAT (Waldman et al., 1998) with ADHD and iii) the use of methylphenidate which blocks DA reuptake into the cell by the DAT as the most common treatment for ADHD.

Besides its role in specific types of behavior, the DAergic mesocortical pathway seems to be particularly important in buffering HPA-response to stress. This circuit frequently shows functional hemispheric asymmetry that can be modulated by early life adversity. For example, DA metabolism is significantly higher in the right infralimbic cortex of handled pups (positive stress) than non-handled, and this has been suggested to underlie, in part, to their superior capacity to adapt to stress and restraint HPA activity (Sullivan and Dufresne, 2006).

It emerges from the above brief overview that ELS may result in either hyper- or hypo-activity of DAergic systems. Thus, increased DA transmission in the mesocortical system and amygdala (a part of the mesolimbic system) may result in schizophrenia and increased fear, respectively, whereas reduced DA activity in mesocorticolimic circuits may lead to memory (hippocampus and frontal cortex) and mood (frontal cortex/ventral striatum) deficits (Figure 1). Notably, hypoactivity in the hippocampus will likely result in increased GC secretion which, in turn will exacerbate neuronal dysfunction and behavioral anomalies. On the other hand, stress-induced hypoactivity in the mesocorticolimbic DAergic system is likely to enhance novelty-seeking and addictive behaviors, a subject that will be dealt with in greater detail in the following section.

ELS targets mesocorticolimbic DAergic circuits: impact on additive behavior

Despite their diverse chemical structures, cellular mechanisms of action and

physiological and behavioral manifestations, all drugs of abuse share a common property: they all act as positive reinforcers and, as a consequence, induce addiction. Increased DA release in the NAcc characterizes drug reinforcement, but also other consumatory behaviors such as sex and food; thus the VTA-NAcc pathway is appropriately also known as the "reward pathway" (Piazza and Le Moal, 1996). Subjective feelings of "pleasure" or hedonia after consummation are experienced as a result of parallel activation of mesocortical DAergic circuits. Though traditionally DA is seen as responsible for the "liking" part of a reward, more recently it has been suggested that DA is not essential/sufficient to mediate changes in hedonic behavior. In fact, DA seems to contribute substantially for incentive salience, i.e., the "wanting" part of the process rather than the "liking" part (Berridge, 2007). Nevertheless, one way or another, DAergic transmission is certainly playing a vital role in the rewarding process. Perusal of the literature indicates that an apparently intricately-regulated balance between hypo- and hyper-DAergic states underlies an individual's cycles of drug-seeking behavior and abuse. Thus, hyper-DAergic states seem to enhance the motivational or rewarding properties of drugs of abuse and hypo-DAergic states appear to enhance drug-seeking behavior in parallel with reductions in the perceived motivational impact of 'natural' rewards such as food and sex (Diana et al., 1998; Diana et al., 1993; Melis et al., 2005; Parsons et al., 1991).

In the context of this review, it is interesting to note that stress or GC in adulthood enhance DA release in the NAcc (Kalivas and Duffy, 1995; Rouge-Pont et al., 1998; Takahashi et al., 1998; Thierry et al., 1976) and increase the strength of excitatory synapses on mesencephalic DA neurons (Saal et al., 2003), while inducing similar patterns of dendritic organization in the NAcc (Liston et al., 2006; Robinson et al., 2001; Robinson and Kolb, 1999). Drugs of abuse and stress display other common biobehavioral features: while repeated exposure to the same (Kalivas and Stewart, 1991) or novel stressors (Dallman et al., 1994) leads to "facilitation" or "sensitization" of behavioral responses, stress as well as drugs of abuse (Robinson and Becker, 1986; Sorg and Kalivas, 1991; Stewart and Badiani, 1993) are accompanied by augmented DA release in the NAcc (Doherty and Gratton, 1992; Kalivas and Stewart, 1991). Several other lines of evidence

derived from animal studies suggest that stress and GC may act, like drugs of abuse, to induce positive reinforcement: i) GC facilitate the psychomotor stimulant effects of cocaine, amphetamine and morphine (Cools, 1991; Marinelli et al., 1994); ii) depletion of GC by adrenalectomy reduces drug and alcohol consumption (Fahlke et al., 1994; Marinelli and Piazza, 2002; Marinelli et al., 1997a; Marinelli et al., 1997b); iii) GC levels before drug self-administration are positively correlated with the extent of low-dose self-administration of cocaine (Goeders and Guerin, 1994; Piazza et al., 1991); and iv) naive rats self-administer GC in a dose-related manner (Piazza et al., 1993).

Addiction is determined by a number of factors other than the intrinsic properties of a given drug. In an interesting series of studies aimed at understanding individual differences in predisposition to drug abuse, Piazza and colleagues found that the liability of rats to self-administer drugs can be predicted by the response of mesolimbic DAergic neurons to stress; specifically, animals that were more sensitive to the DA-releasing actions of stress were more likely to display addictive behavior (Piazza and Le Moal, 1996; Piazza et al., 1991). Polymorphisms in the human DA receptor 2 (Blum et al., 1990; Noble, 2000) and DA receptor 1 (Batel et al., 2008; Huang et al., 2008) have been associated with increased propensity to alcohol and other substances of abuse, gambling and compulsive shopping; however, there is no information available with respect to the physiological responses of the affected individuals to stressful stimuli. Val158Met polymorphism in catechol-O-methyltransferase gene, which is involved in DA degradation, has been associated with schizophrenia, bipolar disorder and also with substance abuse, although some other studies have failed to prove so (Hosak, 2007). Exposure to both, drugs with abuse potential and stress trigger neuroadaptative changes in DAergic circuits that ultimately determine neurochemical and behavioral responses. This indicates that the activity of addiction-related DAergic pathways are subject to programming by lifetime experiences, with the final neurochemical and behavioral phenotype reflecting both, genetics and experiential history.

Early life adversity, i.e. during the ontogeny of mesocorticolimbic DAergic systems, has been repeatedly shown to induce addiction to a variety of drugs of abuse in adult animals; a few examples from the literature follow: i) exposure of

dams to restraint stress leads to persistent behavioral and neurobiological alterations that are associated with increased consumption of psychostimulants in the adult offspring (Kippin et al., 2008); ii) animals stressed during prenatal life display earlier sensitization to the behavioral effects of amphetamine, although their motor responses to the drug do not differ from those of non-stressed animals (Henry et al., 1995); iii) separation of pups from their mothers and/or littermates during the early postnatal period, a procedure that leads to hypersecretion of GC (Ladd et al., 2000; Liu et al., 1997; Mesquita et al., 2007), advances the time of acquisition of cocaine self-administration (Moffett et al., 2006) and enhances cocaine-induced locomotor activity as well as behavioral sensitization (Brake et al., 2004; Kikusui et al., 2005; Li et al., 2003); and iv) MS stress also increases alcohol and drug consumption during adulthood although handling or brief MS – a manipulation that results in reduced GC secretion and responses to stress (de Kloet et al., 1996; Levine, 1967) – decreases voluntary ethanol intake (Huot et al., 2001; Ploj et al., 2003). Though human studies are sparse, it has been shown that childhood adversity is associated with blunted subjective responses to reward-predicting cues as well as dysfunction in left basal ganglia regions implicated in reward-related learning and motivation (Dillon et al., 2009), suggesting that in humans ELS can also change the impact of a reward.

The above examples illustrate the impact that ELS can have on the development of addictive behavior and reinforce the view that the neuronal circuits involved in the regulation of such behavior are particularly vulnerable to programming by stress and GC during the prenatal, perinatal and early postnatal periods. Part of these effects are, as already mentioned, mediated by stress and GC participating in the regulation of the birth and maturation and DAergic cells in the mesolimbic system (Kawamura et al., 2006; Leao et al., 2007). We also noted that the adult progeny of dams stressed during gestation have significantly fewer TH-positive (DAergic) fibers of the NAcc (Leao et al., 2007). Interestingly, these presumably hypo-DAergic animals were recently found to have a greater propensity for developing drug-seeking behaviors (Leão, Rodrigues et al., unpublished observations). The above findings may be explained, at least partly, in terms of hypersensitivity to the DA-releasing effects of drugs of abuse,

evidenced by the increased release of DA in response to amphetamine or cocaine in rats that have either experienced prenatal stress (Kippin et al., 2008; Silvagni et al., 2008) or maternal deprivation stress in the first postnatal days (Hall et al., 1999).

Finally, alterations in the thresholds required for activation of DA type-1 (D1) and type-2 (D2) receptors by DA (Volkow et al., 2004) could represent a potential mechanism through which ELS causes drug-seeking behavior and ultimately, addiction. One hypothetical model predicts that the ratio of D1 to D2 receptors in the NAcc determines the sensitivity to "natural rewards" *vs.* the proclivity to "seek for pleasure" through drug abuse (Volkow et al., 2004). Earlier studies in rats described late gestational stress-induced increases in the expression and ligand binding capacity of D2 receptors in the frontal cortex, hippocampus and core of the NAcc (Berger et al., 2002), with concomitant decreases in the number of D1 receptors in the NAcc. More recently, we observed that the offspring of mothers exposed to exogenous GC in the last trimester of gestation, display diminished DA levels in the NAcc and other mesolimbic structures, an altered D1/D2 ratio and, interestingly, proneness to addictive behaviors (Leão, Rodrigues et al., unpublished observations).

Together, the results summarized above demonstrate that ELS has sustained effects on the morphology and activity of mesolimbic and mesocortical DAergic circuits, accompanied by altered sensitivity and vulnerability to drugs of abuse. In the next section, we will consider the role of the nigrostriatal DAergic pathway which has received relatively little attention in the context of drug abuse. Considering the long-lasting changes in DA receptors expression in several models of early life stress, we may raise the hypothesis that these genes may be transcriptional targets of GCs/stress or that they may undergo epigenetic regulation in response to early life adversity.

A new player in addiction: the nigrostriatal DAergic pathway?

As recently reviewed by Wise (Wise, 2009), the nigrostriatal DAergic system, best known for its role in motor control and Parkinson's disease pathology, also seems to play an important role in addictive disorders. First hints were provided by the observations that electrical stimulation of nigrostriatal

DAergic cells and terminal fields produced rewarding effects (Crow, 1972; Prado-Alcala and Wise, 1984; Wise, 1981) and that selective lesions of the nigrostriatal pathway attenuated drug self-administration (Glick et al., 1975; Linseman, 1976). Those early studies have been backed up by the results of further experimentation (Suto et al., 2004), including the demonstration that intra-nigral infusions of D1 receptor antagonists reduce drug self-administration (Quinlan et al., 2004).

Current views suggest that the contributions of the mesolimbic and nigrostriatal DAergic systems to the development of addiction are distinctly separated in time. Thus, whereas the mesolimbic pathway (especially the NAcc core) is responsible for the rewarding effects of drugs during the initial phases of addiction, the nigrostriatal system assumes an increasingly important role at later stages as drug consumption increases (Everitt et al., 2008; Everitt and Robbins, 2005; Wise, 2009). The NAcc core is important not only for the rewarding effect of drugs of abuse (Wise, 2004) but also mediates the motivational drive or "wanting of a reward" that underlies drug-craving (Berridge, 2007), and assures efficiency of response-outcome associative learning (pavlovian conditioning) (Yin and Knowlton, 2006). However, second-order protocols of drug reinforcement and pharmacological experiments revealed that the dorsal striatum, rather than the NAcc, is essential for drug-seeking behaviour after repetitive drug exposure (Ito et al., 2000). This interpretation is consistent with earlier work which showed that, while dorso-striatal lesions do not affect acquisition of pavlovian responses (Taylor and Robbins, 1986), infusion of DAergic antagonists into the dorsal striatum decreases drug-seeking under second-order drug reinforcement protocols (Vanderschuren et al., 2005). These findings have led to the concept that repetitive exposure to drugs of abuse evolve from being goal-directed behaviors into habit-based actions (Everitt et al., 2008; Everitt and Robbins, 2005; Wise, 2009). Self-administration protocols in monkeys have confirmed the progressive shift from goal-directed (pavlovian) behaviors (facilitated by the NAcc in cooperation with associative cortico-basal ganglia networks) to habit-based (instrumental) actions that depend on the dorsal striatum (in particular, the dorso-lateral striatum, an integral component of the sensorimotor cortico-basal

ganglia pathway (Porrino et al., 2004)).

The new knowledge concerning the contribution of the nigrostriatal DAergic pathway in drug addiction has been now extended to provide further new insights into how stress increases vulnerability to drug abuse behavior. Functional imaging studies in cocaine addicts have revealed a positive correlation between activation of the dorsal striatum by stress and the degree of cocaine craving (Sinha et al., 2005), and our own studies have demonstrated that stress promotes habit-based decisions in rats by increasing activation of the sensorimotor cortico-basal ganglia pathway (Dias-Ferreira et al., 2009); the latter results are reminiscent of the effects of repetitive drug administration.

Albeit several studies have shown that ELS can affect the mesolimbic circuit, the consequences in the nigrostriatal circuit remain poorly studied and understood. Prenatal DEX exposure increases TH+ cell numbers in the substantia nigra, demonstrating that this region can be profoundly affected in terms of DAergic transmission (McArthur et al., 2005). Furthermore, it was shown that ELS can make dopamine neurons from the nigrostriatal pathway to become more susceptible to subsequent insults later in life (Pienaar et al., 2008). Nonetheless, due to the paucity of studies, the direct effect(s) of ELS in the development/maturation of this circuit and its relevance for addiction for example, remains to be determined.

Future perspectives

The available literature, in a rather fragmented way, suggests an association between ELS, DA transmission and mental illness. Yet, it remains to be answer if the DAergic dysfunction is causal, or merely a consequence, of ELS and in several of the psychiatric conditions linked to ELS. Part of the problem relies on "snapshot approach" that is commonly used in the available studies that precludes the understanding of the dynamics of the insult-response-adaptation process. Thus, we believe that one of the priorities in the field should be to perform longitudinal studies that establish a direct link between altered DAergic transmission and specific endophenotypes for each of the pathological conditions in which ELS is implicated. In parallel, a longitudinal multimodal characterization of ELS exposure in the mesolimbic, mesocortical or nigrostriatal

DAergic pathways is needed. If this is achieved, ultimately, we could determine what are the windows of vulnerability of each of these DAergic pathways and which is more affected in each type of ELS. Furthermore, it could help us understand the long-term impact, and the adaptations, of the distinct DA pathways in neuropsychiatric conditions in which ELS is implicated. As an example, for addiction studies, this integrated approach would allow for a better insight on the role of different DA pathways throughout the different phases of addictive behavior. Moreover, this would give insights on how neurons in each of these pathways respond to drugs of abuse and/or stress in both animal models of ELS and human subjects and how these can be therapeutically modulated. Importantly, this approach is useful and applicable to many neuropsychiatric conditions.

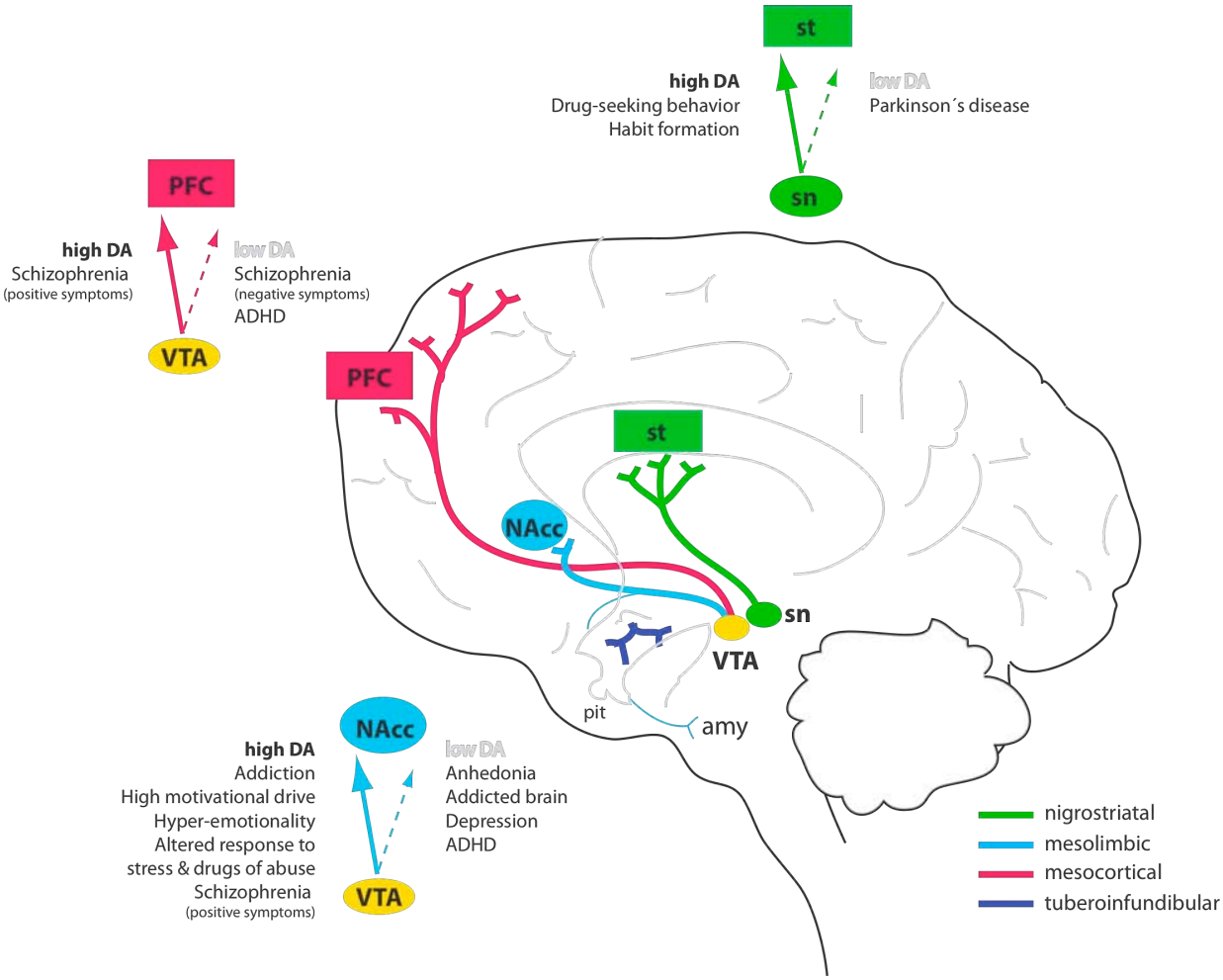
Conclusions

Evidence for the persistent morphological, neurochemical and behavioral impact of elevated GC levels (pharmacologically- or stress-induced) during development illustrates the importance of gene X environment (epigenetic) interactions in the etiology of psychiatric conditions. In light of the ontogenetic development of the mesocorticolimbic and nigrostriatal DAergic systems, reports that prenatal stress or manipulations of the maternal GC *milieu* and postnatal stress (ELS) may be causal to behavioural disorders ascribed to dysfunctional DAergic transmission (e.g. schizophrenia, drug addiction and possibly, depression) are not surprising. Having identified some of the neurobiological substrates that underpin the behavioural anomalies, the immediate challenge is to decipher the molecular and cellular mechanisms that underwrite these changes. Such studies will provide the conceptual basis for devising pharmacological interventions to ameliorate the undesired behavioural outcomes of mal-programmed DAergic circuits. Meanwhile, the existing literature suggests that serious psychiatric conditions in later life are preventable through the judicious use of GC in obstetrics and neonatal medicine, by avoiding stress during pregnancy and by placing emphasis on early parental care.

Legend to Figure

Figure 1. DAergic pathways of the brain. The mesolimbic and mesocortical pathways arise from the ventral tegmental area (VTA), which lies close to the substantia nigra (sn). The mesolimbic pathway projects especially to the nucleus accumbens (NAcc), but also to the amygdala (amy). The mesocortical pathway projects to the prefrontal cortex (PFC). The tuberoinfundibular tract terminates in the hypothalamo-pituitary (pit) unit. The nigrostriatal pathway projects from sn to striatum (st). Altered dopaminergic tone in each of these circuits (either hypo- or hyper-activity) is associated with a particular pathological condition. ADHD: attention deficit hyperactivity disorder.

Figure 1.



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