Universidade do Minho
Escola de Ciências

Sónia Maria de Sousa Borges

Searching for the neurobiological targets through which prenatal glucccorticoids program adult social and affective behaviors

Tese de Mestrado

Mestrado em Genética Molecular

Trabalho efetuado sob a orientação de
Doutora Ana João Rodrigues
Professor Doutor Nuno Sousa
Professora Doutora Dorit Schuller

Outubro de 2012

DECLARAÇÃO

Nome: Sónia Maria de Sousa Borges

Endereço electrónico: sonia.borges25@gmail.com

Telefone: 917937056

Número do Bilhete de Identidade: 13612893

Título dissertação:

Searching for the neurobiological targets through which prenatal glucocorticoids program adult social and affective behaviors

Orientador(es):

Doutora Ana João Rodrigues, Professor Doutor Nuno Sousa, Professora Doutora Dorit Schuller

Ano de conclusão: 2012

Designação do Mestrado ou do Ramo de Conhecimento do Doutoramento: Mestrado em Genética Molecular

Universidade do Minho, 31/10/2012

Assinatura: _____

DECLARAÇÃO

Nome: Sónia Maria de Sousa Borges

Endereço electrónico: sonia.borges25@gmail.com

Telefone: 917937056

Número do Bilhete de Identidade: 13612893

Título dissertação:

Searching for the neurobiological targets through which prenatal glucocorticoids program adult social and affective behaviors

Orientador(es):

Doutora Ana João Rodrigues, Professor Doutor Nuno Sousa, Professora Doutora Dorit Schuller

Ano de conclusão: 2012

Designação do Mestrado ou do Ramo de Conhecimento do Doutoramento: Mestrado em Genética Molecular

Universidade do Minho, 31/10/2012

Assinatura: _____

Para ser grande, sê inteiro: nada

Teu exagera ou exclui.

Sê todo em cada coisa. Põe quanto és

No mínimo que fazes.

Assim em cada lago a lua toda

Brilha, porque alta vive.

Fernando Pessoa, Poemas de Fernando Pessoa

Agradecimentos

Apesar de esta tese de mestrado ter o meu nome como a pessoa responsável pela sua realização, na realidade, este projecto não seria possível sem a ajuda de muitas outras pessoas. Primeiro e principalmente, à Doutora Ana João Rodrigues por me ter ajudado ao longo deste ano e dado a oportunidade de ser a "psicóloga" dos meus ratos, pela sua disponibilidade, e, principalmente, por ter acreditado em mim, pois qualquer grau de sucesso que eu alcancei enquanto estive no laboratório devo-o a ela. Sinto-me honrada por ter trabalhado com ela neste projecto. Um sincero obrigado!

Ao Professor Doutor Nuno Sousa pela sua disponibilidade, pela sua boa disposição e pelo seu interesse no meu trabalho e como estava a decorrer. À Professora Doutora Dorit Schuller pela sua disponibilidade e interesse neste projecto.

Um obrigado às minhas "team colleagues", Bárbara e Carina. À Carina pela sua disponibilidade e pela sua grande ajuda na realização dos western blots e nos testes comportamentais deste projecto. À Bárbara pela sua boa disposição e pela parceria nas inúmeras horas passadas na escrita da tese e por tudo que teve de aturar.

À Adriana por ter me aturado muitas vezes e pelas inúmeras vezes que me elevou o espírito. À Joana Silva, Margarida, Sofia Esteves, Filipa, Miguel Oliveira, Cristina, Ana Ribeiro, Rita, André, Silvina, Mafalda pelo apoio moral demonstrado e pela boa disposição.

Ao grupo de Neurociências pelas discussões científicas e pelo apoio demonstrado durante a realização deste projecto.

Aos meus amigos, principalmente aos meus melhores amigos, Fábio "Furbs" e Susana "Su", por me aturarem desde pequena, por serem meus amigos, por me fazerem rir e pelo apoio demonstrado.

E ao meu irmão pelo apoio demonstrado no meu trabalho.

Por último, às principais pessoas responsáveis por tudo, os meus pais. Obrigado, por sempre me terem ajudado a atingir os meus objectivos, por apoiarem sempre que precisei e por sempre acreditarem em mim, não só ao longo deste ano, mas ao longo da minha vida.

Muito Obrigado!

"**Searching for the neurobiological targets through which prenatal glucocorticoids program adult social and affective behaviors"**

Abstract

Stress activates the hypothalamic-pituitary-adrenal axis and leads to a controlled release of glucocorticoids (GCs) in the blood stream. Due to their numerous effects, synthetic GCs are often prescribed in clinics, as for example in 10% of preterm risk pregnancies in order to accelerate fetal lung maturation. Previous studies have shown that exposure to stress, or administration of GCs during pregnancy can contribute for the development of neuropsychiatric disorders later in life such as anxiety, depression and addiction.

In this work we studied a rat model prenatally exposed to synthetic GC, dexamethasone, at embryonic days 18 and 19. Animals were subjected to standard behavioral tests to assess emotional and social behaviors, and, to further complement these results we measured the emission of ultrasonic vocalization (USVs). iuGC animals are hyperanxious, display enhanced fear response and present depressive-like behavior. In parallel, we hypothesized that emotional impairment observed in iuGC animals could influence social interaction. Indeed, prenatal GC exposure impairs social behavior at different ages, since iuGC animals present low motivation to engage in social play and seem to present *less pleasure* during the interaction. Considering the cross-talk between opioid-endocannabinoid-dopaminergic circuits in both emotional and social behaviors, we further analysed levels of specific receptors in several regions of mesolimbic circuitry. We found prominent changes in Drd2, MOR1 and KOR1 in the nucleus accumbens and amygdala, which may underlie the abovementioned impaired behaviors. As these animals present a significant hypodopaminergic status in the mesolimbic circuitry, together with Drd2 molecular changes, in the last part of the project we decided to treat these animals with L-DOPA, a dopamine precursor. Administration of a therapeutic dose of L-DOPA reverted most of the emotional and social deficits observed in iuGC animals, suggesting a crucial role of dopaminergic transmission in this type of behaviors.

Our results show that prenatal GC administration potentiates emotional and social deficits, probably as a consequence of a deficient dopaminergic signalling in the mesolimbic circuitry. Importantly, we were able to restrain these behaviors by a simple pharmacological approach.

"Explorar os alvos neurobiológicos através dos quais glucocorticoides pré-natais programam comportamentos sociais e afetivos"

Resumo

O stress ativa o eixo hipotálamo-pituitária-adrenal e conduz à libertação de glucocorticoides (GCs) na corrente sanguínea. Devido aos seus efeitos diversos, os GCs sintéticos são usados frequentemente na prática clínica, como por exemplo em gravidezes com risco de parto pré-termo, para promover a maturação pulmonar fetal. Estudos anteriores demonstraram que exposição ao stress, ou a administração de GCs durante a gravidez, pode contribuir para o desenvolvimento de doenças neuropsiquiátricas em adultos, tais como ansiedade, depressão e adição.

Neste trabalho estudamos um modelo em rato de exposição ao GC sintético, dexametasona, nos dias embrionários 18 e 19 (iuGC). Os animais foram submetidos a uma bateria de testes comportamentais para avaliar o comportamento emocional e social, e, de forma a completar estes estudos, também medimos a emissão de vocalizações ultrassónicas (USVs). Os animais iuGC são mais ansiosos, apresentam comportamento do tipo depressivo, e uma resposta ao medo exacerbada.

Em paralelo, avaliamos o comportamento social e vimos que estes animais apresentam uma menor antecipação para iniciar uma interação social e *menos prazer* na mesma. Considerando a interação entre os sistemas opioide-endocanabinoide-dopaminérgico neste tipo de comportamento, decidimos analisar os níveis de determinadas moléculas destes circuitos nestes animais. Identificamos alterações proeminentes no receptor Drd2, no MOR1 e KOR1 no *nucleus accumbens* e na amigdala no grupo iuGC. Uma vez que os iuGC apresentam um estádio hipodopaminergico substancial no circuito mesolimbico juntamente com alterações significativas de Drd2, decidimos tratar os animais com um precursor da dopamina, a L-DOPA. Administração de uma dose terapêutica de L-DOPA reverteu quase todas as alterações comportamentais dos animais iuGC.

Estes resultados mostram que a administração pré-natal de GCs potencia o aparecimento de déficits emocionais e sociais, e que uma simples abordagem farmacológica com o objectivo de aumentar a dopamina nestes animais, foi suficiente para reverter a maioria das alterações comportamentais.

Contents

Abbreviations

11β-HSD – 11-Beta hydroxysteroid dehydrogenase

5-HT - Serotonin

ACTH - Adrenocorticotropic hormone

AH - Anterior hypothalamus

AMY - Amygdala

BDNF - Brain-derived neurotrophic factor

BNST – Bed nucleus of the stria terminalis

CAH - Congenital adrenal hyperplasia

CORT – Corticosteroids

CP - Cerebral peduncle

CPu - Caudate putamen

CRH - Corticotrophin-releasing hormone

DA - Dopamine

DAergic - Dopaminergic

DEX - Dexamethasone

Drd1 - Dopamine receptor 1

Drd2 - Dopamine receptor 2

GC - Glucocorticoid

GR - Glucocorticoid receptor

GREs - Glucocorticoid response elements

HPA - Hypothalamic-pituitary-adrenal

HPT – Hypothalamus

iuGC - *in utero* glucocorticoid

KOR1 - k opioid receptor 1

LC - Locus coeruleus

LDT - Laterodorsal tegmental nucleus

LS - Lateral septum

MOR1 - μ opioid receptor 1

MR - Mineralocorticoid receptor

NAcc - Nucleus accumbens

NEC - Necrotizing enterocolitis

NGFI-A - Nerve growth factor-inducible protein A

NTS - Nucleus tractus solitarius

OXY – Oxytocin

PBN - Parabranchial nucleus

PFC - Prefrontal cortex

PO - Preoptic area

PVN - Paraventricular nucleus

RDS - Respiratory distress syndrome

TH - Tyrosine hydrolase

USV - Ultrasound vocalization

VLM - Ventrolateral medulla

VTA - Ventral tegmental area

1. INTRODUCTION

1. Introduction

1.1. Stress and the Hypothalamic-Pituitary-Adrenal (HPA) axis

Stress is defined as any perturbation of homeostasis of the organism, both psychological or physiological (Goldstein and Kopin, 2007). The stress response is coordinated by the brain; in the presence of a stressor, adrenaline and noradrenaline are released, and this constitutes the foundation of the classic reaction of the "fight-or-flight" response (Juruena et al., 2004). Simultaneously, stress activates the hypothalamic-pituitary-adrenal (HPA) axis. This endocrine response starts with the release of corticotrophin-releasing hormone (CRH) that is secreted by neurons from the paraventricular nucleus (PVN) of the hypothalamus (HPT). The CRH acts on the anterior pituitary, where it stimulates the synthesis and release of adrenocorticotropic hormone (ACTH) into the blood stream. In turn, ACTH induces the synthesis and secretion of corticosteroids in the adrenal cortex (Fig.1). The main corticosteroid is cortisol in humans and corticosterone in rodents (Mesquita et al., 2009).

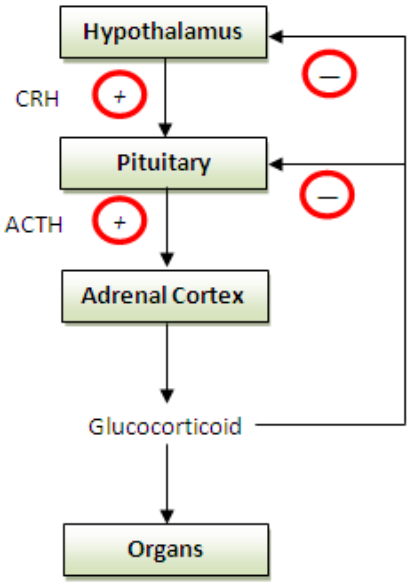


Figure 1 - Regulation and negative feedback of glucocorticoids (GCs) in the hypothalamus-pituitary-adrenal (HPA) axis. Under stressful stimuli the cells of the paraventricular nucleus (PVN) in the hypothalamus release corticotrophin-releasing hormone (CRH). The CRH activates the pituitary, which in turn, releases adrenocorticotropic hormone (ACTH), then it act on the adrenal cortex resulting in an increase in the levels of glucocorticoids in the blood stream. GCs have a negative feedback in order to inhibit further production of GCs.

There are two types of corticosteroids receptors, the high-affinity mineralocorticoid receptor (MR) and the low-affinity glucocorticoid (GC) receptor (GR) (Sanchez et al., 2000). Ligand binding results in the translocation of the receptor to the nucleus, where it regulates the expression of target genes due to the association of the complex receptor-ligand with the glucocorticoid response elements (GREs) in the promoter region of targets genes (Fig.2). Importantly, the GRs can regulate positive or negatively the genes to which they are coupled (Juruena et al., 2004).

The cessation of the HPA response to stress is coordinated by a glucocorticoid negative-feedback process (Fig.1)(Spencer et al., 1998, De Kloet et al., 1998), which is tightly regulated to prevent deleterious effects of both hyper and hypo-cortisolemia.

1.2. The physiological role of GCs

Endogenous GCs (CORT) increase the concentration of glucose and other metabolic fuels; they are also involved in the immune response. GCs have inhibitory effects on both innate and acquired immune response mediated by the T and B cells, and can suppress the effector functions of phagocytes. In short, they are extraordinary efficacious in managing numerous acute disease manifestations of inflammatory and autoimmune disorders (Chatham and Kimberly, 2001). During pregnancy, they are involved in lung maturation (regulation of surfactant production by the lungs), and for the regulation of immune and metabolic functions, and for neuro- and behavioral development (Mesquita et al., 2009).

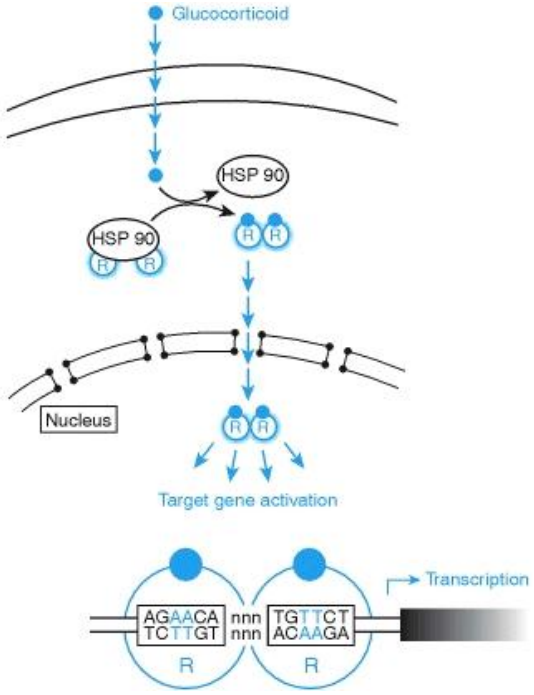


Figure 2 - Scheme of the activation of glucocorticoid receptor (GR). GR in their inactive state are present in the cytoplasm with a multimeric complex of chaperone proteins, including many heat sock proteins. Endogenous or synthetic glucocorticoids act as a ligand; GR-ligand undergoes a conformational change, and becomes active, then dissociates from the complex of chaperone proteins and translocates to the nucleus, where it regulates gene expression by binding to glucocorticoid response elements (GRE). The diagram is adapted from Strachan T., 1999 (Strachan T. and A.P., 1999).

The placenta as well as most foetal tissues expresses GRs, which can bind both endogenous and exogenous GC, from mid-gestation onwards (Johnson et al., 2008). Endogenous GCs have a higher affinity to MR and the expression of these receptors is more confined to specific brain areas and, in rodents, is only present in late gestation (Funder, 1996).

The foetus has limited capacity to degrade synthetic GCs, so, even low levels of GC can have a deleterious impact. The endogenous GCs are much lower in the foetus than in mother circulation due to the existence of the enzyme 11-Beta hydroxysteroid dehydrogenase (11β-HSD), which is located in the syncytiotrophoblast, the site of maternal-foetus exchange (Mesquita et al., 2009). This enzyme converts the GC in 11-keto metabolites, its inactive form. Nonetherless, 11β-HSD is not active throughout the pregnancy, in fact, it was show by McTernan and collaborators, variations of 11β-HSD activity in the end of pregnancy, in rats and

humans (McTernan et al., 2001), that may indicate that increased maternal GCs during this stage can affect the foetus. Moreover, exogenous GCs, such as dexamethasone (DEX) or betamethasone, are poor substrates for 11β-HSD, facilitating the passage through the placenta (White et al., 1997).

Due to their pleiotropic action and ability to cross the placenta, exogenous GCs are widely prescribed, in clinics, to accelerate lung maturation in preterm labour and perinatal period (Crane et al., 2003). Frequently, DEX, a synthetic glucocorticoid that has 25 times more potency than endogenous cortisol (Oliveira et al., 2006), is used in clinics. This seems to reduce neonatal death, respiratory distress syndrome (RDS), intraventricular hemorrhage, necrotizing enterocolitis (NEC) and respiratory support, intensive care admissions and systemic infections in the first 48 hours of life (Miracle et al., 2008). Less frequently, it is used during antenatal period in the foetuses at risk of congenital adrenal hyperplasia (CAH) (New et al., 2001). Although the positive effects of such treatment are undeniable, less is known about the deleterious effects that it can have in the developing brain.

1.3. GCs early in life: molecular, structural and behavioral outcomes

Adversity during early life, including, abuse or neglect, can induce effects on physical and mental health. For example, it increases the risk of development of conduct disorders, personality disorders, major depression, schizophrenia, anxiety and addictive disorders (Agid et al., 1999, Bernet and Stein, 1999, Dube et al., 2003, Heim and Nemeroff, 2001, Young et al., 1997). In contrast, the development of psychiatric disorders is very low in women abused during adulthood (Brown and Moran, 1994), (McCauley et al., 1997). Thus, studies demonstrate that the mechanisms that underlie programming of the adult HPA axis function are dependent on time of exposure, but also, dependent of the dose of GCs.

Some studies report that children exposed prenatally to GC have a reduction in their birth weight, and at the age of 3 present significant behavioural changes (Newnham, 2001). Oliveira and colleagues showed that prenatal administration of endogenous or synthetic (DEX) glucocorticoids can act permissively to accelerate some developmental processes, like eye opening (a measure of neurological development), while it delays others, like body growth (Oliveira et al., 2006), in rats. However, such treatments lead to a long-lasting impairment in

the HPA axis (Matthews et al., 2002). *In utero* exposure to GCs/stress, and consequent impairment in the HPA axis, was found to be associated with long-lasting deficits in cognitive, mood and addictive behaviors (Oliveira et al., 2006, Rodrigues et al., 2011b, Roque et al., 2011, Miracle et al., 2008). Indeed, in human pregnancies, evidence suggests that fetal exposure to synthetic GCs has detrimental effects on birth outcome, childhood cognition and long-term behavior (Seckl, 2004, Crowley, 1995, Miracle et al., 2008). Furthermore, previous work from our lab revealed that prenatal administration of DEX to rats induces a hyperanxious status in the progeny and increases the propensity for developing depression (Roque et al., 2011), together with reward-associated behaviors (Rodrigues et al., 2011b).

Several brain regions seem to be affected by early exposure to GC, however, the hippocampus (Sousa and Almeida, 2002), the prefrontal cortex (Cerqueira et al., 2007) and the bed nucleus of the stria terminalis (BNST) seem to be specially affected (Pego et al., 2008). Previous work showed that rhesus monkeys treated with prenatal DEX present a reduction in hippocampal neuronal number and volumes, until de age of 20 months old (Uno et al., 1990). Indeed, in humans and rats it has been demonstrated that alterations in hippocampus caused by elevated GCs during prenatal period, can be responsible for the development of depression in adulthood (Sheline et al., 1996, Stein et al., 1997). In addition, prenatal GCs administration leads to an increase in the levels of CRH in the amygdala (AMY), which in turn leads to the development of anxiety in adult life (Cratty et al., 1995).

Interestingly, the mesolimbic circuit also known as reward circuit, seems particularly sensitive to early life stress (Leao et al., 2007, Rodrigues et al., 2011a). This pathway involves dopaminergic inputs from the ventral tegmental area (VTA) to the AMY and nucleus accumbens (NAcc), and is activated in consumatory behaviors such as sex, food and drug consumption, and it is generally characterized by increased dopamine (DA) release from the VTA to NAcc (Baier et al., 2012). In this context, it is important to quote that mesolimbic neurons are strongly activated upon stress or GC exposure (Rodrigues et al., 2011a), increasing the strength of excitatory synapses on DA neurons and inducing similar patterns of dendritic organization in the NAcc. This suggests that dopaminergic transmission in the NAcc is GC-dependent (Barrot et al., 2000). McArthur *et al* showed that rats exposed to GC prenatally, significantly increases adult dopamine neuronal numbers in the ventral tegmental area (VTA) (McArthur et al., 2005). These data confirm that mesencephalic dopaminergic neurons are

sensitive to perturbations in circulating GC levels during development (McArthur et al., 2005). On the contrary, previous work from our lab, show that GC treatment induced a reduction in proliferating cells and reduced number of TH-positive cells in the VTA, leading to a decrease in the dopaminergic (DAergic) innervation to the NAcc (Rodrigues et al., 2011a). Such changes may underlie the addictive profile of these animals and partially explain their anxious profile (Oliveira et al., 2006, Leao et al., 2007, Rodrigues et al., 2011a, Rodrigues et al., 2011b).

Though several studies suggest that prenatal GCs affect emotional the status of individuals, less is known about its effects in terms of social behavior, which is the main focus of this thesis.

1.4. Social Play Behavior

Nothing is more familiar to mammalian species than their ordinary, everyday social interactions. Increasing evidence suggests that social support in humans, and affiliative behaviors in animals, can lead to a positive impact on health and decrease mortality from many different causes (DeVries et al., 2003). There are many forms of social behavior, but for the context of this thesis we will only focus on social play behavior. Social play behavior is one of the earliest forms of non-mother-directed social behavior appearing in animals, presenting a significant rewarding effect (Vanderschuren et al., 1997).

In rats, social play behavior exhibits an inverted U-shaped curve, being the highest level around day 30 of life and declining following maturation (Trezza and Vanderschuren, 2008b). Thus, social play is considered the most characteristic social behavior of adolescent animals (Panksepp et al., 1984). In juvenile rats, social interaction with a play partner displays various forms (Vanderschuren et al., 1997). Social play does not have the highest priority and is not displayed unconditionally, only appearing after the primary needs of animals have been satisfied. For example, food deprivation or exposure to intense light conditions suppres social play behavior (Siviy and Panksepp, 1985, Vanderschuren et al., 1995a). Previous studies have identified many behavioral acts during social play, which include pouncing, pinning and social exploration (Baenninger, 1967, Panksepp and Beatty, 1980). Pinning and pouncing are considered to be related with play behavior, while social exploration is not directly related with play (Vanderschuren et al., 1997). The social play behavior starts with one animal approaching

and soliciting another (pouncing), i.e., the soliciting rat attempts to nose or rub the nap of neck of the play partner. After this, pinning may follow. Pinning is defined as one of the animals lying with its dorsal surface on the floor with other animal standing over it. Thus, when a rat attempt to nose the nape of a conspecific, the pounced animal fully rotates to a supine position, pinning is the result (Vanderschuren et al., 1997).

Social interaction is not only rewarding for the animals, but also seems to modulate emotionality, in both rats and humans (Burgdorf et al., 2011). Social interaction profoundly influences the HPA axis activity in humans and other animals. Severe psychosocial stress, or the lack of certainty and control over one's social environment, can lead to chronically elevated HPA axis activity and a deterioration of health (DeVries et al., 2003, DeVries, 2002). This is supported by earlier work that had shown that rats were very susceptible to effects of social isolation during the period of weaning and maturation (Einon and Morgan, 1977, Einon et al., 1978). Moreover, social defeat is one of the most powerful stress paradigms in rodents (McLaughlin et al., 2006). Animals that are deprived of play present abnormal patterns of social, sexual and aggressive behaviors (Gerall et al., 1967, Lore and Flannelly, 1977, Vanderschuren et al., 1997). The offspring of stress exposed pregnant female rats, during the final week of pregnancy, have a diminished quality the social interaction behavior indicative of a reduced social drive (Lee et al., 2007). Another study has shown that administration of DEX to pregnant rats on gestation days 6-8, leads to a decrease in juvenile social play (Kleinhaus et al., 2010).

In summary, stress/GCs may affect the development and/or manifestation of correct social behavior. On the other hand, social deprivation may be a powerful stressor at crucial life stages.

1.5. Modulation of social (and affective) behavior: neurobiological correlates

Social play is the most characteristic expression of social activity, and it is modulated by neural systems involved in reward and motivation, such as the opioid and endocannabinoid sistems, and this seems to rely on a strong interplay with DAergic circuit (Trezza and Vanderschuren, 2008b).

1.5.1. Opioids signalling

The opioid receptors are a group of G-protein-coupled receptors, which has the opiods as ligands. The opioids are found principally in the central and peripheral nervous system and in the gastrointestinal tract (Mansour et al., 1995). Until now, three types of receptors have been identified, referred as μ, k and δ (MOR, KOR and DOR). With the use of selective agonists or antagonists, it became clear that each type of opioid receptor has unique pharmacological properties, is differentially distributed in the central nervous system and has been implicated in a broad range of behaviors and functions, such as, regulation of pain, reinforcement and reward, release of neurotransmitters, and neuroendocrine modulation (Leadem and Yagenova, 1987, Schoffelmeer et al., 1988, Bozarth and Wise, 1984). Each opioid receptor type demonstrates a distinct anatomical distribution. The μ opioid receptor (MOR) is distributed in regions such as the septum, the BNST, the hippocampus, the medial preoptic area, the AMY, the locus coeruleus (LC), the parabranchial nucleus (PBN), the nucleus tractus solitarius (NTS) and the dorsal motor nucleus of the vaugus. The k opioid receptor (KOR) is present in regions such as the NAcc, the BNST, the medial preoptic area, the hypothalamus and the AMY. The δ opioid receptor (DOR) is located in the hippocampus, the AMY and the ventrolateral medulla (VLM) (Mansour et al., 1995).

With respect to the HPA axis, it is known that opioid receptors are able to modify the release of specific hormones, such as, increase the release of corticosteroids or decreasethe release of oxytocin (OXY) (Mansour et al., 1995). There is strong evidence showing that administration of an opioid receptor antagonist induces a greater increment in the HPA axis function in chronically stressed animals when compared with control animals (Drolet et al., 2001), suggesting that opioids have the effect of attenuating stress responses (defensive action of the organism).

Apart from this role in the HPA axis activity, the opioids are important in the modulation of affective and social behaviors (Vanderschuren et al., 1995b). The opioids are released during episodes of social contact and rewarding situations, and can induce odor and place preference (Nelson and Panksepp, 1998, Trezza and Vanderschuren, 2008b). It was also shown by many studies that opioid release results in a powerful attenuation of the reaction to social separation (Nelson and Panksepp, 1998). On the other hand, low basal levels of

opioids induce motivation to seek out social contact (Panksepp et al., 1985, Vanderschuren et al., 1995b).

Therefore, if the rat is treated with morphine it will increase social play. In contrast, an opioid antagonist decreases social play behavior (Trezza and Vanderschuren, 2008b). Actually, depending on the dose of morphine, it can have different effects on social behavior. Morphine treatment powerfully increases pinning, which indicates an increase in the rewarding properties of social play (Vanderschuren et al., 1997). When rats are tested in an unfamiliar environment, pinning and pouncing are initially suppressed, while the rats explore before engaging in social play, although the total levels of play are not affected. A dose of morphine 10 times lower than the dose used to increase social play, is capable to abolish the initial suppression of social play (Vanderschuren et al., 1997).

It has been shown that all types of opioid receptors are involved in reward-related processes. The rewarding effects of opioid drugs are generally mediated by μ-opioid receptors along with evidences that had shown also that the δ-opioid receptor is important in reward processes (Depue and Morrone-Strupinsky, 2005, Le Merrer et al., 2009). Regarding k-opioid receptors stimulation, some reports have shown that it results in dysphoric effects (Mucha and Herz, 1985).

Interestingly, it was found that the three types of opioid receptors have different effects on social behavior. The stimulation of μ- and k-opioid receptors has antagonistic effects. While the stimulation of μ-opioid receptor enhances social behavior, the opposite pattern was observed with k-opioid stimulation (Vanderschuren et al., 1995b). To what concerns the δ-opioid receptor, neither δ-opioid receptor agonist nor δ-opioid receptor antagonists are involved in the regulation of social behavior. Some reports have shown that this is only observed in juvenile rats (Vanderschuren et al., 1995b). In adult rats, δ-opioid receptor agonists have been reported to increase social behavior (Negri et al., 1991).

1.5.2. Cannabinoid signalling

There are currently two subtypes of known cannabinoid receptors, cannabinoid receptor 1 (CB1) and cannabinoid receptor 2 (CB2). The CB1 receptor is expressed principally

in the central nervous system, but also in the lungs, liver and kidneys (Herkenham et al., 1991). Within the brain, there are at least two endogenous ligands that activate the CB1 receptor, namely N-arachidonoylethalamine (anandamine, AEA) and 2-arachidonoyglycerol (2-AG) (Hill and Tasker, 2012). The CB2 receptor is expressed predominantly in the immune system and in the hematopoietic cells, but recent reports also show their expression in the brain (Gong et al., 2006).

With respect to the HPA axis, it is know that the endocannabinoid system is well distributed throughout cortico-limbic and hypothalamic circuitries, which regulates the activation of HPA axis (Gorzalka et al., 2008). The endocannabinoid system has been found to regulate both excitatory and inhibitory neurotransmitter release in the circuitry (Hill et al., 2010). Mice deficient in CB1 receptors or animals treated with a CB1 receptor antagonist present an increase in the basal activity of the HPA axis (Hill and Tasker, 2012). After a period of acute stress, CB1 knock out (KO) mice exhibit a larger peak of ACTH and corticosterone response, suggesting that the loss of CB1 receptor reduces the fast feedback inhibition and increases the magnitude and duration of the HPA axis response to acute stress (Barna et al., 2004). In addition, endocannabinoids (eCBs) play an important role in glucocorticoid-mediated negative feedback (Hill and Tasker, 2012). Interestingly, the endocannabinoid system appears to contribute differently to the feedback loops of GC feedback inhibition of the HPA axis and does so through distinct mechanism. Within the PVN, glucocorticoids cause a rapid release of endocannabinoids, which contribute to a fast-feedback inhibition of the HPA axis. Within the prefrontal cortex (PFC), GCs cause a delayed increase in endocannabinoid mobilization, which contribute to a delayed GC negative feedback of the HPA axis. Combined, this different recruitment of the endocannabinoid system leads to a both short (PVN) and long (PFC), loops of the negative feedback pathway in the brain (Hill and Tasker, 2012).

Besides the modulation of the HPA axis activity, cannabinoid neurotransmission is important in reward processes, and interacts with the opioid system in the modulation of drug, food and social reward (Trezza and Vanderschuren, 2008a, Solinas and Goldberg, 2005, Fattore et al., 2005). Tezza and Vanderschuren have shown that the cannabinoid system has an important role in the modulation of social play behavior, with opposite behavioral results depending on how the endocannabinoid system is stimulated (Trezza and Vanderschuren, 2008a). The direct CB1 cannabinoid receptor agonist and the indirect cannabinoid receptor

agonist exert opposite effects on social behavior (Trezza and Vanderschuren, 2008a). Preclinical reports revealed that both acute and chronic treatment with cannabinoid receptor agonist suppress social behavior (Schneider et al., 2008). In addition, the indirect cannabinoid receptor agonist, which inhibits fatty acid amide hydrolase (FAAH), the enzyme that degrades the endocannabinoid, anandamide, enhances social behavior. Reports have also shown that this indirect cannabinoid receptor agonist is dependent on opioid and dopaminergic neurotransmission, and it is blocked by antagonists of both systems (Trezza and Vanderschuren, 2008a).

Previous reports have exposed that the endocannabinoid and opioid systems interact with each other (Trezza and Vanderschuren, 2008b). Actually, it has been shown that the endocannabinoid system is involved in the reinforcing and dependence-producing properties of opioid drugs. Vanderschuren was the first to show an interaction between these two circuits in the positive modulation of social behavior (Trezza and Vanderschuren, 2008a, Trezza et al., 2012). It was also observed that the increase in social play induced by the indirect cannabinoid receptor agonist was completely block by opioid receptor antagonist, and surprisingly, the increase effect of morphine on social play behavior was reduced by CB1 cannabinoid receptor antagonist (Trezza and Vanderschuren, 2008a).

1.5.3. Dopaminergic signalling

The dopamine (DA) receptors are G-protein-coupled receptors. There are 5 types of DA receptors (Drd 1-5). The Drd1 is the most widespread of the DA receptors and is expressed at higher levels than the other types of DA receptors (Baier et al., 2012). It has been found in the NAcc, AMY, caudate putamen (CPu) and in the prefrontal cortex (PFC). On the other hand, D_5 receptor, also called Drd1-like receptor, is less expressed in the rat brain in comparison to the Drd1 subtype, and it is expressed in the frontal cortex, hippocampus and CPu. The three Drd2-like type of receptors Drd2, Drd3, and Drd4 are all expressed in the NAcc, and olfactory tubercle, amongst other areas (Missale et al., 1998, Xu and Zhang, 2004)

Dopaminergic circuitries can be divided into four main groups: nigrostriatal, mesolimbic, mesocortical, and tuberohypophyseal circuits (Fig.3). The first is the major dopaminergic tract in the brain, and plays an essential role in the control of voluntary motor

movement (Baier et al., 2012). The mesolimbic dopaminergic circuit originates in the cell bodies localized in the VTA and projects to NAcc, AMY and hippocampus. Other group of cell bodies in the VTA project to the prefrontal and perirhinal cortex, forming the mesocortical dopaminergic system. Previous reports have shown that these last two pathways overlap substantially, and are collectively referred as the mesocorticolimbic system, being involved in emotional behaviors including motivation and reward (Chinta and Andersen, 2005). In addition, these neurons have been hypothesized to play a critical role for the action of antipsychotic, antihyperactivity and psychostimulant drugs (Baier et al., 2012). Lastly, the tuberohypophyseal system, which is originated in the hypothalamus and innervates the pituitary and the median eminence, is important in the regulation of the release of pituitary hormones (Baier et al., 2012).

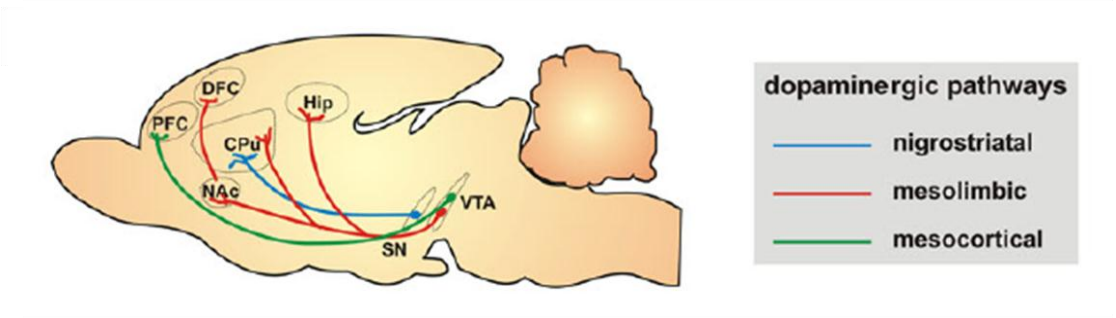


Figure 3 - Schematic representation of dopaminergic pathways. Dopaminergic transmition can be divide in four pathways nigrostriatal, mesolimbic, mesocortical, and tuberohypophyseal systems. The diagram is adapted from Baier CJ, 2012 (Baier et al., 2012).

Work from Kuhn *et al* showed that Drd1 and Drd2 subtypes of DA receptors contribute for the dopaminergic regulation of the HPA axis function, and stimulated increase the HPA axis response (Borowsky and Kuhn, 1992). Besides modulation of the HPA axis, dopamine is also important for the regulation of emotional, affective and social behaviors (Rodrigues et al., 2011b, Vanderschuren et al., 1997), although, the role of the dopaminergic neurotransmission in the modulation of social play is not yet clear. It has been reported an increase and decrease on social play behaviour, after treatment with dopamine receptor agonists(Siviy et al., 1996, Vanderschuren et al., 1997). Part of the rewarding properties of both opioid and cannabinoid drugs involve dopamine-dependent processes, through direct interaction with dopaminoreceptive neurons in the NAcc (Pierce and Kumaresan, 2006). Complementing this,

it was shown by Vanderschuren and colleagues that the effects of the indirect endocannabinoid receptor agonist were blocked by the dopamine receptor antagonist, while those of morphine were not (Trezza and Vanderschuren, 2008a). These results indicate that although the endocannabinoid and opioid systems jointly facilitate social play, they do so through partly dissociable mechanism. The opioid and dopaminergic circuits also seem to interact and modulate the reward behavior (Berridge, 2007, Depue and Morrone-Strupinsky, 2005). Whereas, the mesolimbic dopaminergic system mediates the motivation to a reward, opioids modulate the pleasurable proprieties of a rewarding situation (Berridge, 2003, Kelley et al., 2005, Berridge, 2007). Indeed, there are significant studies showing that the rewarding aspects of social interaction depend on opioid activity (Depue and Morrone-Strupinsky, 2005). Actually, there are many reports evidencing the interaction between opioid and dopaminergic system in social bonding and sexual behavior (Balfour et al., 2004, Young and Wang, 2004).

1.6. Ultrasonic vocalizations – a measure of emotional and social behavior in rodents

Emotional and social behaviors are complex and dynamic and its analysis presents some limitations when using animal models. In the context of this thesis, we decided to complement the behavioral studies with the recording of ultrasonic vocalizations (USVs).

Rats use USVs as a regular feature of their social interactions. Social interactions include aversive and appetitive encounters and the measurement of such USVs can be used an indicative of their emotional status and social capacities (Knutson et al., 1998).

Rat vocalizations are produced through the larynx. Rats use their larynx in two modes: the first, to produce audible sounds of 2-4kHz, by causing vibrations of vocal folds (Nitschke, 1982); And the second, generates USVs, in this case, the larynx is stabilized and used as whistle with a very small orifice created by the vocal cords, which cannot vibrate (I. et al., 2001). This produces the ultrasonic sound by the same principle as a human whistle does, except that the small size of the respiratory tract of the rat produces sound in the ultrasonic range.

It is possible to identify three classes of USVs that the rat uses in different situations. Rat pups show evidence of ultrasonic vocalizations around the 40 kHz in response to situations like separation from their litters and mother, or even when the temperature of the environment drops (Hofer, 1996). Adolescent and adult rats emanate two different kinds of USVs, classified according to their frequency, as low (22 kHz) and high (50 kHz) frequency ultrasonic vocalizations.

In situations of danger, circumstances that originate an increase of anxiety (for example, in the presence of an aggressive opponent, predator, large mammal or when the noise is loud), or even when the rat is expecting an unpleasant stimulus, it emits 22 kHz alarm calls, also called as low frequency vocalizations (Brudzynski, 2009). These kinds of sounds are significant advantages in the present of some predators that do not have the capacity to hear ultrasonic vocalizations. Furthermore, these ultrasounds are propagated more directionally than audible sound, they are difficult to detect and localize, and they dissipate easily in environmental obstacles and weather conditions. And if these sounds are emitted in underground burrows the predators in the ground or in the air do not hear it (Brudzynski, 2009). Such vocalizations are not only emitted during the actual aversive event and in response to stimuli associated with these experiences but also in situations of sexual contact (Knutson et al., 1999). The intensity of an aversive emotional state can be revealed by latencies to pronounce vocalizations, their length and loudness or by the number of calls emitted (Wohr et al., 2005).

High frequency 50 kHz USVs occur in situations associated with a positive status and are representative of a state similar to joy (Knutson et al., 1999). They are expressed in rough-and-tumble play, tickling, exploratory activity, meeting rats after a period of separation or in the initial meeting of resident-intruder pair and in rewarding situations (Brudzynski, 2009, Brudzynski and Chiu, 1995). It was observed that these sounds are also emitted in the presence or anticipation of artificial rewarding situations, like pharmacological stimulus (Maier et al., 2010, Burgdorf et al., 2000).

Besides appetitive situations, 50 kHz USVs also occur after separation from conspecifics during short periods of social isolation. Rats taken out from their home cage and individually exposed to a clean cage, as well as the rat that stays alone in the home cage after the conspecifics has been removed, emit 50 kHz USV (Wohr et al., 2008). Actually, the fact

that the separation from conspecifics elicits 50 kHz USV is an indicative of an affiliative communicative function of positive USV, namely to (re)establish or to maintain social contact.

It is important to mention, that is possible for the rats to emit with a brief difference of time both types of call, but they cannot acoustically mix the 22 kHz and 50 kHz ultrasounds. These mutually exclusive types of vocalization are regulated by two ascending systems that originate in the brainstem tegmentum: the cholinergic system and the mesolimbic dopaminergic system (Fig.4) (Brudzynski, 2009).

The activation of the cholinergic system, which originates in the laterodorsal tegmental nucleus and extends to the basal forebrain and limbic areas (Fig.4, black arrows), induces defensive behavior and increases the number of 22 kHz ultrasonic vocalizations. It was shown that pharmacologic treatments, that activate this system, induce 22 kHz USVs and the use of antagonists inhibits or decreases these USVs, resulting in a negative affective state (Brudzynski, 2001).

The ascending dopaminergic system, that begins in the VTA and ends in the NAcc and other basal forebrain structures (Fig.4, white arrows), when activated induces behavioral activation (increase in locomotor activity and exploration) and increases 50 kHz vocalizations, resulting in a positive affective state. This state includes relevant changes in the somatic, autonomic, and endocrines systems (Brudzynski, 2009).

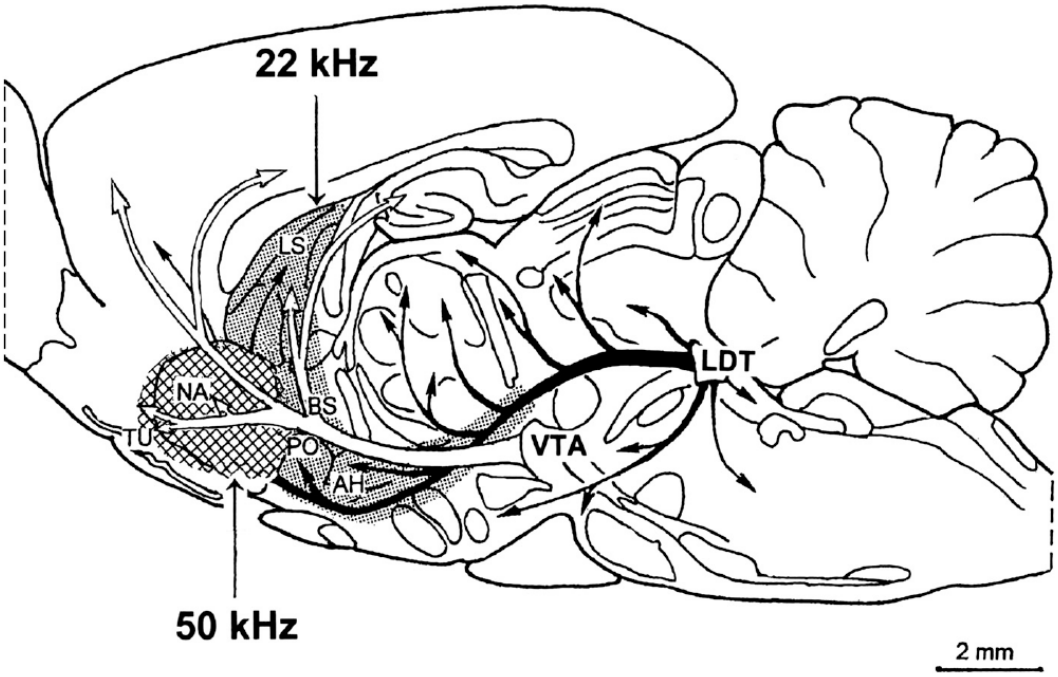


Figure 4 - Two ascending systems are responsible to initiate the ultrasonic vocalizations. The cholinergic system has the origin in the laterodorsal tegmental nucleus (LDT), and release acetylcholine in the anterior hypothalamus (AH), preoptic area (PO), bed nucleus of the stria terminalis (BNST), and lateral septum (LS), and has been involved in 22 kHZ USVs emission. The ventral tegmental area (VTA) is the zone of origin of the dopaminergic system. Release dopamine in the nucleus accumbens (NAcc) and neighboring areas, inducing 50 kHz vocalization. The diagram is adapted from Brudzynski SM. 2009 (Brudzynski, 2009).

Due to the potential of the USVs to ascertain the emotional and motivational status of the animals, in this thesis we decided to complement the behavior studies by measuring this tool.

2. AIMS

2. Aims

Accumulating evidence suggests that high levels of GCs during prenatal period increase the propensity to develop a multiplicity of psychiatric disorders in adulthood, such as anxiety and depression.

This project aims to characterize in detail the emotional, social and affective behaviors of adult animals exposed to GCs at gestation days 18 and 19. To further complement the standard behavioral tests, we will also evaluate the number and type of ultrasonic vocalizations (USVs) to ascertain emotional/effective condition in specific behavioral paradigms. As a last part of the project, we intend to identify the neurobiological correlates of such deficits.

The present thesis had the following objectives:

1. Establish and optimize USV measurement at ICVS;
2. Evaluate emotional and social behaviors of iuGC animals in conjunction with USVs recordings;
3. Ascertain the levels of specific neurotransmitter receptors (opioids, endocannabinoids and dopamine) in the mesolimbic circuits of iuGC animals;
4. Revert the behavioral deficits by pharmacological manipulate of affected circuits.

We expect that this integrative and multimodal analysis will give insight to the mechanisms underlying stress-induced social and emotional dysfunction and will shed some light about the neurotransmitter system(s) affected.

3. MATERIALS AND METHODS

3. Materials and methods

3.1. Animals

All manipulations were conducted in accordance with local regulations (European Union Directive 2010/63/EU) and National Institute of Health guidelines on animal care and experimentation. Pregnant Wistar rats were injected with the synthetic GC, dexamethasone (DEX; iuGC animals), at 1mg kg^{-1} or with saline (CONT; control animals), on days 18 and19 of gestation.

Male offspring were pair housed, according with antenatal treatment, under standard laboratory conditions: artificial 12h light/dark cycle (lights on from 08:00 a.m. to 08:00 p.m.); room temperature 22°C; food and water were provided *ad libitum*.

3.2. Experimental design

Different sets of animals were used during the experimental approach, represented in fig.5. All procedures were performed during the dark period (08:00 p.m.- 02:30 a.m.), except the protocol to assess maternal behavior, the forced swimming test and the light/dark box test.

Animals were assessed for social behavior at different ages (Fig.5). In adulthood, we analysed their emotional status using different behavioral paradigms to assess anxiety, depressive-like behavior and fear. A set of animals was sacrificed and brain areas (AMY, NAcc and HPT) were collected to be used for western blot analysis.

Finally, we tried to reverse the phenotype by pharmacological manipulation.

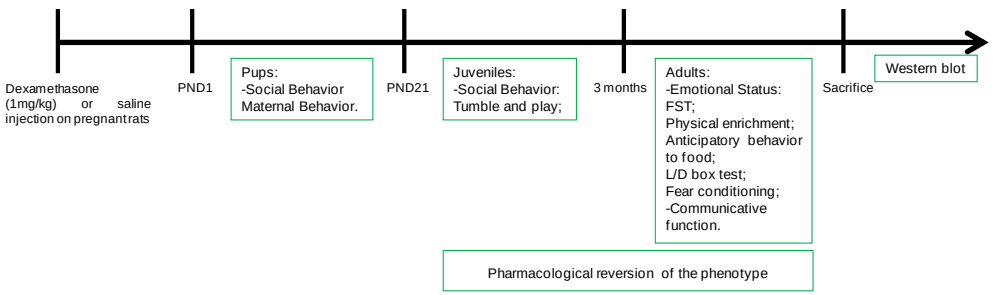


Figure 5 - Schematic representation of the experimental approach.

3.3. Behavioral characterization

3.3.1. Emotional Status

3.3.1.1. Forced swimming test

The forced swimming test (FST) apparatus consists in a glass cylindrical tank, which was filled with water (24 ± 1°C) to the depth enough so the animals could not support themselves by placing the paws or tail on the base of the cylinders. The water was cleaned between trials.

On the first day (pre-test day) and second day (test day), rats were placed inside the cylinder for 5 min (Roque et al., 2011). On both days, UVS and behavior was recorded but only the second day was scored. The characteristics assessed were climbing time, immobility time (when the rat is floating in the water), and latency to be immobile.

3.3.1.2. Physical Enrichment

The apparatus consisted in an arena with 43.2x43.2 cm, with transparent acrylic walls and white floor (MedAssociates). Adult rats were individually habituated to the arena for 10 min with toys for two consecutive days. On the testing day, the animals were social isolated for 3.5 h before testing (Trezza and Vanderschuren, 2008b). After this period, the animals were individually placed in the arena with toys for 10 min. Immediately after, the ultrasonic vocalizations were recorded. Before each trial, the apparatus was cleaned with Ethanol 10%, in order to remove odor from the previous animal.

3.3.1.3. Anticipatory behavior to food (feeding sessions)

The feeding sessions protocol was carried out for seven days as described previously (Burgdorf et al., 2000). Testing occurred in cages similar to their home cages. The testing box measured 30x20x30 cm, with a steel grid and approximately 3 cm of wood shavings covering the floor. During 6 consecutive days, the subjects were allowed to access food for 1hr per day. In the beginning of the test, the subjects were placed individually in the testing cage. After 3 min (basal situation), a white light located 10cm above the floor was turned on and it stayed on

('cue exposure') during 2 min, until the beginning of the feeding session, when they received a recipient full of food. On the seventh day, after the 2 min with a light on, instead of receiving food, an empty recipient was placed inside the testing box ('extinction'). USVs were recorded throughout the days, during the cue exposure and extinction periods. After the seventh day, the subjects were allowed to access food *ad libitum*.

3.3.1.4. Light/dark box test

The light/dark box test (L/D test) was performed inside the open field arena (43.2x43.2 cm, transparent acrylic walls and white floor) (MedAssociates Inc., St.Albans, Vermont). The animals were individually placed in the center of the illuminated part. A dark compartment was attached to one side with an opening facing the center of the open field. The distance travelled and time spent in each compartment were recorded in a single-trial of 10 min.

3.3.1.5. Confined cage test

The confined cage test was performed in a non-restrictive Plexiglas cylinder (inner diameter 8.8 cm, length 22.2 cm), mounted on a Plexiglas platform and placed in a ventilated, sound-attenuated chamber (SR-LAB, San Diego Instruments, San Diego, CA, USA). Inside of the cylinder was placed a stainless steel grid, through which an electric current could be passed (shock chamber). The microphone and a video camera were placed inside the sound attenuated chamber. The protocol was realized in two consecutive days, where the animals were placed inside of the shock chamber for 11 min. The USVs and percentage of total time freezing (total time which the animal didn't move) were measured. Percentage of total time freezing was calculated as the probability of the time freeze of an animal by the total time in the chamber. Chambers were cleaned between tests (ethanol 10 %) in order to remove olfactory cues.

3.3.1.6. Fear conditioning

The fear conditioning test was performed in the same apparatus as the confined cage test. The protocol was realized in three consecutive days (Borta et al., 2006). During the first day (habituation), each animal was placed in the sock chamber for 11 min. On conditioning day (second day), each subject was positioned again inside of the shock chamber. After the first 3 min, where no light or shock was given, the animal was exposed to six lights on/shock pairings, each followed by an inter-stimulus interval (ISI) of 60s. This shock (0,4mA ± 0,1) was administered during 500ms, after the period (20s) with the light on. Following conditioning, animals return to their home cages.

In the following day (test day), the animals were again placed in the shock chamber for 11 min. After the initial phase of 3 min, the light (3 W, incandescent light bulb) was presented six times for 20s each but not shock was given, again with an ISI of 60s. During all procedures USVs and behavior (freezing) were recorded. Percentage of total time freezing was calculated as the probability of the time freeze of an animal by the total time in the chamber. Chambers were cleaned between tests, as mentioned before.

3.3.2. Social behavior

3.3.2.1. Maternal Behavior

Maternal behavior was assessed on postnatal days (PND) 3, 7 and 15. The mothers and pups (litters) were measured together in their home cage (basal situation), during 5 min (Hofer and Shair, 1978). The procedure was performed during the light phase (08:00 a.m.) and during dark phase (08:00 p.m.).

3.3.2.2. Tumble and play

Tumble and play test (Trezza and Vanderschuren, 2008b) was performed in cages similar to their home cages, which measured 30x20x30 cm, with approximately 2 cm of wood shavings covering the floor. For behavior recordings a camera with zoom lens was used. The characteristics of the social behavior scored were: pouncing (this is an index of play solicitation, i.e., when one of the animals is attempting to nose or rub the nape of the neck of the test

partner), pinning (when one of the animals is lying with is dorsal surface on the floor with the one animal on top of him) and social exploration (when animals are sniffing any part of the body, including the anogenital area, of the test partner). Play response to solicitation was calculated as the probability of an animal of being pinned in response to pouncing by the test partner.

Juvenile rats were socially isolated for 3.5 h before testing. Each animal was then placed into the test cage with their sibling pair for 15 min. This protocol was performed three times. On the first trial, two familiar animals living in the same cage (from the same exposure group) were tested (familiar pairs). On the second trial, two unfamiliar animals (from the same exposure group) were tested (unfamiliar pairs). Lastly, we tested social interaction between one CONT animal and one iuGC animal (CONT-iuGC pairs). Besides behavior, we also measured the positive USVs during 15 min of the test, immediately after the animals were placed in the test cage.

3.3.2.3. Communicative function

In order to assess the social interaction in adult animals, we isolated one animal from the home cage and individually exposed it to a clean new cage for 15 min. During this time we recorded the USVS from the two animals, i.e., from the one isolated and the remaining animal in the home cage. After this period, animals were reunited and, once again, USVs were recorded, along with their social behavior (pinning, pouncing and social exploration) throughout the 15 min of the test.

3.3. Western Blot

Specific areas (AMY, NAcc and HPT) were collected by macrodissection, and subsequently frozen at -80°C. For western blot, lysis buffer (50 mM Tris pH 7.4, 50 mM EDTA, 50 mM NaCl, 100 mM phenylmethylsulfonyl fluoride, complete protease inhibitors) was added to each frozen area. After, samples were manually homogenized and centrifuged at 13 000 r.p.m. for 10 min at 4°C. The supernatant was collected and quantified using the Bradford method. Fifty μg of protein was loaded into a SDS-polyacrylamide gel, and gel was then

transferred to a nitrocellulose membrane. After, membranes were incubated overnight at 4°C with the primary antibodies: rabbit anti-k opioid receptor 1 (1:2500, Invitrogen), rabbit anti-μ opioid receptor 1 (1:1000, Invitrogen), rabbit anti-dopamine D1 receptor (1:2500, Abcam), rabbit anti-dopamine D2 receptor (1:1000, Abcam) and rabbit anti-endocannabinoid receptor 1 (1:500, kindly provided by Ken McKenzie). After incubation with the secondary antibodies, membranes were developed with luminol reagent (SantaCruz). Films were analyzed using ImageJ software.

3.4. Pharmacological reversal of behavioral alterations

L-DOPA/carbidopa (Sinemet, Merck, NJ, USA) was administered daily, 4h before testing, at a dose of 24 mg kg^{-1} (in water) by oral gavage.

The administration of L-DOPA/carbidopa started three days before the animals being tested for emotional behavior (FST, food sessions- anticipation behavior, fear conditioning), social and affective behaviors (tumble and play) and also for communicative function.

3.5. USVs analysis

An Ultrasound Microphone (CM16/CMPA, Avisoft Bioacoustics, Berlin, Germany) sensitive to frequencies of 10-200 kHz, was used, 20 cm above the floor, in all experiences. It was connected via an Avisoft UltrasoundGate 416H (Avisoft Biocoustics) to a personal computer. Vocalization was recorded using the Avisoft-Recorder (version 5.1.04) with the following settings: sampling rate: 250000; format: 16 bit. For acoustical analysis, recordings were transferred to Avisoft SASLab Pro (version 5.1.22, Avisoft Bioacoustics). This program was used in order to produce spectrograms of USVs by conducting a fast Fourier transformation (256 FFT-length, 100% frame, Hamming window filter, 50% time window overlap). These spectrograms had a frequency resolution ~1.2 kHz and a temporal resolution ~0.4ms.

Twenty-kHz call detection was provided by an automated threshold-based algorithm (threshold: -40 dB) and a hold time mechanism (hold time: 20 ms). A lower-cut-off-frequency of

18 kHz was use to reduce background noise. For detection of 40 kHz was used an automated threshold-based algorithm of -50 dB, an hold time of 10 ms and a high pass of 30 kHz (lower-cut-off-frequency). In support of 50 kHz call detection, it was used the same threshold (-40 dB) as the 20 kHz USVs, but a hold time of 5 ms and a lower-cut-off-frequency of 40 kHz.

Calls were also inspected manually to ensure that, when necessary, USVs not detected automatically could be subsequently included in the automatic parameter analysis. Various parameters, including peak frequency, peak amplitude and bandwidth, which were determined for each element of the entire spectrogram, were determined automatically. Temporal parameters determined included latency to call, call duration and the duration of intervals between subsequent calls. Finally, the total number of calls emitted was scored.

3.6. Statistical analysis

Statistical significance was determined using the GraphPad Prism 5 software. All data were represented as mean + SEM. Data were verified for Gaussian distribution. Unpaired t-tests was use to determine whether CONT and iuGC animals differ in USVs production and behavior during tumble and play, EPM, FST, L/D box, and fear conditioning tests. When no Gaussian distribution was assumed, we used a nonparametric test (Mann-Whitney U-test).

An two-way ANOVA for repeated measurements was used to test the differences between groups throughout time during the anticipatory behavior to food and physical enrichment tests. Significance is referred as * for $P < 0.05$, ** for $P < 0.01$, *** for $P < 0.001$.

4. RESULTS

4. Results

4.1. Effects of iuGC treatment on emotional behavior of offspring

Previous works from our lab have shown that *in utero* glucocorticoid (iuGC) exposure impairs emotional behavior, leading to an anxious phenotype, depressive-like behavior and drug-seeking behavior (Oliveira et al., 2006, Pego et al., 2008, Rodrigues et al., 2011b, Roque et al., 2011). To further characterize these animals, we implemented a new technique at the ICVS, which is the measurement of USVs (ultrasonic vocalizations), allowing to combine behavioral assessment and USVs emissions.

Depressive-like-behavior

In order to assess depressive-like behavior, animals performed the forced swimming test (FST). As expected, iuGC animals presented an increase in total time immobile (Fig.6, t=4.095, P=0.0005), decreased latency to immobility (Fig.6, t=2.207, P=0.038) and reduced climbing (Fig.6, t=3.387, P=0.0027) when compared with CONT animals.

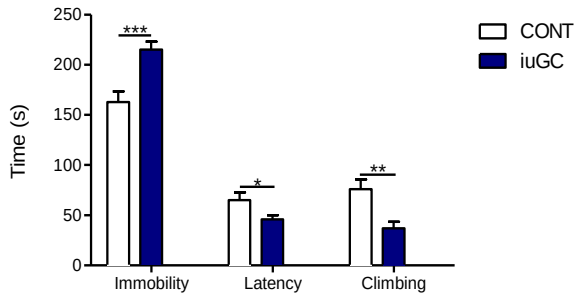


Figure 6 - Prenatal administration of iuGC leads to depressive-like-behavior in the FST test. iuGC present increased immobility time, decrease latency to immobility and reduced climbing time. n_{CONT}=12; n_{iuGC}=12; CONT=Controls; iuGC=*in utero* glucocorticoids; *P<0.05, ** P<0.01, *** P<0.001.

Depression leads to a lack of motivation and diminished pleasure feelings (Frank et al., 2007, Der-Avakian and Markou, 2012). In order to assess motivation/pleasure, we analysed

the emission of USVs in an enriched environment in socially isolated animals. In addition, we also measured USVs in response to a cue that anticipates food in food deprived animals.

During the enrichment test (arena with toys), iuGC animals emitted less 50 kHz USVs when compared with CONT (Fig.7, $F_{1,41}$= 5.58, *P*=0,023), this difference was more accentuated in the initial day.

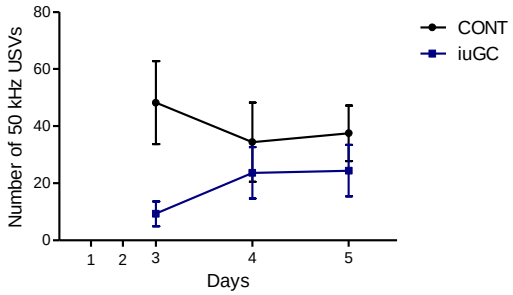


Figure 7 - Prenatal administration of iuGC leads to a diminished affective state in a novel enriched environment. iuGC emitted less 50 kHz USVs than CONT animals, throughout time. n_{CONT}=8; n_{iuGC}=8; CONT=Controls; iuGC=*in utero* glucocorticoids.

Relative to anticipatory behavior to food, animals were exposed to a cue, in this case a light, for 6 consecutive days, that predicted a bowl of food, and during this period the USVs were measured. Both groups increased the emission of positive calls over time (Fig.8A, $F_{5,82}$=5.83, *P*=0,0001), suggesting that they learned the task. Over the days, number of USVs was substantially different between groups, with the iuGC animals emitting significantly less USVs than CONT animals (Fig.8A, $F_{1,82}$=13,95, *P*=0.0003). On the seventh day, we also measured the USVs during the extinction period, i.e., after the light was off, the animal was presented with an empty recipient. Comparing both groups during cue exposure and extinction period, we observe that CONT rats emitted less in the second period (Fig.8B, *t*=2.659, *P*=0.0187), whereas the iuGC subjects emitted approximately the equal number of calls in the two periods (Fig.8B, *t*=0.7442, *P*=0.4691).

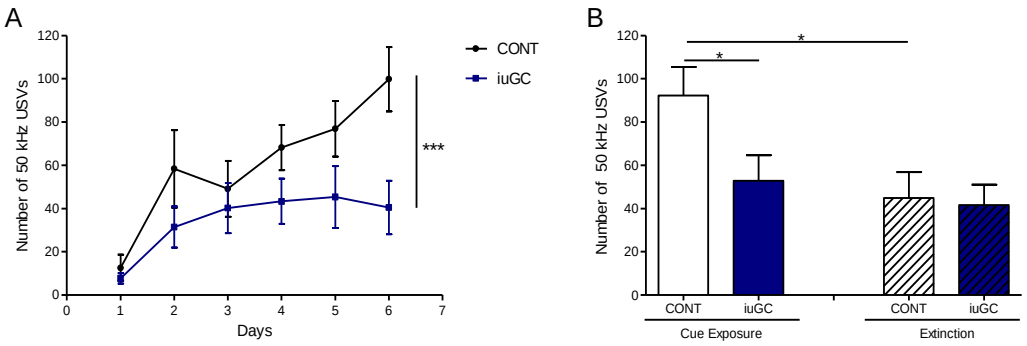


Figure 8 - Prenatal administration of iuGC leads to a lack of motivation/pleasurable feelings in anticipation to food. (A) Number of positive calls over the six consecutive days, during cue exposure, emitted by iuGC and CONT rats. Both animals increase the emission of positive calls over time although iuGC emitted less than CONT animals. (B) Number of 50 kHz USVs, during cue exposure and extinction periods (7th day). iuGC animals emitted almost the same number of USVs in the two periods, while the CONT emitted significantly less in the second period when compared with the first. n_{CONT}=8; n_{iuGC}=8; CONT=Controls; iuGC=*in utero* glucocorticoids; **P*<0.05, ** *P*<0.01, *** *P*<0.001.

Anxiety

In order to assess if iuGC treated animals presented an anxious phenotype, we performed the light/dark box test. iuGC spend more time in the dark box than CONT animals (Fig.9A, *t*=2.215, *P*=0.0407). Regarding the distance travelled during the test, no differences were found (Fig.9B, dark: *t*=1.384, *P*=0.1854, light: *t*=1.825, *P*=0.0867).

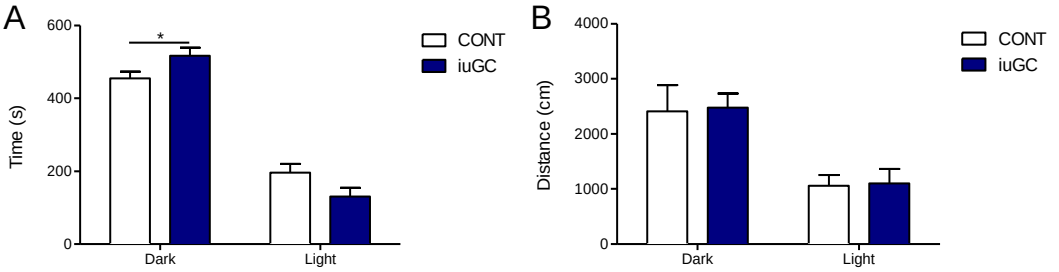


Figure 9 - Prenatal administration of dexamethasone leads to the development of anxious phenotype. Time (A) and distance (B) covered during the light/dark box test. iuGC animals spent more time in the dark box compared

with CONT, concerning the distance no differences were found. n_{CONT}=10; n_{iuGC}=10; CONT=Controls; iuGC=*in utero* glucocorticoids; *P<0.05, ** P<0.01, *** P<0.001.

An additional paradigm was performed in order to assess the anxious phenotype. Adult animals were placed inside of a novel and confined cage for 11 min. Control animals emitted very few negative 22 kHz calls, but iuGC animals emitted significantly more (Fig.10A, U=9, P=0.0202). Regarding freezing behavior, no differences were found (Fig.10B, t=0.06417, P=0.9498). After habituation to the test box, iuGC animals no longer emitted 22 kHz vocalizations (Fig.10A), and a slight decrease in the percentage of freezing was observed (Fig.10B, U=25.5, P= 0.8162).

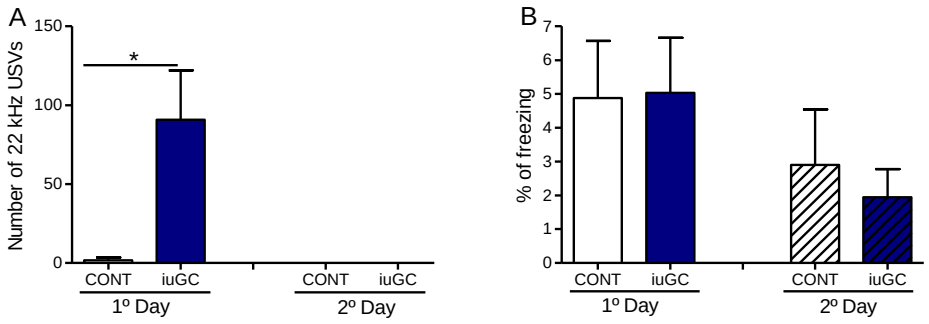


Figure 10 - Prenatal administration of dexamethasone leads to the development of an anxious phenotype. Number of 22 kHz calls (A) and percentage of freezing time (B) in a novel and confined cage. iuGC animals in an anxiogenic environment emitted more negative calls than CONT, while no differences were found in the freezing behavior, on the first day. In the second day, both groups did not emit any 22 kHZ calls, and no differences were found in the percentage of freezing. n_{CONT}=8; n_{iuGC}=8; CONT=Controls; iuGC=*in utero* glucocorticoids; *P<0.05, ** P<0.01, *** P<0.001.

Fear

We also assessed fear behavior, by conditioning the animals to a cue (light) paired with electric shocks. After the first 3 min of cage habituation, we did not observe either emission of negative calls or any time immobile. During the period of 6 light/shock pairs, as expected, both groups emitted several 22 kHz calls and spent more time immobile than in the initial phase, but iuGC to a greater extent (Fig.11A, t=2.611, P=0.0242; Fig.11C, t=2.355, P=0.0336, respectively). On the following day, the test day, animals were exposed to a cue again, but no

sock was given. In the initial phase (0-3 min), both animals spent slightly more time immobile and emitted more negative vocalizations than on the day before (Fig.11D and Fig.10B, respectively). On the next phase, cue exposure elicited more negative USVs and increased freezing time in both groups, but once more, iuGC rats were over reactive (Fig.11B, t=3,441, P=0.0063; Fig.11D, t=2.858, P=0.0126, respectively).

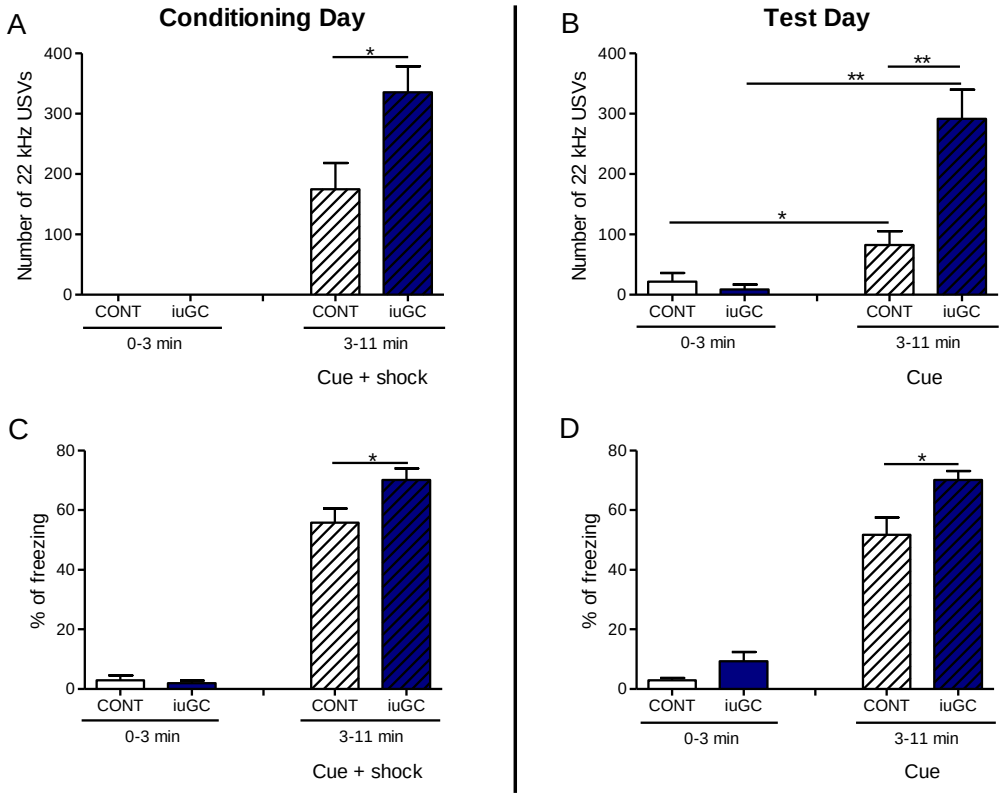


Figure 11 - Prenatal administration of GC leads to accentuated fear behavior. Number of 22 kHz USVs emitted during conditioning day (A) and test day (B). Percentage of time freezing during conditioning day (C) and test day (D). In the conditioning day, after the initial phase, the animals were submitted to 6 pairs of light/shock, both number of 22 kHz calls and percentage of freezing time increase in both groups, with a higher increase in iuGC animals. On the test day, animals were exposed 6 times to cue but without shock. We observe an increase in the number of USVs and in freezing time, and iuGC animals remain hyper-reactive. n_{CONT}=8; n_{iuGC}=8; CONT=Controls; iuGC=*in utero* glucocorticoids; *P<0.05, ** P<0.01, *** P<0.001.

By analyzing the number of 22 kHz USVs during time in the test day, it was possible to confirm that iuGC animals emitted more negative calls than control group throughout the session (Fig.12). Following the first time that light was ON, both groups increased the number

of vocalizations, but after the second cue exposure, iuGC animals emitted more USVs and remained hyper-reactive over time (Fig.12). The pattern of 22 kHz USV emission in both groups was interesting since immediately after the lights were turned off, both groups emitted more negative vocalizations, indicative of the consolidated association with the electric shock (Fig.12).

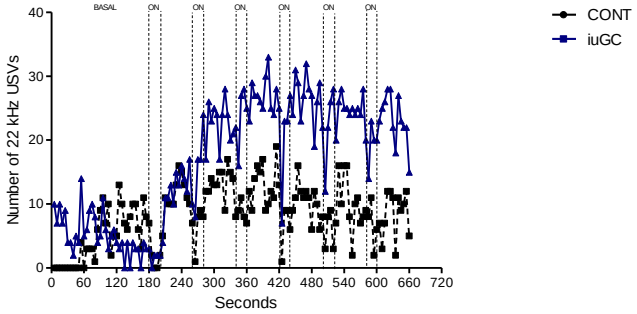


Figure 12 - Prenatal administration of iuGC leads to enhanced fear response. Number of 22 kHz of USVs over time emitted during the test da; the iuGC emitted more 22 kHz calls than CONT animals after the second cue exposure and remain over-reactive until the end of the test. n_{CONT}=8; n_{iuGC}=8; CONT=Controls; iuGC=*in utero* glucocorticoids;

4.2. Effects of iuGC treatment on social behavior of offspring

In order to asses if iuGC treatment affects social interaction, we performed different social paradigms. The first protocol consisted in analysing pup-mother interaction through 40 kHz USVs recording. When the protocol was performed in the light period (08:00 a.m.) no differences were found between groups, except on day 7 (Fig. 13A, $F_{1,36}$=0.14, P=0.7079, day 7: t=2.656, P<0.05). Relatively to the dark phase of the cycle (08:00 p.m.), once again, we did not observe differences between groups (Fig. 13B, $F_{1,32}$=1.85, P=0.1835).

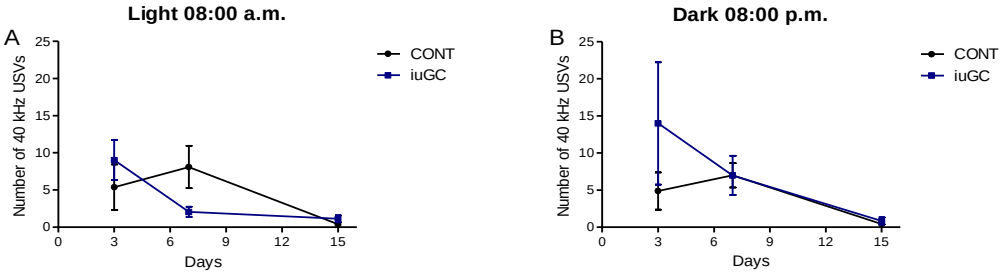


Figure 13 - Effect of iuGC treatment on pups behavior. Number of 40 kHz USVs during light phase (A) and dark phase (B). No differences were found between groups, in both periods, during 5 min of protocol. Light 08:00 a.m.: day 3 - n_{CONT}=5; n_{iuGC}=5; day 7 - n_{CONT}=7; n_{iuGC}=10; day 15 - n_{CONT}=7; n_{iuGC}=10; Dark 08:00 p.m.: day 3 - n_{CONT}=4; n_{iuGC}=4; day 7 - n_{CONT}=6; n_{iuGC}=9; day 15 - n_{CONT}=7; n_{iuGC}=10; CONT=Controls; iuGC=*in utero* glucocorticoids.

To evaluate social behavior in juveniles, we performed the tumble and play protocol. Familiar (one rat with is sibling pair) iuGC pairs emited less USVs when compared with familiar control animals (Fig.14, t=2.375, P=0.0336). In the case of unfamiliar pairs, they presented the same trend as familiar pairs (Fig.14, t=1.802, P=0.0893). Surprisingly, the CONT-iuGC pairs elicited less positive 50 kHz vocalizations than the unfamiliar CONT-CONT pair and unfamiliar iuGC-iuGC pair (Fig.14, t=4.124, P=0.0004, t=1.850, P=0.0778, respectively).

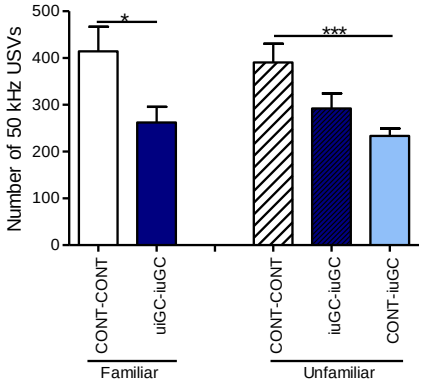


Figure 14 - GC exposure leads to a decrease in a number of positive calls during tumble and play protocol. Both familiar and unfamiliar iuGC-iuGC emitted less than CONT-CONT pairs. Interestingly, the CONT-iuGC present a decrease in the 50 kHz emission compared with unfamiliar iuGC-iuGC and CONT-CONT pairs. $n_{CONT-CONT}$= 11; $n_{iuGC-iuGC}$= 8; $n_{CONT-iuGC}$= 16; CONT=Controls; iuGC=*in utero* glucocorticoids; **P*<0.05, ** *P*<0.01, *** *P*<0.001.

We also evaluated other parameters of social behavior, namely pouncings (play solicitation), pinnings (acceptance to play) and social exploration, during the 15 min of the test. Familiar iuGC pairs have a trend to solicit less and performed less pinnings that CONT pairs (Fig.15A, t=1.525, P=0.1513; Fig.15B, t=2.973, P=0.0108, respectively). Similarly, unfamiliar iuGC pairs requested less to play (Fig.15A, t=3.032, P=0.0075), and pinned less when compared with unfamiliar CONT pairs (Fig.15B, t=3.424, P=0.0032). This was complemented by analysis of the percentage of positive responses to play solicitation. Once again, iuGC presented a decrease in the percentage of play responses when compared with CONT pairs (Fig.15C, Familiar: t=2.615, P=0.0187, Unfamiliar: t=2.220, P=0.0403). Surprisingly, CONT-iuGC pairs pounced and pinned more than iuGC pairs (Fig.15A, t=6.439, P<0.0001; Fig.15B t=3.634, P=0.0015, respectively). Interestingly, CONT-iuGC pairs spent more time in social exploration than unfamiliar CONT-CONT (Fig.15D, t=2.882, P=0.0080). Furthermore, no major differences were found in social exploration time (Fig.15D) and latency to the first occurrence of social interaction (Fig.15E).

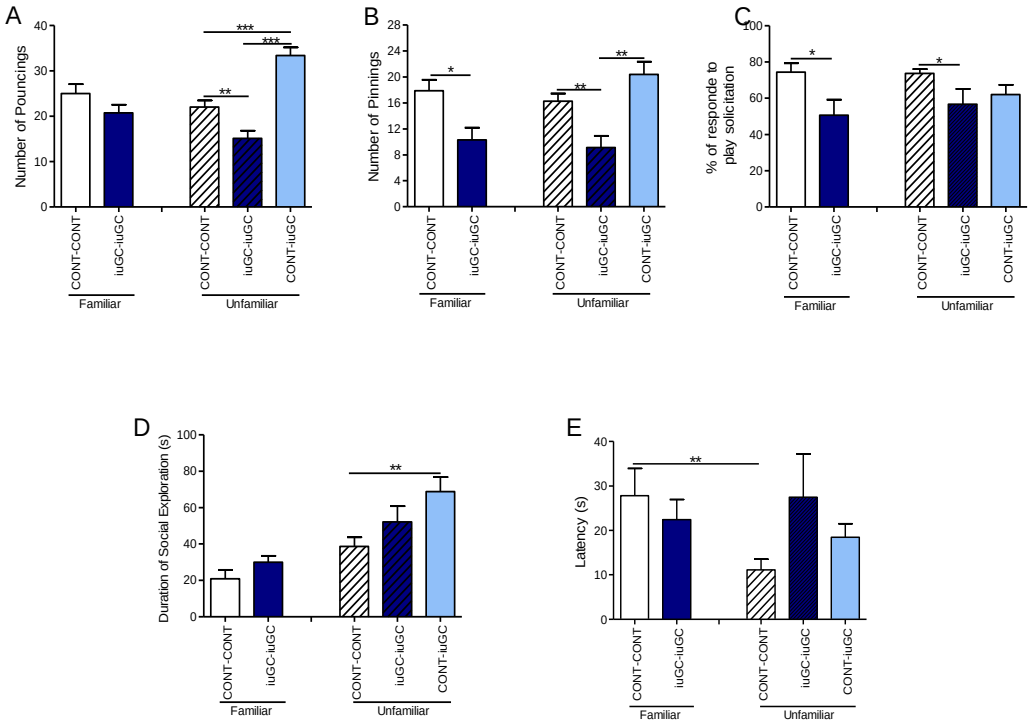


Figure 15 - Prenatal administration of dexamethasone leads impaired juvenile social behavior. Number of pouncings (A), pinnings (B) and percentage of response to play solicitation (C), duration of social exploration (D) and latency (E) to perform one of the parameters analysed during tumble and play protocol. Familiar iuGC pairs, present a trend to solicit less, while, the unfamiliar iuGC pair solicit statistically less than CONT animals. iuGC

pairs, both familiar and unfamiliar, present less pinning and a inferior response to play solicitation when compared with the CONT pairs. Unexpectedly, CONT-iuGC pairs present an increase in pouncing, pinning and spend more time in social exploration than iuGC and CONT pairs. No major differences were found in latency. $n_{CONT-CONT}$= 11; $n_{iuGC-iuGC}$= 8; $n_{CONT-iuGC}$= 16; CONT=Controls; iuGC= *in utero* glucocorticoids; **P*<0.05, ** *P*<0.01, *** *P*<0.001.

Analyzing the responses in terms of percentage of play solicitation and acceptance, in the CONT-iuGC pairs, we could observe that iuGC animals displayed inferior play solicitation in comparison with CONT animals (Fig. 16A; *t*=5.637, *P*<0.0001), and a trend for a decrease of pinning responses (Fig. 16B; *t*= 2.014, *P*=0.0530). In conformity, iuGC animals presented a significant reduction in the response to play solicitation when compared to controls (Fig. 16C; *t*=2.163, *P*=0.0389). Thus, iuGC animals were not only likely to initiate a play session but were also less likely to respond when solicited by a partner. No differences were found in social exploration (data not shown).

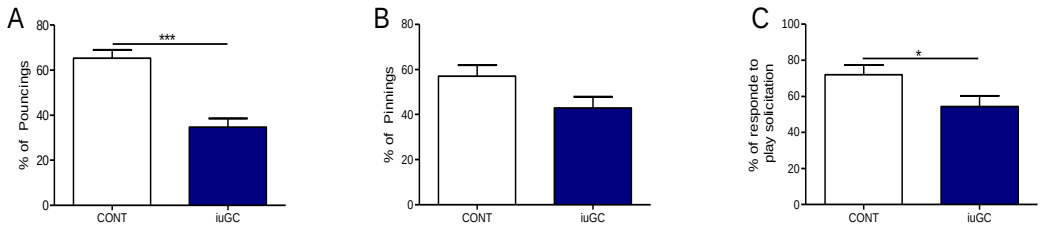


Figure 16 - Prenatal administration of iuGC leads to impaired juvenile social behavior. Percentage of pouncings (A), pinnings (B) and response to play solicitation (C) during tumble and play, concerning the CONT-iuGC pairs. The iuGC animals pounce and pin less than CONT, leading to a lower percentage of response to play solicitation. n_{CONT}=16; n_{iuGC}=16; CONT=Controls; iuGC=*in utero* glucocorticoids; **P*<0.05, ** *P*<0.01, *** *P*<0.001.

4.3. Effects of iuGC treatment on adult social behavior

Besides appetitive situations, 50 kHz USVs are also emitted after short periods of separation from conspecifics, as a communicative function. Separated animals, which can see each other, one in a new cage, other in the home cage, emit 50 kHz USVs, suggesting an affiliative communicative function of 50 kHz USVs, namely to (re)establish or to maintain social

contact (Wohr et al., 2008). During this separation, iuGC animals had a trend to emit less 50 kHz USVs than CONT animals (Fig.17A, new cage: t=2.452, P=0.0578, home cage: U=6.5, P=0.7715). After this period of separation, animals were reunited and social behavior was measured. Regarding the USVs, we did not find differences between pairs (Fig.17A, t=0.4292, P=0.6828). Once again, the iuGC-iuGC pairs pounced (Fig.17B, t=2.598, P=0.0408) and pinned less compared to CONT pairs (Fig.17C, U=0.5, P=0.0408). In agreement, response to play solicitation was lower in iuGC animals (Fig.17D, U=1, P=0.0591). No statistical differences were found in latency to first approach and duration of social exploration, though it was observed a trend for an increased latency iuGC pairs (Fig.17F, t=1.501, P=0.1840) and reduced social exploration (Fig.17E, U=3, P=0.2).

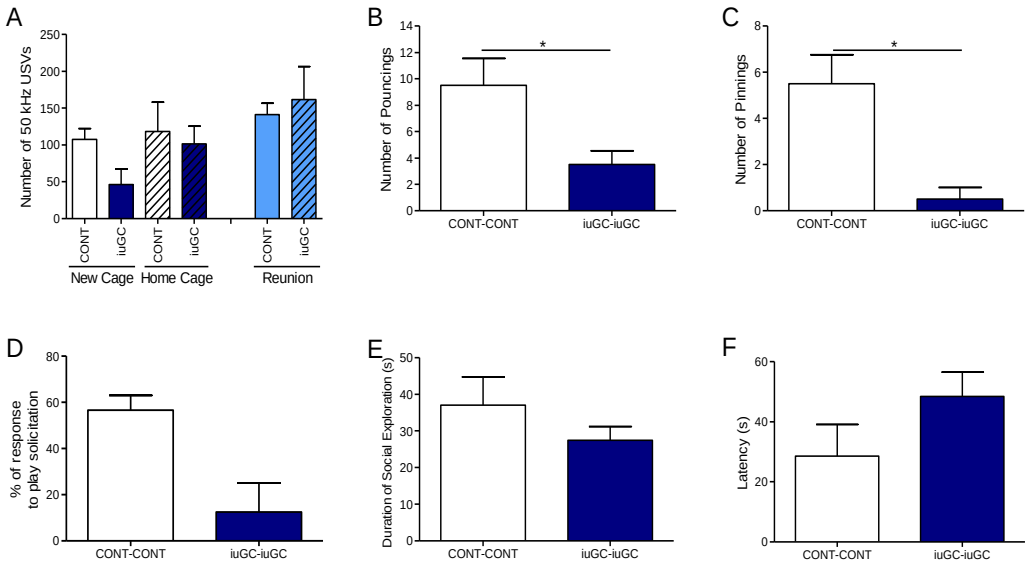


Figure 17 - Prenatal administration of GC leads to an impairment in adult social behavior. Number of 50 kHz USVs during a period of separation and reunion (A), characteristics of social behavior, pouncing (B), pinning (C), response to play solicitation (D), duration of social exploration (E) and (F) latency to the first occurrence of social interaction. No differences were found in USVs measurements. Regarding the social behavior, iuGC animals present a reduction in the number of pouncing, pinning and in the percentage of response to play solicitation when compared with CONT pairs. n_{CONT}=4; n_{iuGC}=4; CONT=Controls; iuGC=*in utero* glucocorticoids; * P<0.05, ** P<0.01, *** P<0.001.

4.4. Effects of iuGC treatment on endocannabinoid, opioid and dopaminergic circuits

As mentioned before, opioid/endocannabinoid/dopaminergic circuits are important for emotional behaviors. In order to assess if iuGC treatment affects these circuits, we analysed, by western blot the expression of opioid, endocannabinoid and dopamine receptors in three different areas (AMY, NAcc and HPT).

In the AMY, we found no statistical differences regarding the levels of dopamine receptor 1 (Drd1). The non-glycosylated form (45 kDa) and the glycosylated receptor (75 kDa) of dopamine receptor 2 (Drd2), were more expressed in iuGC animals (Fig.18C, 45 kDa: t=2.678, P=0.0215, 75 kDa: U=40, P=0.0240). Concerning the opioid receptors, the levels of μ opioid receptor type 1 (MOR1) and k opioid receptor type 1 (KOR1) were higher in iuGC when compared with CONT animals (Fig.18E, U=1, P=0.0023; Fig.18G, t=4.8, P=0.001, respectively). The endocannabinoid receptor type 1 (eCB1), appeared not to be affected in the AMY of iuGC animals. No differences were found in the 53 kDa isoform of the receptor (Fig.18I, t=0.1943, P=0.8498) and in the glycosylated (64 kDa) receptor (Fig.18I, t=0.03838, P=0.9701).

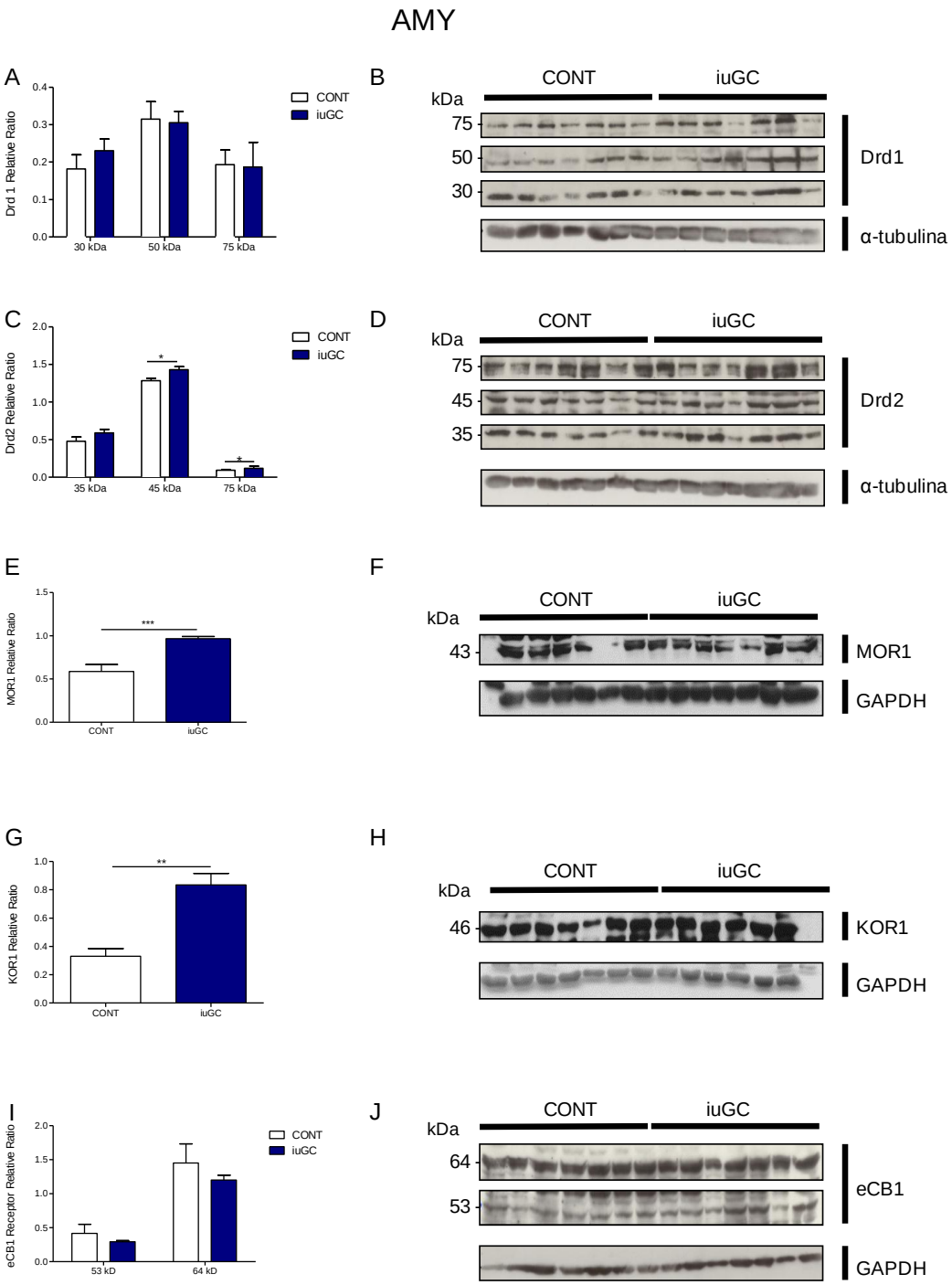


Figure 18 - Expression of dopamine, opioid and endocannabinoid receptors in the amygdala (AMY). No differences were found in the expression of Drd1 (A) and eCB1 (I). The levels of expression of Drd2 (C), MOR1 (E), KOR1 (G) were different in both groups, with a higher expression in the iuGC animals. Representative immunoblot, of Drd1 (B), Drd2 (D), MOR1 (F), KOR1 (H) and eCB1 (J). n_{CONT}=7; n_{iuGC}=7; CONT=Controls; iuGC=*in utero* glucocorticoids; **P*<0.05, ** *P*<0.01, *** *P*<0.001.

In the NAcc, no differences were found regarding Drd1 expression (Fig.19A). Drd2 precursor (35 kDa), and the non-glycosylated form (45 kDa), were more expressed in iuGC than in CONT animals (Fig.19C, 35 kDa: U=12, *P*<0.0001, 45 kDa: *t*=3.090, *P*=0.0094, respectively). The MOR1 presented a trend to be reducely expressed (Fig.19E, *t*=1.527, *P*=0.1549), while KOR1 was highly expressed in the iuGC rats (Fig.19G, *t*=2.656, *P*=0.0224),

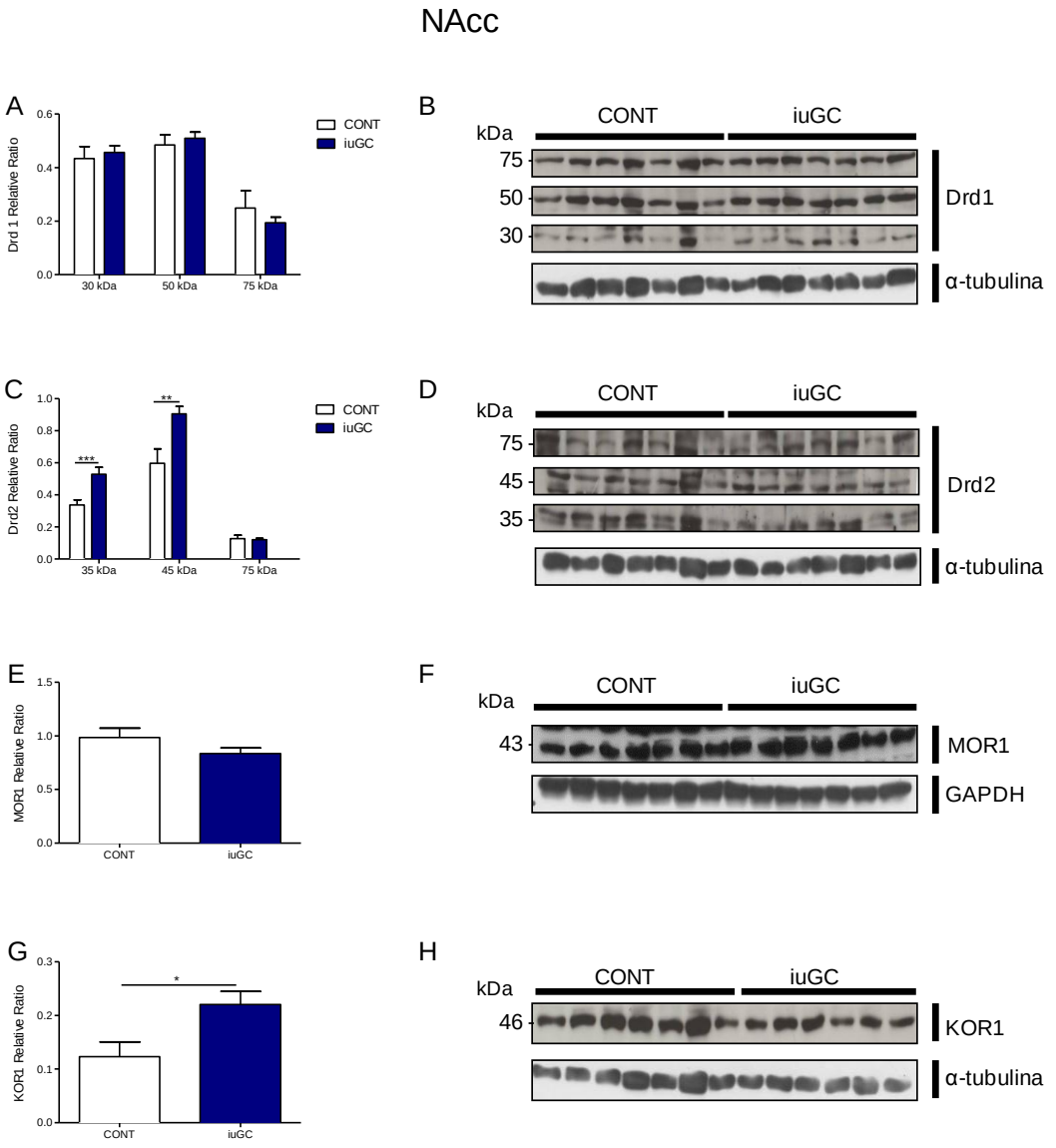


Figure 19 - Impaired dopamine receptor 2 (Drd2) and k opioid receptor 1 (KOR1) expression, on the nucleus accumbens (NAcc), of iuGC animals. No differences were found in the expression of Drd1 (A) and MOR1 (E) in the NAcc. Representative immunoblot, of Drd1 (B), Drd2 (D), MOR1 (F) and KOR1 (H). n_{CONT}=7; n_{iuGC}=7 (in KOR1 n_{iuGC}=6); CONT=Controls; iuGC=*in utero* glucocorticoids; **P*<0.05, ** *P*<0.01, *** *P*<0.001.

In the HPT, we found no differences in Drd1 (Fig.20A), nor Drd2 (Fig.20C, 45 kDa: t=0.3241, P=0.7515; 75 kDa: t=0.5173, P=0.6143). In the same way, MOR1 and KOR1 were similar between groups (Fig.20E, U=75, P=0.4520; Fig.19G, t=1.280, P=0.2124, respectively). Due to technical problems the expression of eCB1 receptors was not analyzed in the NAcc and HPT.

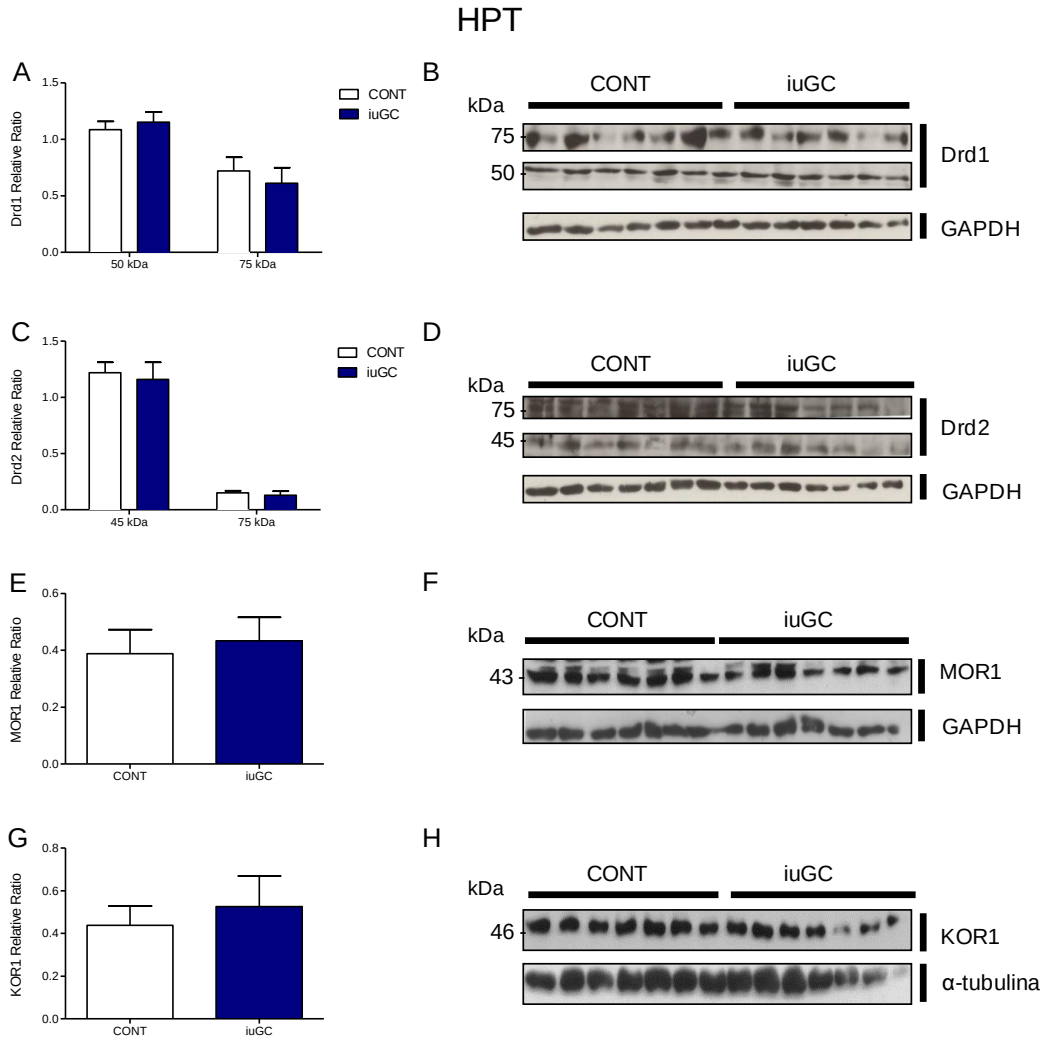


Figure 20 – Hypothalamic levels of Drd1 (A), Drd2 (C), MOR1 (E), and KOR1 (G). No differences were found. Representative immunoblot, of Drd1 (B), Drd2 (D), MOR1 (F) and KOR1 (H) in control and iuGC animals. n_{CONT}=7; n_{iuGC}=7; CONT=Controls; iuGC=*in utero* glucocorticoids.

4.5. Dopaminergic modulation of the emotional condition in iuGC animals

Considering some results showing that L-DOPA treatment could revert some of the behavioral alterations of iuGC animals (Rodrigues et al.,2011b), and considering the fact that the dopaminergic circuits seem to be crucial for both emotional and social behaviors, we decided to treat the animals with L-DOPA. The administration of L-DOPA/carbidopa (24mg/kg) by oral gavage (4h before tests), started three days before the behavioral tests, in order to normalize dopamine levels as previously shown (Rodrigues et al., 2011b). Besides the results from animals under L-DOPA treatment, we will present side by side the results of the basal conditions (previously shown in the sections 4.1 to 4.3). Due to lack of time, procedures in basal situation and under L-DOPA treatment were performed with different sets of animals, except for the L/D box test.

Depressive-like-behavior

To assess if dopamine normalization could revert the depressive-like behavior, we submitted animals to the FST test. L-DOPA treatment was able to fully revert the depressive-like behavior observed previously (Fig.21B, immobility: t=0.3583, P=0.7243; latency: U=44, P=0.6842; climbing: t=1.249, P=0.2287).

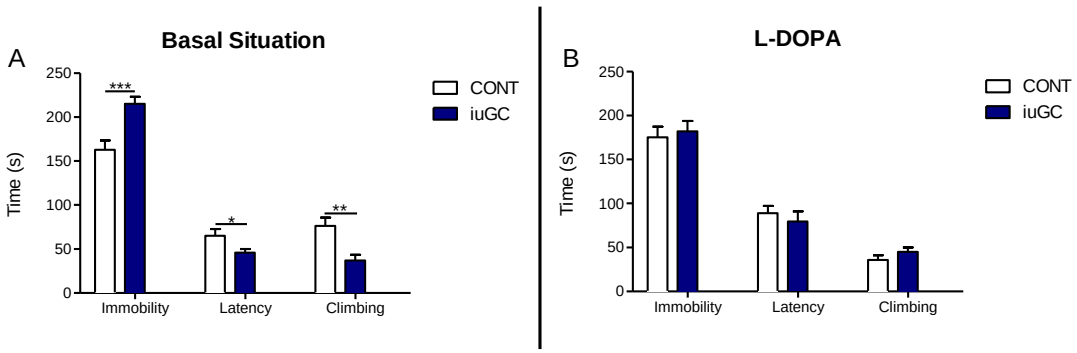


Figure 21 - Reversion of depressive-like-behavior of iuGC animals by L-DOPA treatment. The graph represents the total time immobile, latency to stay immobile and total time climbing of CONT and iuGC animals, without (A) and under L-DOPA treatment (B). No differences were found between groups, in L-DOPA treated animals (B). (A) is reproduced from Fig.6. n_{CONT}=10; n_{iuGC}=10; CONT=Controls; iuGC=*in utero* glucocorticoids; * P <0.05, ** P <0.01, *** P<0.001.

We also performed the food anticipation paradigm with the subjects treated with L-DOPA. The curves of both groups were very different (Fig.22B, $F_{1,108}$=18,79, P< 0.0001); iuGC animals emitted much more positive calls than CONT animals. On the seventh day, we also measured the USVs during the extinction period and we found no major differences (Fig.22D, cue exposure: t=6731, P=0.5094, extinction: t=0.7340, P=0.4730).

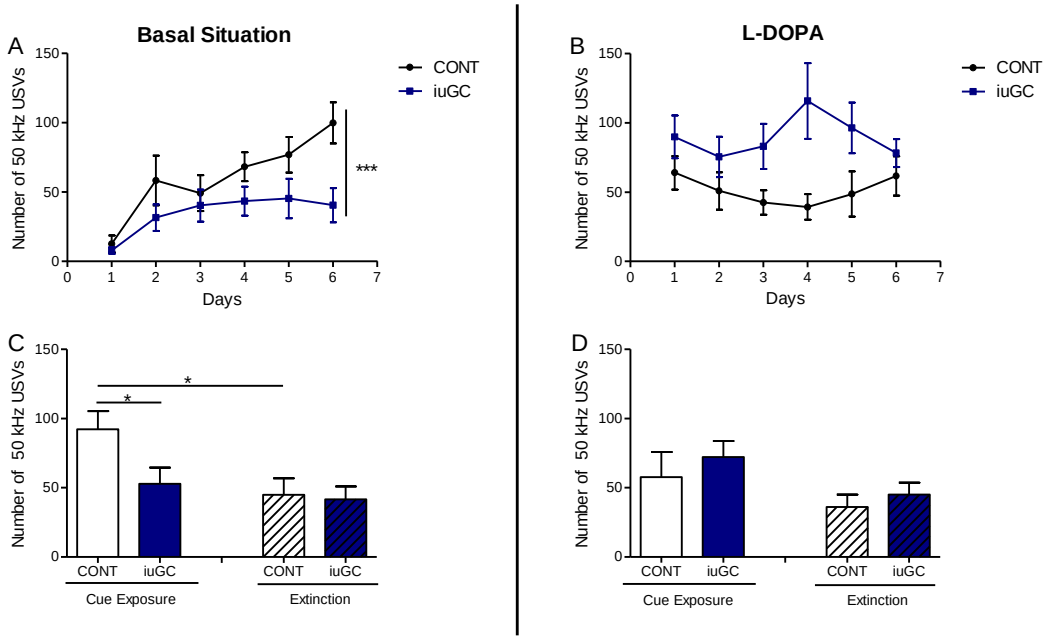


Figure 22 - Effects of L-DOPA administration in USVs emission in anticipation to food. Number of positive 55 kHz calls over the six consecutive days, under basal conditions (A) and L-DOPA treatment (B). In basal situation, iuGC emitted less than CONT animals. After L-DOPA treatment, iuGC emitted more than CONT animals over time. Number of 50 kHz USVs on seventh day, during cue exposure and extinction periods, under basal situation (C) and under L-DOPA treatment (D). During cue exposure iuGC animals emitted less than CONT under basal situation. While with L-DOPA treatment, no major differences were found in both periods. (A) and (C) are reproduced from Fig.8. Basal situation: n_{CONT}=8; n_{iuGC}=8; L-DOPA treatment: n_{CONT}=10; n_{iuGC}=10; CONT=Controls; iuGC=*in utero* glucocorticoids; *P<0.05, ** P<0.01, *** P<0.001.

Anxiety

We also analysed the effect of L-DOPA treatment on anxious behavior. Regarding the light/dark box test, no differences were found in the time spent (Fig.23B, dark: t=1.228,

P=0.2385; light: *t*=1.587, *P*=0.1334) and distance travelled (Fig.23A, dark: *t*=1.384, *P*=0.1854; light: *t*=1.825, *P*=0.0867) in the dark and light compartments.

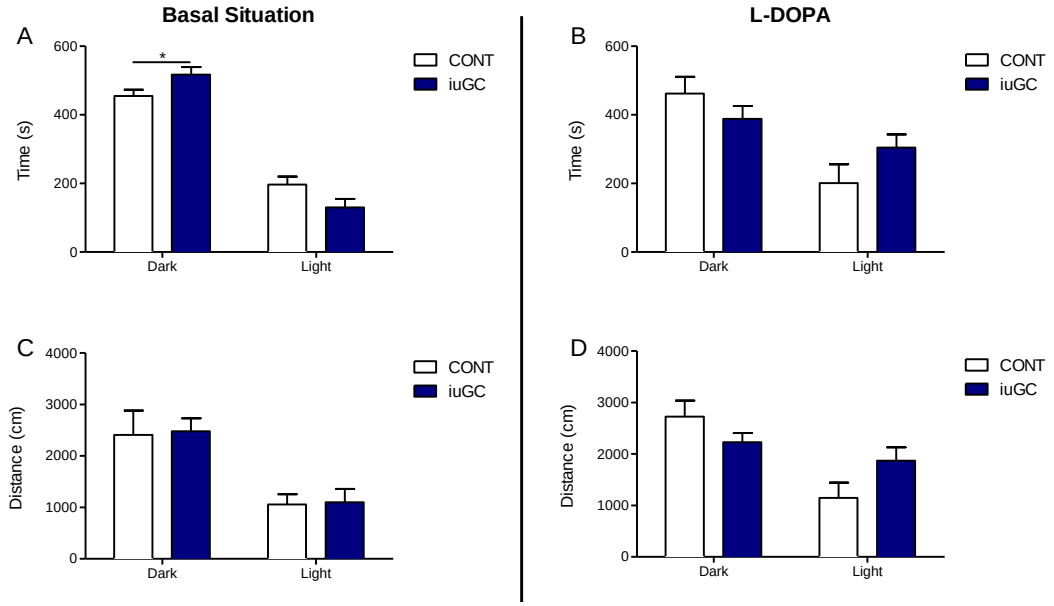


Figure 23 - Effects of L-DOPA administration on light/dark box test. Time spent in the dark and light compartment, under basal situation (A) and under L-DOPA treatment (B). In basal situation, iuGC animals spent more time in the dark compartment than compared with CONT; with L-DOPA treatment no differences were found. Distance covered in the light/dark box test in a basal situation (C) and under L-DOPA treatment (D). No differences were found in both conditions. (A) and (C) are reproduced from Fig.9. Basal situation: n_{CONT}=8; n_{iuGC}=8; L-DOPA treatment: n_{CONT}=10; n_{iuGC}=10; CONT=Controls; iuGC=in utero glucocorticoids; * *P*<0.05, ** *P*<0.01, *** *P* <0.001.

In treated animals both CONT and iuGC groups did not emit any aversive 22 kHz calls during confinement (data not shown). In accordance, both groups displayed extremely reduced freezing (Fig.24, U=22, *P*=0.7658).

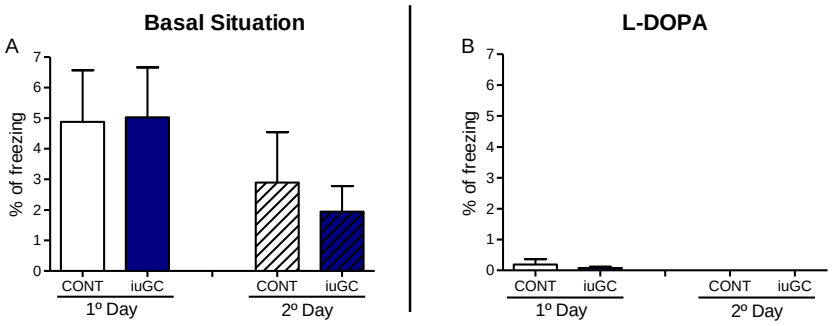


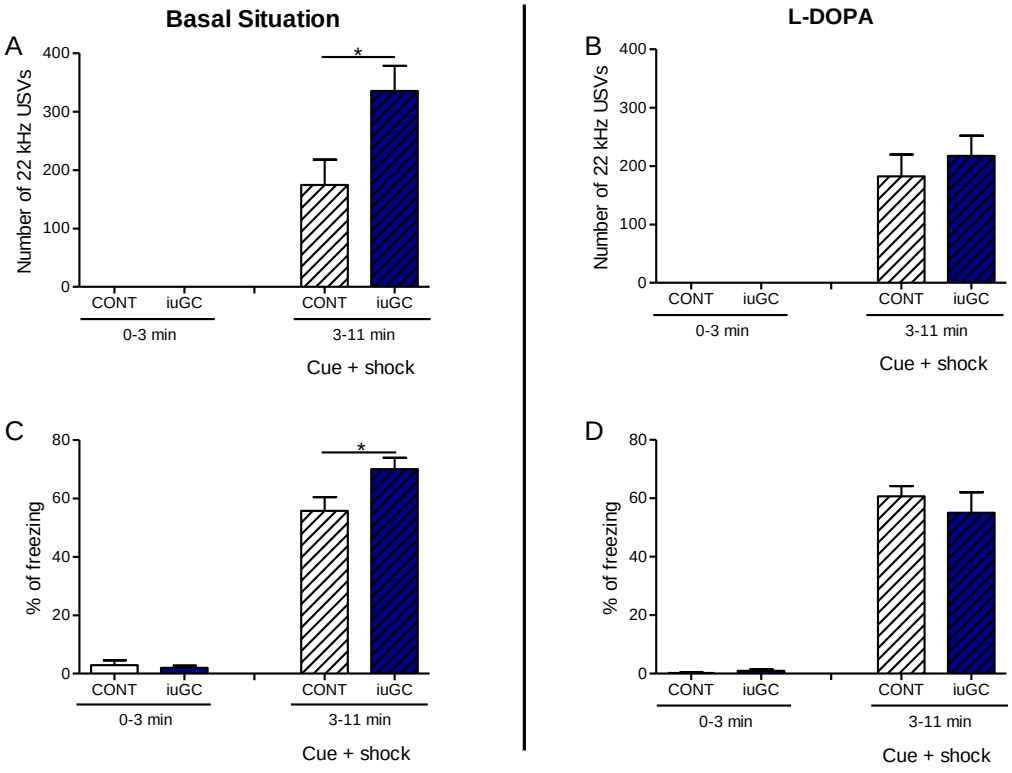
Figure 24 - Reversion of anxious phenotype of iuGC animals by L-DOPA treatment. Freezing behavior performed in a novel and confined cage during 11 min by each group, in a basal situation (A) and under L-DOPA treatment (B). In basal situation no differences were found on both days. Under L-DOPA treatment, both groups present reduced freezing, once again, no differences were found between groups on day 1. In the second day, animals do not stay immobile. (A) is reproduced from Fig.10B. Basal situation: n_{CONT}=8; n_{iuGC}=8; L-DOPA treatment: n_{CONT}=8; n_{iuGC}=8; CONT=Controls; iuGC=*in utero* glucocorticoids.

Fear

L-DOPA treatment was able to revert the increased 22 kHz USVs emission during conditioning day (Fig.25B, t=0.6813, P=0.5076), as well as the augmented freezing percentage (Fig.25D, t=0.7117, P=0.4884).

On the testing day, no major differences were found in L-DOPA treated animals but this was more likely a result of changes in control animals rather than in iuGC animals (USVs: Fig.25F, t=0.6409, P=0.5319; Freezing: Fig.25H, t=0.2744, P=0.7884).

Conditioning day



Test day

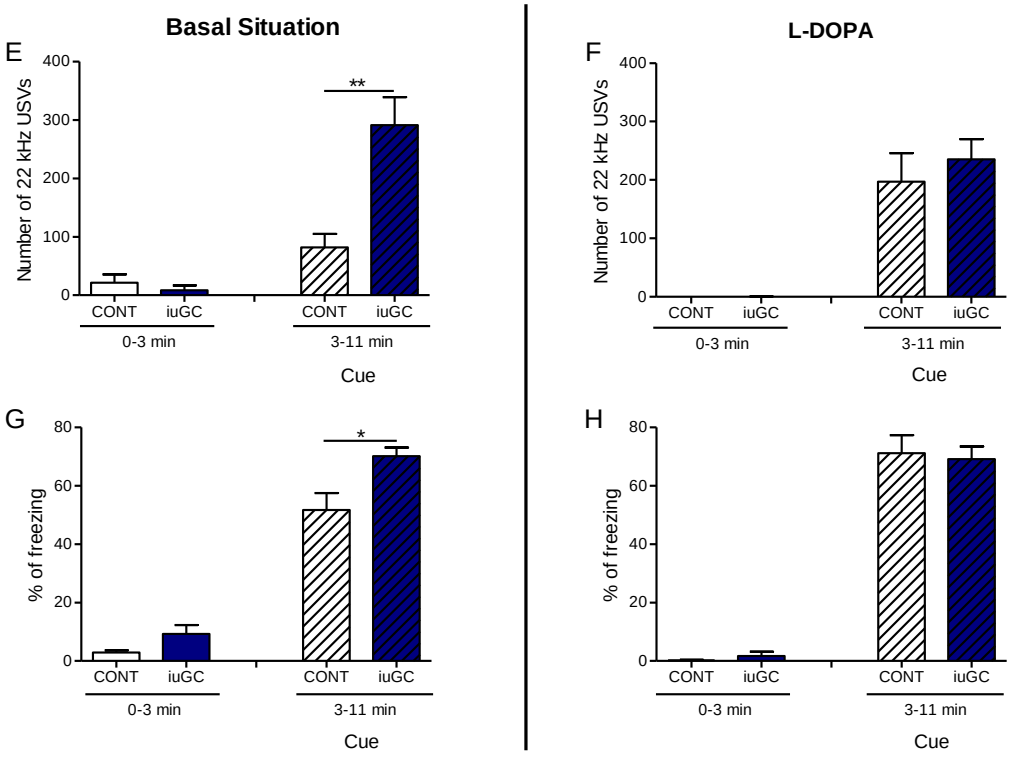


Figure 25 - Effect of L-DOPA treatment on fear conditioning paradigm. Number of 22 kHz USVs emitted during conditioning day in basal situation (A) and under L-DOPA treatment (B). In the conditioning day, after the initial phase, the animals were submitted to 6 pairs of light/shock. In basal situation, iuGC animals emitted more than CONT (A), while with L-DOPA treatment no differences were found between groups (B). Regarding percentage of time that animal remained immobile on the conditioning day, under basal condition, iuGC animals remained more time immobile compared with CONT (C), while under L-DOPA treatment no differences were found (D). On the test day, after the first 3 min, animals were exposed 6 times to light but no shock was given. Once again, iuGC animals emitted more negative 22 kHz calls than CONT, under basal situation (E). With L-DOPA treatment no differences were found (F). In basal situation, iuGC animals remained more time immobile compared with CONT (G). Under L-DOPA treatment no differences were found between groups (H). (A),(C),(E) and (F) are reproduced from Fig.11. Basal situation: n_{CONT}=8; n_{iuGC}=8; L-DOPA treatment: n_{CONT}=8; n_{iuGC}=8; CONT=Controls; iuGC=*in utero* glucocorticoids; **P*<0.05, ** *P*<0.01, *** *P*<0.001.

On the testing day, in the initial 100 seconds we detected no emission of negative USVs. Following the first time that the lights were ON, both groups increased the number of vocalizations to a similar level (Fig.26).

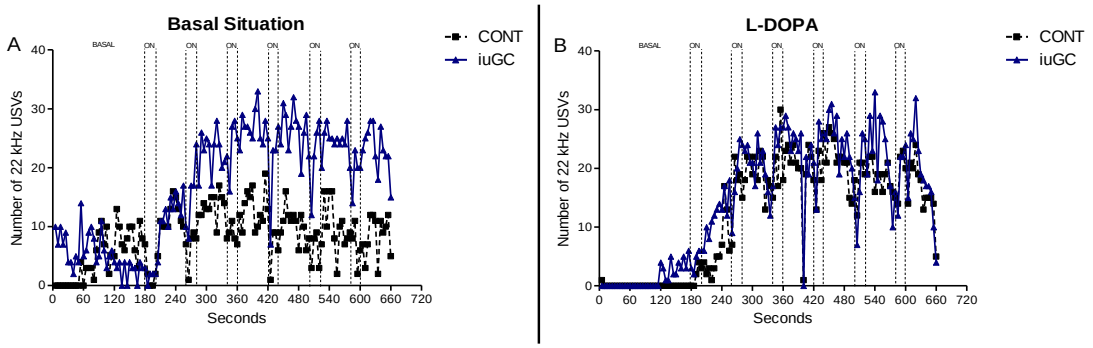


Figure 26 - Effects of L-DOPA administration on both groups in fear conditioning paradigm. Number of 22 kHz of USVs over time emitted during the test day under basal conditions (A) and L-DOPA treatment (B). The iuGC animals emitted more negative 22 kHz calls than CONT animals after the second cue exposure and remain until the end of the test (A) in a basal situation. With L-DOPA treatment, both groups increase the number of 22 kHz USVs similarly. (A) is reproduced from Fig.12. Basal situation: n_{CONT}=8; n_{iuGC}=8; L-DOPA treatment: n_{CONT}=8; n_{iuGC}=8; CONT= Controls; iuGC=*in utero* glucocorticoids.

4.6. Dopaminergic manipulation of social behavior in iuGC animals

To further ascertain if it is possible to revert the social behavior impairments observed during tumble and play test, we treated juvenile animals with L-DOPA using a similar protocol as described for adult animals. During the tumble and play protocol we found no major differences between CONT and iuGC pairs, both in familiar and unfamiliar conditions (Fig.27B, familiar pairs: t=0.4343, P=0.670; unfamiliar pairs: t=0.3815, P=0.7085).

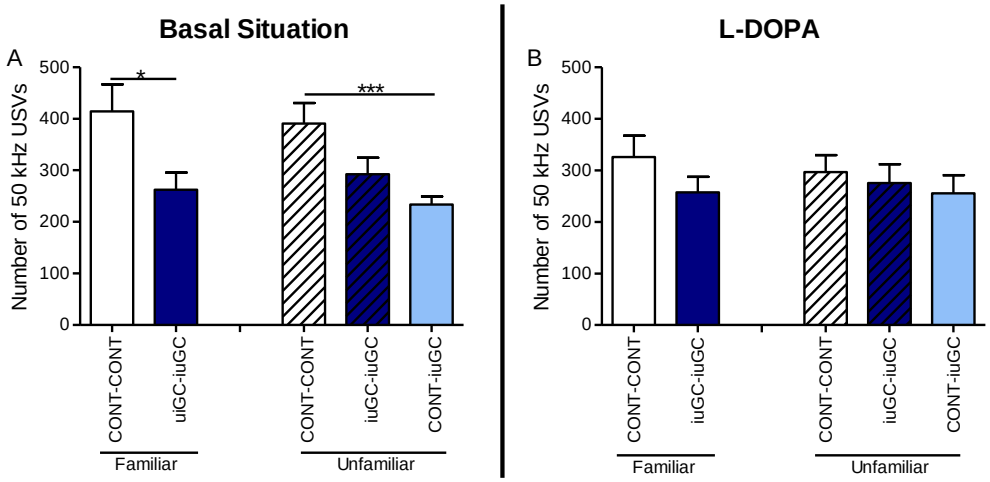


Figure 27 – Dopaminergic manipulation of social behavior. Number of positive 50 kHz USVs emitted during tumble and play protocol, in basal situation (A) and under L-DOPA treatment (B). Social play elicited less positive calls in iuGC-iuGC pairs when compared with CONT-CONT pairs, in a basal situation. Under L-DOPA treatment no differences were found between all groups tested. (A) is reproduced from Fig.14. Basal situation $n_{CONT\text{-}CONT}$= 11; $n_{iuGC\text{-}iuGC}$= 8; $n_{CONT\text{-}iuGC}$= 16; L-DOPA treatment: $n_{CONT\text{-}CONT}$= 8; $n_{iuGC\text{-}iuGC}$= 8; $n_{CONT\text{-}iuGC}$= 8; CONT= Controls; iuGC=*in utero* glucocorticoids, *P<0.05, ** P<0.01, *** P<0.001.

Similarly, no major differences were found between groups in terms of social behavior. Both groups presented similar patterns of poucning (Fig.28D, familiar: U=47, P=0.5967; unfamiliar: t=0.7253, P=0.4802), pinning (Fig.28F, familiar: t=0.5424, P=0.5939, unfamiliar: t=1.007, P=0.3308) and percentage of response to play solicitation (Fig.28H, familiar: t=0.9496, P=3556; unfamiliar: t=1.887, P=0.08). Interestingly, we found an effect of L-DOPA treatment on the latency to perform social play behavior, all groups take less time to engage in social play, especially the unfamiliar iuGC pairs.

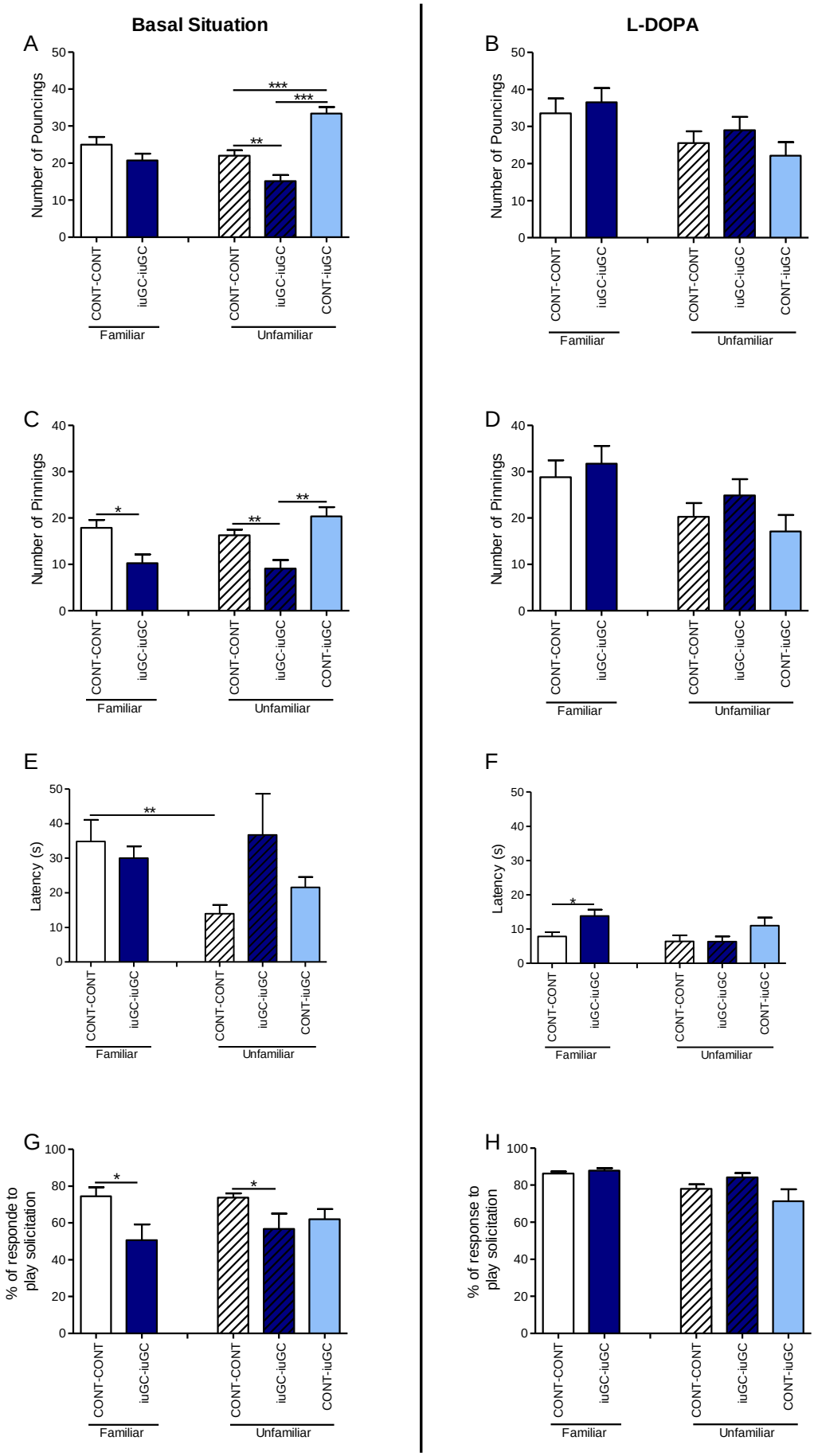

Basal Situation
L-DOPA
A
B
C
D
E
F
G
H
Number of Pouncings
Number of Pinnings
Latency (s)
% of responde to play solicitation
% of response to play solicitation
CONT-CONT
iuGC-iuGC
CONT-iuGC
Familiar
Unfamiliar
*
**
***

Figure 28 - Reversion of social behavior impairment observed in the iuGC treated animals by L-DOPA administration. In a basal situation, the iuGC pairs pounce less (A), pin less (C) and having as a result inferior percentage of response to play solicitation (G), when compared with CONT pairs. No differences were found in the number of pouncings (B), pinnings (D) and percentage of response to play solicitation (H) between groups, under L-DOPA treatment. Interestingly, the latency to engage in social play is inferior under L-DOPA treatment (F), especially unfamiliar iuGC pairs, while in a basal situation no differences were found (E). (A),(C),(E) and (G) are reproduced from Fig.15. Basal situation $n_{CONT\text{-}CONT}$= 11; $n_{iuGC\text{-}iuGC}$= 8; $n_{CONT\text{-}iuGC}$= 16; L-DOPA treatment: $n_{CONT\text{-}CONT}$= 8; $n_{iuGC\text{-}iuGC}$= 8; $n_{CONT\text{-}iuGC}$= 8; CONT= Controls; iuGC=*in utero* glucocorticoids; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

Regarding the CONT-iuGC pairs no major differences were found between groups, in terms of percentage of solicitation to play (pouncings: Fig.29B, t=0.6452, P=0.5292) and acceptance (pinning: Fig.29D, t=1.501, P=0.1555) and in the response to play solicitation (Fig. 29F; t=0.8006, P=0.4367).

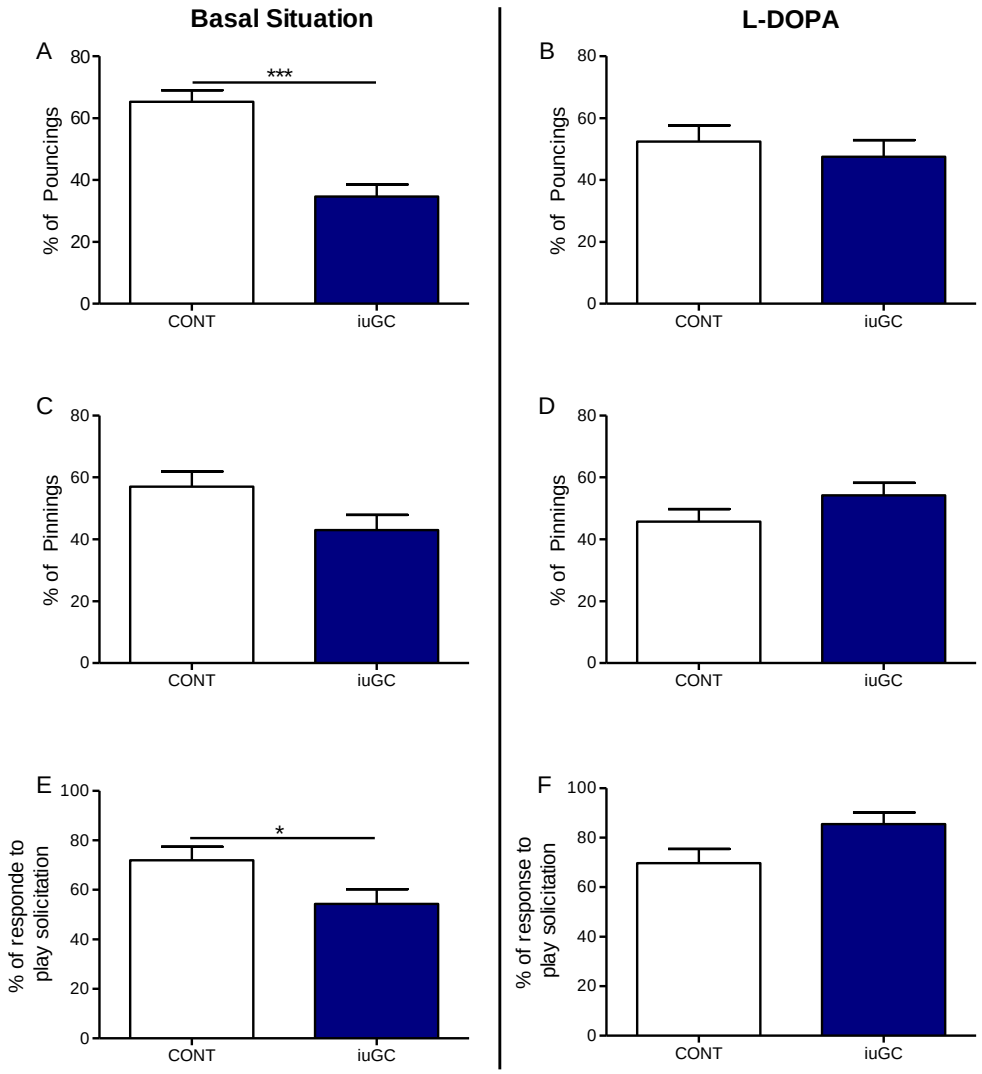


Figure 28 - Reversion of social behavior impairment observed in the iuGC treated animals, by L-DOPA administration. iuGC animals perform less solicitation to play (A) and respond less (C) having as consequence a lower percentage of response to play solicitation (E) when compared with CONT subject, under basal conditions. With L-DOPA treatment, no differences were found between animals, in percentage of pouncing (B), pinning (D) and response to play solicitation (F). (A),(C) and (E) are reproduced from Fig.16. Basal situation: $n_{CONT-iuGC}$= 16; L-DOPA treatment: $n_{CONT-iuGC}$= 8; CONT=Controls; iuGC= *in utero* glucocorticoids; **P* <0.05, ** *P* <0.01, *** *P* <0.001.

5. DISCUSSION

5. Discussion

5.1. Stress effects in affective state

Stress can have remarkably different outcomes, depending on the time of exposure, type, length and intrinsic (epi/genetic) factors of the individual. Early life stress has a powerful programming effect and leads to the appearance of several neuropsychiatric conditions later in life, especially those involving the reward circuitry such as anxiety, depression and reward disorders (e.g. addiction) (Roque et al., 2011, Oliveira et al., 2006, Rodrigues et al., 2011b).

Psychological resilience is known as a relatively stable personality trait characterized by the capability to bounce back from negative experiences and by flexible adaptation to the ever-changing demands of life (Block and Kremen, 1996). It is well-known that positive affective states, as studied longitudinally in humans, confer resilience to depression and anxiety and lead to an increase in overall health and a decrease in mortality from all causes (Lyubomirsky et al., 2005). In addition, individuals with high levels of happiness or trait positive affect appear to be more likely to approach reward activities, especially social ones. On the other hand, socialization is itself a factor that elicits highest levels of positive affect state (Stone et al., 2006).

In humans, positive affective states are measured primarily via subjective self-report and behaviorally by facial/vocal displays. In laboratory animals this is not possible, we can only rely on empirical observations. However, it has been proposed that ultrasonic vocalizations are potential tools to measure when animals are in a positive affective state. For example, several studies show that 50 kHz USVs are correlated with positive affective state in rats. Social interaction, anticipation to food, drugs of abuse and electrical brain stimulation reward, increase number of positive 50 kHz calls, in another word, increase positive affective state (Burgdorf et al., 2000, Burgdorf et al., 2001, Burgdorf et al., 2008). As expected, these calls are decreased by aversive stimuli such as social defeat, frustative non-rewarding situations, sickness and foot shock (Burgdorf et al., 2000, Burgdorf et al., 2008).

By measuring the USVs in combination with standard behavioral tests, we showed that *in utero* glucocorticoid exposed animals present a prominent anxious phenotype, depressive-like behavior and emotional over-reactivity (enhanced fear response).

Depressive-like behavior

Repeated stress and HPA axis hyperactivation have been associated with the development of depression (Tsigos and Chrousos, 2002). iuGC animals have an impairment in the HPA activity (Oliveira et al., 2006), which could potentiate the appearance of depressive-like behavior. We performed two different behavioral tests to ascertain depressive-like behavior of iuGC animals – the FST and USV emission in anticipation to a rewarding event (food). During the FST, we observe an increase in immobility time and decreased time to immobility, suggesting that prenatal dexamethasone treatment leads to a depressive-like state, as previously reported (Roque et al., 2011). While immobility and latency are considered an indicator of depression, several articles suggest that climbing behavior is a defensive-escape response to potentially dangerous situations than a characteristic of depressive-like-behavior. A similar increase in the immobility time was observed in other models of prenatal DEX exposure (dexamethasone in drinking water from day 15 of pregnancy) (Hauser et al., 2009), suggesting that this is a common trait of GC exposed animals.

One other core symptoms of depression is anhedonia, i.e., is the inability to obtain pleasure from enjoyable experiences, such as eating, play, sexual behavior or social interactions (Moreau, 1997). More recent studies have highlighted the need to consider different aspects of pleasant behavior, such as motivation or desire to engage in an activity ("motivational anhedonia") as compared to the level of enjoyment of the activity itself ("consummatory anhnedonia") (Treadway and Zald, 2011). When assessed for anhedonia, in the anticipation to food paradigm, iuGC animals emitted less positive USVs than CONT rats, throughout time when exposed to a cue that predict food. Note that at day 1 no differences were found, as expected, since the animals still do not associate the stimuli with the reward.

These results may indicate that *in utero* glucocorticoid exposure leads to a diminished motivational status. Indeed it has been shown that prior chronic corticosterone exposure to mice or rats leads to reduction in food-motivated instrumental acquisition and performance,

i.e., lack of motivational drive (Olausson et al., 2012). We also evaluated the response of the animals to a physical enriched environment, which could be seen as a measure of consummatory anhnedonia, although some validation studies are still required. While CONT animals decreased the number of positive USVs throughout days, potentially indicating that they lost "interest" in the environment (habituation), iuGC animals seem to increase calls, which may be a result of their anxious behavior, though more studies need to be performed.

Anxiety

Depression has been linked to stress exposure and is frequently comorbidily associated with anxiety (Aina and Susman, 2006, Tsigos and Chrousos, 2002), suggesting an overlapping pathway of neuronal (dys)function between these affective disorders. Avoidance of the open arms in the elevated plus maze (EPM) is considered the goal standard index of anxiety-related behavior and it has been previously shown that iuGC animals avoid open arms (Oliveira et al., 2006). In order to further confirm the anxious phenotype, we perform the light/dark box test. iuGC group presents a decrease in the time spend in the anxiogenic light compartment, indicative of higher anxiety levels. In addition, we also observe that iuGC subjects emit more 22kHz aversive vocalizations than controls in a novel and confined cage, importantly, that these differences are eliminated with habituation. These data are accordingly with information shown by Borta *et al* that rats that are highly anxious tented to vocalize more often and present more freezing time (though we were unable to measure this parameter) during an aversive stimulus, than rats that display low anxiety-like behavior (Borta et al., 2006).

Fear

In the fear conditioning paradigm that we used, flight or avoidance responses to signaled shock is considered to reflect fear rather than anxiety. We observe that iuGC animals emitted more negative calls than CONT when presented with paired cue-shock stimuli. This may suggest different pain sensitivity, which is in accordance with preceding literature showing that chronic stress induces a hypersensitivity to painful stimuli (Metz et al., 2001, D'Amato and Pavone, 2012), but may also reflect a general emotional over-reactivity in these animals. In

further support of this last idea, iuGC animals also emit more negative calls and present an increase in freezing percentage in response to cue that predict a harmful stimulus and during inter-stimulus-interval. Both groups emitted negative USVs in a context associated with such painful experience and iuGC animals present a trend for increased contextual emission. Besides the fact that USVs reflect the animals affective state, some reports hypothesized that they also have a communicative function (Wohr et al., 2008). Indeed, the 22 kHz calls in response to a predator, might serve as "alarm calls", which determines the probability of their emission in the presence of listeners, namely rats from the same colony. However, in our experiments, fear conditioning protocol was performed inside of a ventilated, sound-attenuated chamber in a room with no other animals present, which suggests that rather than alarm calls, these 22kHz USVs are emitted due to a negative affective state of the animal.

These results show that early life stress modulates the response to a novel and painful/anxiogenic stimulus, which is supported by previous works that showed that prenatal stress impair the organism's resilience to stress in adulthood (Oliveira et al., 2006).

5.2. Impact of prenatal stress on social/affective behaviors

Charles Darwin wrote: 'Happiness is never better exhibited than by young animals, such as puppies, kittens, lambs, &c., when playing together, like our own children' (Darwin, 1871/1936)

All mammals engage in some form of social play behavior, spend time in these activities with no understandable meaning than having fun, suggesting that social play is important for happiness/positive affective state. In humans, it was found that happy individuals are more likely to enjoy their leisure activities and social interactions, and to be more satisfied with their activities in general (Lyubomirsky et al., 2005).

We hypothesized that the emotional and motivational status of the animal could influence social interaction, thus we assessed iuGC animals in different social paradigms. In maternal-pup induced vocalizations, we did not find major differences between groups, except for day 7, where iuGC litters emitted significantly less than controls. This may indicate some degree of social deficit, although more studies need to be performed, such as for example pup-

mother vocalization upon separation and reunion. In juvenile animals, social deficits were obvious and remained until adulthood. In the tumble and play protocol, juvenile iuGC rats presented a trend to a reduced number of 50 kHz calls when playing with their familiar pair in comparison with CONT pairs, which may indicate some degree of anhedonia. Concerning the unfamiliar pairs, social interaction with a novel partner may elicit negative feelings - indicative of anxiety, and, together with the lack of motivation, may further impair social behavior. The unfamiliar iuGC pairs play less and emit less quantity of positive USVs, opposite pattern was observed in CONT pairs. Regarding the CONT-iuGC pair, once again, the iuGC pounce significantly less and present a decrease in play response in comparison with CONT partners. This is in agreement with data suggesting that social behavior and vocalization emission is greatly dependent on the partner (Trezza and Vanderschuren, 2008b, Wright et al., 2010). In adulthood, this play protocol involves some constraints because animals already present aggressive behavior and fight instead of playing to establish dominance/submissive hierarchy (Vanderschuren et al., 1997). Thus we assessed communicative function, another indirect measure of social interaction. Isolated iuGC animals have a trend to emit less calls than controls whereas animals that remain in their home cage present no differences. However, upon reunion, iuGC group presents remarkably different social interaction, presenting a decrease in social behavior. These results are further supported by previous findings that indicate that early gestational exposure to dexamethasone in marmots (Hauser et al., 2007), in pig sows (hydrocortisone) (Kranendonk et al., 2006) and in rats (Kleinhaus et al., 2010) reduces social affiliative behavior in the offspring.

These findings showing that *in utero* glucocorticoids causes a reduction in motivational drive/lack of pleasurable feelings in rewarding situations such as social interaction, are in further support for the depressive-like behavior/anhedonia status observed iuGC animals.

Altogether, these results may be relevant in the context of neuropsychiatric conditions that present impaired social behavior such as schizophrenia and autism (Cannon et al., 2008, Trezza and Vanderschuren, 2008b), in which both have been proposed to have a neurodevelopmental cause, and where stress may aggravate/potentiate the symptoms (Gillott and Standen, 2007, Seghers and Docherty, 2009).

5.3. Endocannabionid, opioid and dopaminergic correlates

Mesolimbic dopaminergic transmission is regulated at several levels, and converging evidence shows a strong overlap and interdependence with endocannabinoid system (Hill and McEwen, 2010, Gorzalka et al., 2008). As mentioned before the endocannabinoid/opioid/dopaminergic circuits are extremely important in the modulation of emotional and social behaviors.

It is known that endocannabinoid and dopaminergic system have overlapping circuits, but this is not observed between endocannabinoid and opioid system, actually, it has been proposed that they have partially distinct neural mechanisms (Trezza and Vanderschuren, 2008b). Endocannabinoid system is important for the regulation of the HPA axis, like mentioned before, but also, its antagonism leads to development of anxiety and depressive-like behavior (Parolaro et al., 2010). It is known that activation of endocannabinoid receptor type 1 (eCB1) receptors in the prefrontal cortex and ventral hippocampus induces an anxiolytic response (Moreira et al., 2009, Rubino et al., 2008), while his activation on the amygdala and dorsal hippocampus raises an anxiogenic response (Rubino et al., 2008, Roohbakhsh et al., 2007). In the amygdala of iuGC animals we did not find significant differences in eCB1. Unfortunately, due to technical problems, we were unable to quantify CB1 in other relevant areas (NAcc and HPT). Recent work has shown that endocannabinoids in the amygdala and NAc are crucial for social behaviors (Trezza et al., 2012). Interestingly, results obtained by our group showing that endocannabinoid, anandamine (AEA), is remarkably reduced in the NAcc of these animals, may explain their altered social (and emotional?) behavior. To validate this idea, we could administer in loco or systemically endocannabinoids in iuGC animals and observe if we were able to ameliorate the phenotype.

The interaction between endocannabinoid system and opioid was widely study in rats in the modulation of reward processes. The endocannabinoid system has been shown to be involved in the reinforcing and dependence-producing properties of opioid drugs, and μ-opioid receptors have been implicated in the motivational effects of cannabinoids (Fattore et al., 2005, Maldonado et al., 2006). In addition, stimulation of μ opioid receptors (MOR) in the AMY enhances approach and food consumatory behaviours at cues (Mahler and Berridge, 2009). Surprisingly, and contrary to what we expected, iuGC animals present a significantly increased

expression of MOR in the AMY. Such de-regulation can explain the alterations in the motivation towards food, though these results are somehow contra intuitive.

Concerning the k opioid receptors (KOR), it is known that dysregulation of KOR signalling within neural circuits involved in mood and motivation contributes to depression and anxiety disorders (Knoll et al., 2011, Carlezon et al., 2009, Tejeda et al., 2012). In addition, fear conditioning and extinction regulate KOR mRNA expression in the AMY; and KOR antagonist in the basolateral amygdala can produce anxiolytic-like effects (Knoll et al., 2011), so, in theory, the observed higher levels of KOR could help explain the anxiogenic profile of these animals.

It has been shown that MOR and KOR agonists have different effects on social behavior. The stimulation of MOR, but not KOR, enhances social play (Vanderschuren et al., 1995b). Relatively, to the δ opioid receptor, only when in juvenile rats, are not involved in the regulation of social behavior (Vanderschuren et al., 1995b). Low levels of opioids in this brain region could explain a reduced stimulation of MOR and consequent social impairment. The current observation of increased levels of MOR may be a compensatory mechanism due to low levels of ligand, though this hypothesis needs to be validated.

Regarding the NAcc, it is known that KOR activation in this brain region produces anhedonia without affecting the magnitude of fear (Muschamp et al., 2011). We also observe higher levels of KOR in this brain region, potentially explaining the observed anhedonia.

Dopaminergic input in emotional behavior is undeniable but this input was less studied in the context of social behavior. Confirming previous results, we found that the mesolimbic dopaminergic circuit is significantly affected in iuGC animals. In our model, no differences were found in dopamine D1 receptor (Drd1), while we observe high levels of dopamine D2 receptor (Drd2) expression when compared with CONT animals in the NAcc and amygdala, as previously reported (Oliveira et al., 2012, Rodrigues et al., 2011b); such changes could underlie the enhanced fear conditioning of iuGC animals and the anhedonia. Previous works demonstrate that antagonism of Drd1 and Drd2 in the AMY may mediate the formation and the retention of newly-acquired fear associations, though our animals do not seem to present any change in this aspect of fear behavior.

5.4. Impact of dopaminergic manipulation

Considering Drd2 changes and the reported hypodopaminergic status in iuGC animals (Rodrigues et al., 2011b), and the importance of correct dopaminergic tone for social and emotional manifestations, we decided to explore whether the behavioral impairments could be reverted by dopamine normalization. We administered L-3,4-dihydroxyphenylalanine (L-DOPA), a precursor of dopamine, by oral gavage daily. L-DOPA was given 4h prior to the behavioral tests to ensure the absence of side effects commonly associated with this drug. L-DOPA crosses the protective blood-brain barrier, and when it enters in the central nervous system, it is converted into dopamine by the enzyme DOPA decarboxylase.

We found that systemic administration of L-DOPA reverts most of the emotional and social/affective deficits in iuGC group. L-DOPA reverted the depressive-like behavior in the FST and increased substantially the emission of positive calls in anticipation to food. This data is in accordance with previous reports that shown that patients with Parkinson's disease (which reveal a depression like-behavior and anhedonia) during pramipexole (dopamine agonists) treatment present a significantly reduction in depression and anhedonia symptoms in addition to motor improvement (Constantinescu, 2008, Barone et al., 2010). Moreover, an anti-depressive effect was proposed for L-DOPA in Parkinson´s disease patients (Maricle et al., 1995) though contradictory reports are also found (Cools, 2006).

Though we were expecting a positive effect in the depressive-like behavior, less/none effect was expected in the anxiogenic phenotype. Remarkably, L-DOPA treatment before the L/D box test abolished all the differences between groups; animals placed in an anxiogenic environment (confined cage) no longer emit 22 kHz calls emission and present no freezing. Importantly, it is known that dopamine D2 receptor is involved in the inhibition of 22 kHz ultrasonic vocalization (Bartoszyk, 1998) in a model of anxiety (Sanchez, 2003). L-DOPA supplementation and consequent normalization of Drd2 (Rodrigues et al., 2011b) could explain the decrease in USVs emission. Though we were not expecting this effect, previous literature showed that dopamine D2/D3 receptor agonist (ropinirole) had an anxiolytic-like activity in three models of anxiety (rat, mouse and marmoset) (Rogers et al., 2000).

iuGC treatment impairs fear conditioning in adulthood, which is consistent with deficits found in the amygdalar function and memory consolidation for emotional experiences (Oliveira

et al., 2006). Surprisingly, our results are somehow contradictory since they show enhanced fear response in our animals. A possible explanation is the type of protocol that is slightly different in the two cases, or the fact that we are measuring different aspects of fear behavior. While Oliveira and colleagues showed impaired fear conditioning by a decrease in fear potentiated startle (augmentation of startle reflex by a fear stimulus), we are observing a "more direct response" to a cue predicting a fearful stimulus. Under L-DOPA treatment, iuGC group no longer presents differences from control group (both USVs and freezing %). These results are in agreement with previous articles that shown that dopamine D2 and dopamine D2/3 receptor agonist are among the most potent inhibitor of footshock-induced ultrasonic vocalization (Bartoszyk, 1998). However, it is important to stress that control animals treated with L-DOPA emit much more negative USVs than non-treated animals. Since dopamine is crucial for the establishment and learning of fear conditioning, we might suppose that control animals (with normal dopaminergic input) boosted with more dopamine may present an overt response in the fear paradigm.

In social behavior, L-DOPA did not have a major impact in the positive 50 kHz emission, however, play behavior increased substantially compared with animals in basal situation and, importantly, no differences were found between groups. When rats are tested for social play in a new cage, two different stimuli compete for their attention: an unfamiliar cage and unfamiliar test partner. When the animal is placed inside of the box with the unfamiliar test partner, it will first explore the unfamiliar test cage before engaging in social play, which leads to a temporary suppression of social behavior, although total levels of social play measured during the 15 min are not affected (Vanderschuren et al., 1995a). Interestingly, under L-DOPA treatment, the latency to engage in social play behavior is slightly lower, especially unfamiliar iuGC-iuGC pairs, when compared with animals on basal situation. Concisely, L-DOPA treated animals prefer to engage immediately in playful interactions with their partners instead of first investigating the new environment.

In further support of dopaminergic importance in social behavior, it was shown that treatment with blockers of dopaminergic transmission, such as chlorpromazine or haloperidol, as well as low doses of apomorphine, decreases social play (Vanderschuren et al., 1997). Some works reveal that treatment with dopamine D3 receptor agonist do not affect social play, while the treatment with dopamine D2 receptor agonist has biphasic effects depending on the

dose: low levels increase, although higher levels decrease social play (Vanderschuren et al., 1997). Since L-DOPA is a precursor, it will modulate the activity of more than one type of dopamine receptor, so an interesting approach would be to administer D2 specific agonists/antagonists to confirm if the behavioral impairment of iuGC animals occurs through this receptor. In addition it would be interesting to investigate the correlation between dopaminergic and opioid circuitry by eventually combining agonists/antagonists of these two pathways to ascertain their relevance and interconnectivity in emotional and social behaviors.

In summary, we have found that prenatal glucocorticoids exposure leads to long-lasting anxious and depressive-like behavior and to impaired fear response, together with deficits in social interaction. Most of these behavioral deficits were reverted with dopamine normalization by L-DOPA administration by oral gavage. Moreover, our findings support the USVs measurement technique as a powerful tool to assess rodent emotional and affective status.

6. FUTURE PERPECTIVES

6. Futures perspectives

As future experiments, we aim to better understand the key role and interplay between dopaminergic, opioid and endocannabinoid circuitries in both emotional and social behaviors.

These goals can be achieved using several strategies:

a) Administration of endocannabinoid, opioid and/or dopaminergic agonist/antagonists to further study the involvement of these neurotransmitters in the behavioural deficits observed (systemically and *in loco* in target brain regions of mesolimbic circuit).

b) Study other dimensions of social interaction such as aggressive and sexual behaviors.

c) Analyse social behaviour in other stress models (maternal separation, chronic stress) in order to understand the role of stress and the importance of the window of stress exposure for the development of social deficits.

7. REFERENCES

7. References

AGID, O., SHAPIRA, B., ZISLIN, J., RITSNER, M., HANIN, B., MURAD, H., TROUDART, T., BLOCH, M., HERESCO-LEVY, U. & LERER, B. 1999. Environment and vulnerability to major psychiatric illness: a case control study of early parental loss in major depression, bipolar disorder and schizophrenia. *Mol Psychiatry,* 4**,** 163-72.

AINA, Y. & SUSMAN, J. L. 2006. Understanding comorbidity with depression and anxiety disorders. *J Am Osteopath Assoc,* 106**,** S9-14.

BAENNINGER, L. P. 1967. Comparison of behavioural development in socially isolated and grouped rats. *Anim Behav,* 15**,** 312-23.

BAIER, C. J., KATUNAR, M. R., ADROVER, E., PALLARES, M. E. & ANTONELLI, M. C. 2012. Gestational restraint stress and the developing dopaminergic system: an overview. *Neurotox Res,* 22**,** 16-32.

BALFOUR, M. E., YU, L. & COOLEN, L. M. 2004. Sexual behavior and sex-associated environmental cues activate the mesolimbic system in male rats. *Neuropsychopharmacology,* 29**,** 718-30.

BARNA, I., ZELENA, D., ARSZOVSZKI, A. C. & LEDENT, C. 2004. The role of endogenous cannabinoids in the hypothalamo-pituitary-adrenal axis regulation: in vivo and in vitro studies in CB1 receptor knockout mice. *Life Sci,* 75**,** 2959-70.

BARONE, P., POEWE, W., ALBRECHT, S., DEBIEUVRE, C., MASSEY, D., RASCOL, O., TOLOSA, E. & WEINTRAUB, D. 2010. Pramipexole for the treatment of depressive symptoms in patients with Parkinson's disease: a randomised, double-blind, placebo-controlled trial. *Lancet Neurol,* 9**,** 573-80.

BARROT, M., MARINELLI, M., ABROUS, D. N., ROUGE-PONT, F., LE MOAL, M. & PIAZZA, P. V. 2000. The dopaminergic hyper-responsiveness of the shell of the nucleus accumbens is hormone-dependent. *Eur J Neurosci,* 12**,** 973-9.

BARTOSZYK, G. D. 1998. Anxiolytic effects of dopamine receptor ligands: I. Involvement of dopamine autoreceptors. *Life Sci,* 62**,** 649-63.

BERNET, C. Z. & STEIN, M. B. 1999. Relationship of childhood maltreatment to the onset and course of major depression in adulthood. *Depress Anxiety,* 9**,** 169-74.

BERRIDGE, K. C. 2003. Pleasures of the brain. *Brain Cogn,* 52**,** 106-28.

BERRIDGE, K. C. 2007. The debate over dopamine's role in reward: the case for incentive salience. *Psychopharmacology (Berl),* 191**,** 391-431.

BLOCK, J. & KREMEN, A. M. 1996. IQ and ego-resiliency: conceptual and empirical connections and separateness. *J Pers Soc Psychol,* 70**,** 349-61.

BOROWSKY, B. & KUHN, C. M. 1992. D1 and D2 dopamine receptors stimulate hypothalamo-pituitary-adrenal activity in rats. *Neuropharmacology,* 31**,** 671-8.

BORTA, A., WOHR, M. & SCHWARTING, R. K. 2006. Rat ultrasonic vocalization in aversively motivated situations and the role of individual differences in anxiety-related behavior. *Behav Brain Res,* 166**,** 271-80.

BOZARTH, M. A. & WISE, R. A. 1984. Anatomically distinct opiate receptor fields mediate reward and physical dependence. *Science,* 224**,** 516-7.

BROWN, G. W. & MORAN, P. 1994. Clinical and psychosocial origins of chronic depressive episodes. I: A community survey. *Br J Psychiatry,* 165**,** 447-56.

BRUDZYNSKI, S. M. 2001. Pharmacological and behavioral characteristics of 22 kHz alarm calls in rats. *Neurosci Biobehav Rev,* 25**,** 611-7.

BRUDZYNSKI, S. M. 2009. Communication of adult rats by ultrasonic vocalization: biological, sociobiological, and neuroscience approaches. *ILAR J,* 50**,** 43-50.

BRUDZYNSKI, S. M. & CHIU, E. M. 1995. Behavioural responses of laboratory rats to playback of 22 kHz ultrasonic calls. *Physiol Behav,* 57**,** 1039-44.

BURGDORF, J., KNUTSON, B. & PANKSEPP, J. 2000. Anticipation of rewarding electrical brain stimulation evokes ultrasonic vocalization in rats. *Behav Neurosci,* 114**,** 320-7.

BURGDORF, J., KNUTSON, B., PANKSEPP, J. & IKEMOTO, S. 2001. Nucleus accumbens amphetamine microinjections unconditionally elicit 50-kHz ultrasonic vocalizations in rats. *Behav Neurosci,* 115**,** 940-4.

BURGDORF, J., KROES, R. A., MOSKAL, J. R., PFAUS, J. G., BRUDZYNSKI, S. M. & PANKSEPP, J. 2008. Ultrasonic vocalizations of rats (Rattus norvegicus) during mating, play, and aggression: Behavioral concomitants, relationship to reward, and self-administration of playback. *J Comp Psychol,* 122**,** 357-67.

BURGDORF, J., PANKSEPP, J. & MOSKAL, J. R. 2011. Frequency-modulated 50 kHz ultrasonic vocalizations: a tool for uncovering the molecular substrates of positive affect. *Neurosci Biobehav Rev,* 35**,** 1831-6.

CANNON, T. D., CADENHEAD, K., CORNBLATT, B., WOODS, S. W., ADDINGTON, J., WALKER, E., SEIDMAN, L. J., PERKINS, D., TSUANG, M., MCGLASHAN, T. & HEINSSEN, R. 2008. Prediction of psychosis in youth at high clinical risk: a multisite longitudinal study in North America. *Arch Gen Psychiatry,* 65**,** 28-37.

CARLEZON, W. A., JR., BEGUIN, C., KNOLL, A. T. & COHEN, B. M. 2009. Kappa-opioid ligands in the study and treatment of mood disorders. *Pharmacol Ther,* 123**,** 334-43.

CERQUEIRA, J. J., MAILLIET, F., ALMEIDA, O. F., JAY, T. M. & SOUSA, N. 2007. The prefrontal cortex as a key target of the maladaptive response to stress. *J Neurosci,* 27**,** 2781-7.

CHATHAM, W. W. & KIMBERLY, R. P. 2001. Treatment of lupus with corticosteroids. *Lupus,* 10**,** 140-7.

CHINTA, S. J. & ANDERSEN, J. K. 2005. Dopaminergic neurons. *Int J Biochem Cell Biol,* 37**,** 942-6.

CONSTANTINESCU, R. 2008. Update on the use of pramipexole in the treatment of Parkinson's disease. *Neuropsychiatr Dis Treat,* 4**,** 337-52.

COOLS, R. 2006. Dopaminergic modulation of cognitive function-implications for L-DOPA treatment in Parkinson's disease. *Neurosci Biobehav Rev,* 30**,** 1-23.

CRANE, J., ARMSON, A., BRUNNER, M., DE LA RONDE, S., FARINE, D., KEENAN-LINDSAY, L., LEDUC, L., SCHNEIDER, C. & VAN AERDE, J. 2003. Antenatal corticosteroid therapy for fetal maturation. *J Obstet Gynaecol Can,* 25**,** 45-52.

CRATTY, M. S., WARD, H. E., JOHNSON, E. A., AZZARO, A. J. & BIRKLE, D. L. 1995. Prenatal stress increases corticotropin-releasing factor (CRF) content and release in rat amygdala minces. *Brain Res,* 675**,** 297-302.

CROWLEY, P. A. 1995. Antenatal corticosteroid therapy: a meta-analysis of the randomized trials, 1972 to 1994. *Am J Obstet Gynecol,* 173**,** 322-35.

D'AMATO, F. R. & PAVONE, F. 2012. Modulation of nociception by social factors in rodents: contribution of the opioid system. *Psychopharmacology (Berl),* 224**,** 189-200.

DARWIN, C. 1871/1936. *The Descent of Man and Selection in Relation to Sex.,* New York, Random House.

DE KLOET, E. R., VREUGDENHIL, E., OITZL, M. S. & JOELS, M. 1998. Brain corticosteroid receptor balance in health and disease. *Endocr Rev,* 19**,** 269-301.

DEPUE, R. A. & MORRONE-STRUPINSKY, J. V. 2005. A neurobehavioral model of affiliative bonding: implications for conceptualizing a human trait of affiliation. *Behav Brain Sci,* 28**,** 313-50; discussion 350-95.

DER-AVAKIAN, A. & MARKOU, A. 2012. The neurobiology of anhedonia and other reward-related deficits. *Trends Neurosci,* 35**,** 68-77.

DEVRIES, A. C. 2002. Interaction among social environment, the hypothalamic-pituitary-adrenal axis, and behavior. *Horm Behav,* 41**,** 405-13.

DEVRIES, A. C., GLASPER, E. R. & DETILLION, C. E. 2003. Social modulation of stress responses. *Physiol Behav,* 79**,** 399-407.

DROLET, G., DUMONT, E. C., GOSSELIN, I., KINKEAD, R., LAFOREST, S. & TROTTIER, J. F. 2001. Role of endogenous opioid system in the regulation of the stress response. *Prog Neuropsychopharmacol Biol Psychiatry,* 25**,** 729-41.

DUBE, S. R., FELITTI, V. J., DONG, M., CHAPMAN, D. P., GILES, W. H. & ANDA, R. F. 2003. Childhood abuse, neglect, and household dysfunction and the risk of illicit drug use: the adverse childhood experiences study. *Pediatrics,* 111**,** 564-72.

EINON, D. F. & MORGAN, M. J. 1977. A critical period for social isolation in the rat. *Dev Psychobiol,* 10**,** 123-32.

EINON, D. F., MORGAN, M. J. & KIBBLER, C. C. 1978. Brief periods of socialization and later behavior in the rat. *Dev Psychobiol,* 11**,** 213-25.

FATTORE, L., DEIANA, S., SPANO, S. M., COSSU, G., FADDA, P., SCHERMA, M. & FRATTA, W. 2005. Endocannabinoid system and opioid addiction: behavioural aspects. *Pharmacol Biochem Behav,* 81**,** 343-59.

FRANK, L., MATZA, L. S., HANLON, J., MANNIX, S., REVICKI, D. A., FELTNER, D. & MORLOCK, R. J. 2007. The patient experience of depression and remission: focus group results. *J Nerv Ment Dis,* 195**,** 647-54.

FUNDER, J. W. 1996. Mineralocorticoid receptors in the central nervous system. *J Steroid Biochem Mol Biol,* 56**,** 179-83.

GERALL, H. D., WARD, I. L. & GERALL, A. A. 1967. Disruption of the male rat's sexual behaviour induced by social isolation. *Anim Behav,* 15**,** 54-8.

GILLOTT, A. & STANDEN, P. J. 2007. Levels of anxiety and sources of stress in adults with autism. *J Intellect Disabil,* 11**,** 359-70.

GOLDSTEIN, D. S. & KOPIN, I. J. 2007. Evolution of concepts of stress. *Stress,* 10**,** 109-20.

GONG, J. P., ONAIVI, E. S., ISHIGURO, H., LIU, Q. R., TAGLIAFERRO, P. A., BRUSCO, A. & UHL, G. R. 2006. Cannabinoid CB2 receptors: immunohistochemical localization in rat brain. *Brain Res,* 1071**,** 10-23.

GORZALKA, B. B., HILL, M. N. & HILLARD, C. J. 2008. Regulation of endocannabinoid signaling by stress: implications for stress-related affective disorders. *Neurosci Biobehav Rev,* 32**,** 1152-60.

HAUSER, J., DETTLING-ARTHO, A., PILLOUD, S., MAIER, C., KNAPMAN, A., FELDON, J. & PRYCE, C. R. 2007. Effects of prenatal dexamethasone treatment on postnatal physical, endocrine, and social development in the common marmoset monkey. *Endocrinology,* 148**,** 1813-22.

HAUSER, J., FELDON, J. & PRYCE, C. R. 2009. Direct and dam-mediated effects of prenatal dexamethasone on emotionality, cognition and HPA axis in adult Wistar rats. *Horm Behav,* 56**,** 364-75.

HEIM, C. & NEMEROFF, C. B. 2001. The role of childhood trauma in the neurobiology of mood and anxiety disorders: preclinical and clinical studies. *BIOL PSYCHIATRY,* 49**,** 1023-39.
HERKENHAM, M., LYNN, A. B., JOHNSON, M. R., MELVIN, L. S., DE COSTA, B. R. & RICE, K. C. 1991. Characterization and localization of cannabinoid receptors in rat brain: a quantitative in vitro autoradiographic study. *J Neurosci,* 11**,** 563-83.

HILL, M. N. & MCEWEN, B. S. 2010. Involvement of the endocannabinoid system in the neurobehavioural effects of stress and glucocorticoids. *Prog Neuropsychopharmacol Biol Psychiatry,* 34**,** 791-7.

HILL, M. N., PATEL, S., CAMPOLONGO, P., TASKER, J. G., WOTJAK, C. T. & BAINS, J. S. 2010. Functional interactions between stress and the endocannabinoid system: from synaptic signaling to behavioral output. *J Neurosci,* 30**,** 14980-6.

HILL, M. N. & TASKER, J. G. 2012. Endocannabinoid signaling, glucocorticoid-mediated negative feedback, and regulation of the hypothalamic-pituitary-adrenal axis. *Neuroscience,* 204**,** 5-16.

HOFER, M. A. 1996. Multiple regulators of ultrasonic vocalization in the infant rat. *Psychoneuroendocrinology,* 21**,** 203-17.

HOFER, M. A. & SHAIR, H. 1978. Ultrasonic vocalization during social interaction and isolation in 2-weeek-old rats. *Dev Psychobiol,* 11**,** 495-504.

I., S., WEISZ D. J., YANG B.Y. , FUNG K. & A., A. 2001. The mechanism of ultrasonic vocalization in the rat. . *Soc Neurosci Abstr,* 27.

JOHNSON, R. F., RENNIE, N., MURPHY, V., ZAKAR, T., CLIFTON, V. & SMITH, R. 2008. Expression of glucocorticoid receptor messenger ribonucleic acid transcripts in the human placenta at term. *J Clin Endocrinol Metab,* 93**,** 4887-93.

JURUENA, M. F., CLEARE, A. J. & PARIANTE, C. M. 2004. [The hypothalamic pituitary adrenal axis, glucocorticoid receptor function and relevance to depression]. *Rev Bras Psiquiatr,* 26**,** 189-201.

KELLEY, A. E., BALDO, B. A., PRATT, W. E. & WILL, M. J. 2005. Corticostriatal-hypothalamic circuitry and food motivation: integration of energy, action and reward. *Physiol Behav,* 86**,** 773-95.

KLEINHAUS, K., STEINFELD, S., BALABAN, J., GOODMAN, L., CRAFT, T. S., MALASPINA, D., MYERS, M. M. & MOORE, H. 2010. Effects of excessive glucocorticoid receptor stimulation during early gestation on psychomotor and social behavior in the rat. *Dev Psychobiol,* 52**,** 121-32.

KNOLL, A. T., MUSCHAMP, J. W., SILLIVAN, S. E., FERGUSON, D., DIETZ, D. M., MELONI, E. G., CARROLL, F. I., NESTLER, E. J., KONRADI, C. & CARLEZON, W. A., JR. 2011. Kappa opioid

receptor signaling in the basolateral amygdala regulates conditioned fear and anxiety in rats. *BIOL PSYCHIATRY,* 70**,** 425-33.

KNUTSON, B., BURGDORF, J. & PANKSEPP, J. 1998. Anticipation of play elicits high-frequency ultrasonic vocalizations in young rats. *J Comp Psychol,* 112**,** 65-73.

KNUTSON, B., BURGDORF, J. & PANKSEPP, J. 1999. High-frequency ultrasonic vocalizations index conditioned pharmacological reward in rats. *Physiol Behav,* 66**,** 639-43.

KRANENDONK, G., HOPSTER, H., FILLERUP, M., EKKEL, E. D., MULDER, E. J. & TAVERNE, M. A. 2006. Cortisol administration to pregnant sows affects novelty-induced locomotion, aggressive behaviour, and blunts gender differences in their offspring. *Horm Behav,* 49**,** 663-72.

LE MERRER, J., BECKER, J. A., BEFORT, K. & KIEFFER, B. L. 2009. Reward processing by the opioid system in the brain. *Physiol Rev,* 89**,** 1379-412.

LEADEM, C. A. & YAGENOVA, S. V. 1987. Effects of specific activation of mu-, delta- and kappa-opioid receptors on the secretion of luteinizing hormone and prolactin in the ovariectomized rat. *Neuroendocrinology,* 45**,** 109-17.

LEAO, P., SOUSA, J. C., OLIVEIRA, M., SILVA, R., ALMEIDA, O. F. & SOUSA, N. 2007. Programming effects of antenatal dexamethasone in the developing mesolimbic pathways. *Synapse,* 61**,** 40-9.

LEE, P. R., BRADY, D. L., SHAPIRO, R. A., DORSA, D. M. & KOENIG, J. I. 2007. Prenatal stress generates deficits in rat social behavior: Reversal by oxytocin. *Brain Res,* 1156**,** 152-67.

LORE, R. & FLANNELLY, K. 1977. Rat societies. *Sci Am,* 236**,** 106-11, 113-6.

LYUBOMIRSKY, S., KING, L. & DIENER, E. 2005. The benefits of frequent positive affect: does happiness lead to success? *Psychol Bull,* 131**,** 803-55.

MAHLER, S. V. & BERRIDGE, K. C. 2009. Which cue to "want?" Central amygdala opioid activation enhances and focuses incentive salience on a prepotent reward cue. *J Neurosci,* 29**,** 6500-13.

MAIER, E. Y., MA, S. T., AHRENS, A., SCHALLERT, T. J. & DUVAUCHELLE, C. L. 2010. Assessment of ultrasonic vocalizations during drug self-administration in rats. *J Vis Exp*.

MALDONADO, R., VALVERDE, O. & BERRENDERO, F. 2006. Involvement of the endocannabinoid system in drug addiction. *Trends Neurosci,* 29**,** 225-32.

MANSOUR, A., FOX, C. A., AKIL, H. & WATSON, S. J. 1995. Opioid-receptor mRNA expression in the rat CNS: anatomical and functional implications. *Trends Neurosci,* 18**,** 22-9.

MARICLE, R. A., NUTT, J. G. & CARTER, J. H. 1995. Mood and anxiety fluctuation in Parkinson's disease associated with levodopa infusion: preliminary findings. *Mov Disord,* 10**,** 329-32.

MATTHEWS, S. G., OWEN, D., BANJANIN, S. & ANDREWS, M. H. 2002. Glucocorticoids, hypothalamo-pituitary-adrenal (HPA) development, and life after birth. *Endocr Res,* 28**,** 709-18.

MCARTHUR, S., MCHALE, E., DALLEY, J. W., BUCKINGHAM, J. C. & GILLIES, G. E. 2005. Altered mesencephalic dopaminergic populations in adulthood as a consequence of brief perinatal glucocorticoid exposure. *J Neuroendocrinol,* 17**,** 475-82.

MCCAULEY, J., KERN, D. E., KOLODNER, K., DILL, L., SCHROEDER, A. F., DECHANT, H. K., RYDEN, J., DEROGATIS, L. R. & BASS, E. B. 1997. Clinical characteristics of women with a history of childhood abuse: unhealed wounds. *JAMA,* 277**,** 1362-8.

MCLAUGHLIN, J. P., LI, S., VALDEZ, J., CHAVKIN, T. A. & CHAVKIN, C. 2006. Social defeat stress-induced behavioral responses are mediated by the endogenous kappa opioid system. *Neuropsychopharmacology,* 31**,** 1241-8.

MCTERNAN, C. L., DRAPER, N., NICHOLSON, H., CHALDER, S. M., DRIVER, P., HEWISON, M., KILBY, M. D. & STEWART, P. M. 2001. Reduced placental 11beta-hydroxysteroid dehydrogenase type 2 mRNA levels in human pregnancies complicated by intrauterine growth restriction: an analysis of possible mechanisms. *J Clin Endocrinol Metab,* 86**,** 4979-83.

MESQUITA, A. R., WEGERICH, Y., PATCHEV, A. V., OLIVEIRA, M., LEAO, P., SOUSA, N. & ALMEIDA, O. F. 2009. Glucocorticoids and neuro- and behavioural development. *Semin Fetal Neonatal Med,* 14**,** 130-5.

METZ, G. A., SCHWAB, M. E. & WELZL, H. 2001. The effects of acute and chronic stress on motor and sensory performance in male Lewis rats. *Physiol Behav,* 72**,** 29-35.

MIRACLE, X., DI RENZO, G. C., STARK, A., FANAROFF, A., CARBONELL-ESTRANY, X. & SALING, E. 2008. Guideline for the use of antenatal corticosteroids for fetal maturation. *J Perinat Med,* 36**,** 191-6.

MISSALE, C., NASH, S. R., ROBINSON, S. W., JABER, M. & CARON, M. G. 1998. Dopamine receptors: from structure to function. *Physiol Rev,* 78**,** 189-225.

MOREAU, J. L. 1997. [Validation of an animal model of anhedonia, a major symptom of depression]. *Encephale,* 23**,** 280-9.

MOREIRA, F. A., AGUIAR, D. C., CAMPOS, A. C., LISBOA, S. F., TERZIAN, A. L., RESSTEL, L. B. & GUIMARAES, F. S. 2009. Antiaversive effects of cannabinoids: is the periaqueductal gray involved? *Neural Plast,* 2009**,** 625469.

MUCHA, R. F. & HERZ, A. 1985. Motivational properties of kappa and mu opioid receptor agonists studied with place and taste preference conditioning. *Psychopharmacology (Berl),* 86**,** 274-80.

MUSCHAMP, J. W., VAN'T VEER, A., PARSEGIAN, A., GALLO, M. S., CHEN, M., NEVE, R. L., MELONI, E. G. & CARLEZON, W. A., JR. 2011. Activation of CREB in the nucleus accumbens shell produces anhedonia and resistance to extinction of fear in rats. *J Neurosci,* 31**,** 3095-103.

NEGRI, L., NOVIELLO, V. & ANGELUCCI, F. 1991. Behavioural effects of deltorphins in rats. *Eur J Pharmacol,* 209**,** 163-8.

NELSON, E. E. & PANKSEPP, J. 1998. Brain substrates of infant-mother attachment: contributions of opioids, oxytocin, and norepinephrine. *Neurosci Biobehav Rev,* 22**,** 437-52.

NEW, M. I., CARLSON, A., OBEID, J., MARSHALL, I., CABRERA, M. S., GOSECO, A., LIN-SU, K., PUTNAM, A. S., WEI, J. Q. & WILSON, R. C. 2001. Prenatal diagnosis for congenital adrenal hyperplasia in 532 pregnancies. *J Clin Endocrinol Metab,* 86**,** 5651-7.

NEWNHAM, J. P. 2001. Is prenatal glucocorticoid administration another origin of adult disease? *Clin Exp Pharmacol Physiol,* 28**,** 957-61.

NITSCHKE, W. 1982. *Acoustic Behavior in the Rat: Research, Theory, and Applications,* New York, Praeger Publishers

OLAUSSON, P., KIRALY, D. D., GOURLEY, S. L. & TAYLOR, J. R. 2012. Persistent effects of prior chronic exposure to corticosterone on reward-related learning and motivation in rodents. *Psychopharmacology (Berl)*.

OLIVEIRA, M., BESSA, J. M., MESQUITA, A., TAVARES, H., CARVALHO, A., SILVA, R., PEGO, J. M., CERQUEIRA, J. J., PALHA, J. A., ALMEIDA, O. F. & SOUSA, N. 2006. Induction of a hyperanxious state by antenatal dexamethasone: a case for less detrimental natural corticosteroids. *BIOL PSYCHIATRY,* 59**,** 844-52.

OLIVEIRA, M., RODRIGUES, A. J., LEAO, P., CARDONA, D., PEGO, J. M. & SOUSA, N. 2012. The bed nucleus of stria terminalis and the amygdala as targets of antenatal glucocorticoids: implications for fear and anxiety responses. *Psychopharmacology (Berl),* 220**,** 443-53.

PANKSEPP, J. & BEATTY, W. W. 1980. Social deprivation and play in rats. *Behav Neural Biol,* 30**,** 197-206.

PANKSEPP, J., JALOWIEC, J., DEESKINAZI, F. G. & BISHOP, P. 1985. Opiates and play dominance in juvenile rats. *Behav Neurosci,* 99**,** 441-53.

PANKSEPP, J., SIVIY, S. & NORMANSELL, L. 1984. The psychobiology of play: theoretical and methodological perspectives. *Neurosci Biobehav Rev,* 8**,** 465-92.

PAROLARO, D., REALINI, N., VIGANO, D., GUIDALI, C. & RUBINO, T. 2010. The endocannabinoid system and psychiatric disorders. *Exp Neurol,* 224**,** 3-14.

PEGO, J. M., MORGADO, P., PINTO, L. G., CERQUEIRA, J. J., ALMEIDA, O. F. & SOUSA, N. 2008. Dissociation of the morphological correlates of stress-induced anxiety and fear. *Eur J Neurosci,* 27**,** 1503-16.
PIERCE, R. C. & KUMARESAN, V. 2006. The mesolimbic dopamine system: the final common pathway for the reinforcing effect of drugs of abuse? *Neurosci Biobehav Rev,* 30**,** 215-38.

RODRIGUES, A. J., LEAO, P., CARVALHO, M., ALMEIDA, O. F. & SOUSA, N. 2011a. Potential programming of dopaminergic circuits by early life stress. *Psychopharmacology (Berl),* 214**,** 107-20.

RODRIGUES, A. J., LEAO, P., PEGO, J. M., CARDONA, D., CARVALHO, M. M., OLIVEIRA, M., COSTA, B. M., CARVALHO, A. F., MORGADO, P., ARAUJO, D., PALHA, J. A., ALMEIDA, O. F. & SOUSA, N. 2011b. Mechanisms of initiation and reversal of drug-seeking behavior induced by prenatal exposure to glucocorticoids. *Mol Psychiatry*.

ROGERS, D. C., COSTALL, B., DOMENEY, A. M., GERRARD, P. A., GREENER, M., KELLY, M. E., HAGAN, J. J. & HUNTER, A. J. 2000. Anxiolytic profile of ropinirole in the rat, mouse and common marmoset. *Psychopharmacology (Berl),* 151**,** 91-7.

ROOHBAKHSH, A., MOGHADDAM, A. H., MASSOUDI, R. & ZARRINDAST, M. R. 2007. Role of dorsal hippocampal cannabinoid receptors and nitric oxide in anxiety like behaviours in rats using the elevated plus-maze test. *Clin Exp Pharmacol Physiol,* 34**,** 223-9.

ROQUE, S., OLIVEIRA, T. G., NOBREGA, C., BARREIRA-SILVA, P., NUNES-ALVES, C., SOUSA, N., PALHA, J. A. & CORREIA-NEVES, M. 2011. Interplay between Depressive-Like Behavior and the Immune System in an Animal Model of Prenatal Dexamethasone Administration. *Front Behav Neurosci,* 5**,** 4.

RUBINO, T., GUIDALI, C., VIGANO, D., REALINI, N., VALENTI, M., MASSI, P. & PAROLARO, D. 2008. CB1 receptor stimulation in specific brain areas differently modulate anxiety-related behaviour. *Neuropharmacology,* 54**,** 151-60.

SANCHEZ, C. 2003. Stress-induced vocalisation in adult animals. A valid model of anxiety? *Eur J Pharmacol,* 463**,** 133-43.

SANCHEZ, M. M., YOUNG, L. J., PLOTSKY, P. M. & INSEL, T. R. 2000. Distribution of corticosteroid receptors in the rhesus brain: relative absence of glucocorticoid receptors in the hippocampal formation. *J Neurosci,* 20**,** 4657-68.

SCHNEIDER, M., SCHOMIG, E. & LEWEKE, F. M. 2008. Acute and chronic cannabinoid treatment differentially affects recognition memory and social behavior in pubertal and adult rats. *Addict Biol,* 13**,** 345-57.

SCHOFFELMEER, A. N., RICE, K. C., JACOBSON, A. E., VAN GELDEREN, J. G., HOGENBOOM, F., HEIJNA, M. H. & MULDER, A. H. 1988. Mu-, delta- and kappa-opioid receptor-mediated inhibition of neurotransmitter release and adenylate cyclase activity in rat brain slices: studies with fentanyl isothiocyanate. *Eur J Pharmacol,* 154**,** 169-78.

SECKL, J. R. 2004. Prenatal glucocorticoids and long-term programming. *Eur J Endocrinol,* 151 Suppl 3**,** U49-62.
SEGHERS, J. P. & DOCHERTY, N. M. 2009. Cognitive impairments, emotion, stress, and language in schizophrenia. *Psychiatry Res,* 170**,** 97-102.

SHELINE, Y. I., WANG, P. W., GADO, M. H., CSERNANSKY, J. G. & VANNIER, M. W. 1996. Hippocampal atrophy in recurrent major depression. *Proc Natl Acad Sci U S A,* 93**,** 3908-13.
SIVIY, S. M., FLEISCHHAUER, A. E., KERRIGAN, L. A. & KUHLMAN, S. J. 1996. D2 dopamine receptor involvement in the rough-and-tumble play behavior of juvenile rats. *Behav Neurosci,* 110**,** 1168-76.

SIVIY, S. M. & PANKSEPP, J. 1985. Energy balance and play in juvenile rats. *Physiol Behav,* 35**,** 435-41.

SOLINAS, M. & GOLDBERG, S. R. 2005. Motivational effects of cannabinoids and opioids on food reinforcement depend on simultaneous activation of cannabinoid and opioid systems. *Neuropsychopharmacology,* 30**,** 2035-45.

SOUSA, N. & ALMEIDA, O. F. 2002. Corticosteroids: sculptors of the hippocampal formation. *Rev Neurosci,* 13**,** 59-84.

SPENCER, R. L., KIM, P. J., KALMAN, B. A. & COLE, M. A. 1998. Evidence for mineralocorticoid receptor facilitation of glucocorticoid receptor-dependent regulation of hypothalamic-pituitary-adrenal axis activity. *Endocrinology,* 139**,** 2718-26.

STEIN, M. B., KOVEROLA, C., HANNA, C., TORCHIA, M. G. & MCCLARTY, B. 1997. Hippocampal volume in women victimized by childhood sexual abuse. *Psychol Med,* 27**,** 951-9.

STONE, A. A., SCHWARTZ, J. E., SCHKADE, D., SCHWARZ, N., KRUEGER, A. & KAHNEMAN, D. 2006. A population approach to the study of emotion: diurnal rhythms of a working day examined with the Day Reconstruction Method. *Emotion,* 6**,** 139-49.

STRACHAN T. & A.P., R. 1999. Chapter 8, Human gene expression. *Human Molecular Genetics.* 2nd ed. New York: Wiley-Liss.

TEJEDA, H. A., SHIPPENBERG, T. S. & HENRIKSSON, R. 2012. The dynorphin/kappa-opioid receptor system and its role in psychiatric disorders. *Cell Mol Life Sci,* 69**,** 857-96.

TREADWAY, M. T. & ZALD, D. H. 2011. Reconsidering anhedonia in depression: lessons from translational neuroscience. *Neurosci Biobehav Rev,* 35**,** 537-55.

TREZZA, V., DAMSTEEGT, R., MANDUCA, A., PETROSINO, S., VAN KERKHOF, L. W., PASTERKAMP, R. J., ZHOU, Y., CAMPOLONGO, P., CUOMO, V., DI MARZO, V. & VANDERSCHUREN, L. J. 2012. Endocannabinoids in Amygdala and Nucleus Accumbens Mediate Social Play Reward in Adolescent Rats. *J Neurosci,* 32**,** 14899-14908.

TREZZA, V. & VANDERSCHUREN, L. J. 2008a. Bidirectional cannabinoid modulation of social behavior in adolescent rats. *Psychopharmacology (Berl),* 197**,** 217-27.
TREZZA, V. & VANDERSCHUREN, L. J. 2008b. Cannabinoid and opioid modulation of social play behavior in adolescent rats: differential behavioral mechanisms. *Eur Neuropsychopharmacol,* 18**,** 519-30.

TSIGOS, C. & CHROUSOS, G. P. 2002. Hypothalamic-pituitary-adrenal axis, neuroendocrine factors and stress. *J Psychosom Res,* 53**,** 865-71.

UNO, H., LOHMILLER, L., THIEME, C., KEMNITZ, J. W., ENGLE, M. J., ROECKER, E. B. & FARRELL, P. M. 1990. Brain damage induced by prenatal exposure to dexamethasone in fetal rhesus macaques. I. Hippocampus. *Brain Res Dev Brain Res,* 53**,** 157-67.

VANDERSCHUREN, L. J., NIESINK, R. J., SPRUIJT, B. M. & VAN REE, J. M. 1995a. Influence of environmental factors on social play behavior of juvenile rats. *Physiol Behav,* 58**,** 119-23.

VANDERSCHUREN, L. J., NIESINK, R. J., SPRUIJT, B. M. & VAN REE, J. M. 1995b. Mu- and kappa-opioid receptor-mediated opioid effects on social play in juvenile rats. *Eur J Pharmacol,* 276**,** 257-66.

VANDERSCHUREN, L. J., NIESINK, R. J. & VAN REE, J. M. 1997. The neurobiology of social play behavior in rats. *Neurosci Biobehav Rev,* 21**,** 309-26.

WHITE, P. C., MUNE, T., ROGERSON, F. M., KAYES, K. M. & AGARWAL, A. K. 1997. 11 beta-Hydroxysteroid dehydrogenase and its role in the syndrome of apparent mineralocorticoid excess. *Pediatr Res,* 41**,** 25-9.

WOHR, M., BORTA, A. & SCHWARTING, R. K. 2005. Overt behavior and ultrasonic vocalization in a fear conditioning paradigm: a dose-response study in the rat. *Neurobiol Learn Mem,* 84**,** 228-40.

WOHR, M., DAHLHOFF, M., WOLF, E., HOLSBOER, F., SCHWARTING, R. K. & WOTJAK, C. T. 2008. Effects of genetic background, gender, and early environmental factors on isolation-induced ultrasonic calling in mouse pups: an embryo-transfer study. *Behav Genet,* 38**,** 579-95.

WRIGHT, J. M., GOURDON, J. C. & CLARKE, P. B. 2010. Identification of multiple call categories within the rich repertoire of adult rat 50-kHz ultrasonic vocalizations: effects of amphetamine and social context. *Psychopharmacology (Berl),* 211**,** 1-13.

XU, M. & ZHANG, J. 2004. Molecular genetic probing of dopamine receptors in drug addiction. *Curr Opin Drug Discov Devel,* 7**,** 703-8.

YOUNG, E. A., ABELSON, J. L., CURTIS, G. C. & NESSE, R. M. 1997. Childhood adversity and vulnerability to mood and anxiety disorders. *Depress Anxiety,* 5**,** 66-72.

YOUNG, L. J. & WANG, Z. 2004. The neurobiology of pair bonding. *Nat Neurosci,* 7**,** 1048-54.