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Effects of prenatal exposure to glucocorticoids in impulsivity and novelty-seeking behavior

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"Those who do not know the torment of the unknown cannot have the joy of discovery"

Claude Bernard, 1813 - 1878

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Effects of prenatal exposure to glucocorticoids in impulsivity and novelty-seeking behavior

Abstract

Early life stress (ELS) or elevated levels of glucocorticoids (GCs) may result in persistent effects in the central nervous system that can lead to maladaptive behavior in adult life and increase the vulnerability to develop psychiatric disorders, such as anxiety, depression or drug addiction.

In clinics, synthetic GCs are often prescribed in pregnancies in risk of pre-term labor, to ensure fetal lung maturation. Regardless of its beneficial effect, elevated levels of GCs during this period can lead to deleterious and permanent effects on brain function and development. The mesocorticolimbic dopaminergic circuit, also known as the 'reward' system, seems to be a key target of stress/GCs, since it has been shown that animals exposed to GC *in utero* (iuGC) have structural and molecular alterations in several brain regions of this pathway. Importantly, such changes may underlie the observed addictive-like behavior of iuGC animals.

Vulnerability for addictive behavior may be modulated by individual emotional condition and/or specific personality traits. iuGC animals present anxious and depressive-like behavior, but less is known about their novelty-seeking and impulsivity traits, two behavioral dimensions that contribute substantially to drug-seeking behavior. In this perspective, we evaluated iuGC animals in novelty-dependent behavioral tests. iuGC animals did not present major differences in novelty-induced locomotor activity nor in general exploratory behavior (Novelty Place Preference and Novel Object Recognition). However, iuGC rats explore familiar and novel objects similarly in the 24h retention time test, suggesting long-term memory impairment. In addition, we assessed impulsive action, using the 5-Choice Serial Reaction Time Task (5-CSRTT) and impulsive choice, using the Delay Discounting (DD) test. Whereas no significant differences were found in 5-CSRTT performance, iuGC animals present alterations in the DD task. Since DD task is strongly dependent on the prefrontal cortex (PFC) and also the amygdala, we analyzed the neuronal activation pattern of these brain regions upon task performance. iuGC treatment induced significant changes in c-fos positive cell density in the orbitofrontal cortex, medial PFC and less so in the amygdala, which could potentially explain the observed behavioral differences. Altogether, our results suggest that iuGC treatment does not seem to affect novelty-seeking behavior, but it has an effect in impulsive choice, which may contribute for the observed enhanced drug-seeking behavior.

Efeitos da exposição pré-natal a glucocorticóides na impulsividade e comportamento de procura de novidade

Resumo

Exposição a stress pré-natal ou a níveis elevados de glucocorticóides (GC) podem resultar em alterações persistentes no sistema nervoso central, aumentando a vulnerabilidade para o desenvolvimento de doenças psiquiátricas tais como a ansiedade, depressão ou dependência de drogas.

Na clínica, os GCs sintéticos são vulgarmente prescritos em casos de risco de parto prematuro, para assegurar a maturação pulmonar fetal. Independentemente do seu efeito benéfico, níveis elevados de GCs durante este período podem levar a efeitos nefastos e permanentes na função e desenvolvimento cerebral. O circuito mesocorticolímbico, conhecido como o sistema de recompensa, parece ser um alvo preferencial do stress /GCs, uma vez que foi demonstrado que animais expostos *in útero* a GCs (iuGC) têm alterações estruturais e moleculares neste circuito. De salientar que tais mudanças podem ser a base do *comportamento aditivo* observado nestes animais.

A vulnerabilidade para o *comportamento aditivo* pode ser modulada pela condição emocional e/ou características da personalidade do indivíduo. Os animais iuGC são ansiosos e apresentam características do tipo depressivo, mas pouco se sabe sobre o seu efeito em características como a procura de sensação/novidade e impulsividade, duas dimensões comportamentais que contribuem substancialmente para o *comportamento aditivo*. Nesta perspectiva, estes animais foram avaliados em testes comportamentais baseados na resposta à *novidade*. Os animais iuGC não apresentam diferenças substanciais na atividade locomotora induzida pela *novidade* nem no comportamento exploratório geral (Novelty Place Preference e Novel Object Recognition). Contudo, os indivíduos iuGC exploram objectos familiares e novos de forma semelhante no teste com tempo de retenção de 24h. Adicionalmente, avaliámos a *acção impulsiva*, através do 5-Choice Serial Reaction Time Task (5-CSRTT) e a *escolha impulsiva*, utilizando o teste Delay Discounting (DD). O grupo iuGC apresenta alterações na tarefa DD, mas não na performance no 5-CSRTT. Como o teste DD é dependente do córtex pré-frontal (PFC) e da amígdala, foi analisado o padrão de activação neuronal destas regiões usando imunohistoquímica para c-fos. O tratamento iuGC induziu alterações na densidade de células c-fos positivas no córtex orbito frontal e PFC medial, com menor efeito na amígdala, o que pode ajudar a explicar as diferenças observadas. Em suma, os nossos resultados sugerem que o tratamento iuGC não parece afetar o comportamento exploratório, mas tem um efeito deletério na memória de longo prazo. Em simultâneo, os animais iuGC apresentam maior escolha impulsiva, o que pode contribuir para a maior vulnerabilidade para comportamentos *aditivos*.

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Abbreviations

5-CSRTT: 5-Choice Serial Reaction Time Task

5-HT: 5-Hidroxytryptamine/Serotonin

11 β-HSD: 11 Beta-hydroxysteroid dehydrogenase

ACC: Anterior Cingulate Cortex

ACTH: Adrenocorticotrophin Hormone

ADHD: Attention Deficit Hyperactivity Disorder

AVP: Arginine Vasopressin

BET: Betamethasone

BLA: Basolateral nucleus of the Amygdala

BNST: Bed nucleus of the Stria Terminalis

CeA: Central nucleus of the Amygdala

CNS: Central Nervous System

CONT: Control

CRH: Corticotropin-Releasing Hormone

CRF: Continuous Reinforcement

CPP: Conditioned Place Preference

DA: Dopamine

DD: Delay Discounting

DEX: Dexamethasone

DLS: Dorsolateral Striatum

dlOFC: dorsolateral Orbitofrontal Cortex

D1: Dopamine Receptor 1

D2: Dopamine Receptor 2

D3: Dopamine Receptor 3

ELS: Early Life Stress

GC: Glucocorticoid

GR: Glucocorticoid Receptor

HPA: Hypothalamic – Pituitary – Adrenocortical

HI: High Impulsive

HNP: High Novelty Preferring

HR: High Responder

ILC: Infralimbic Cortex

ITI: Inter-trial Interval

iuGC: *in utero* Glucocorticoid

LNP: Low Novelty Preferring

lOFC: lateral Orbitofrontal Cortex

LR: Low Responder

mPFC: medial Prefrontal Cortex

MR: Mineralocorticoid Receptor

mRNA: messenger Ribonucleic Acid

NAcc: Nucleus Accumbens

NE: Norepinephrine

NIH: National Institute of Health

NOR: Novel Object Recognition

NPP: Novelty Place Preference

OF: Open Field

OFC: Orbitofrontal Cortex

PFC: Prefrontal Cortex

PLC: Prelimbic Cortex

PBS: Phosphate–Buffered Saline

SAM: Sympathetic-Adrenomedullary

SD: Stimulus duration

vOFC: ventral Orbitofrontal Cortex

VTA: Ventral Tegmental Area

Chapter 1 – Introduction

1. Introduction

The maintenance of a dynamic equilibrium, or homeostasis, during exposure to internal or external threats, is required for life (Chrousos and Gold, 1992; Charmandari *et al*, 2005). These threats or stressors induce a cascade of reactions that reflect in a complex and constant adaptation of several endocrine, autonomic and behavioral systems that help preserve viability through change or allostasis (McEwen and Seeman, 1999).

The stressor type influences the repertoire of neuronal populations that perceive it, as well as the molecular mediators that are involved in the adaptive response. A physical stressor, such as trauma, recruits brainstem and hypothalamic regions (Ulrich-Lai and Herman, 2009; Joëls and Baram, 2009), whereas a psychological one, e.g., social embarrassment, recruits brain regions that mediate emotion (the amygdala and the prefrontal cortex (PFC)), learning and memory (hippocampus) and decision making (PFC) (De Kloet *et al*, 2005; McEwen, 2007; Joëls and Baram, 2009). Though the type of stressor may differ, physical stressors often have psychological features and vice versa. In addition, the age of an individual affects the magnitude of the stress response, since the molecular cascades that are activated by stress in the hypothalamus and hippocampus in adult life differ from those activated in the infant phase, changing again with aging (Lupien *et al*, 2009; Joëls and Baram, 2009). The duration of the stress also impacts the elicited response. The system is able to adapt with one type of stressor, whereas chronic stress can cause inhibition of neurogenesis, disruption of neuronal plasticity and neurotoxicity. Other aspects, such as sex and even genetic factors can also contribute to the diversity and impact of the stressors in the brain (Joëls and Baram, 2009).

Studies in both animals and humans have shown that the brain is particularly sensitive to stress in the prenatal period and early childhood, since it undergoes important changes during these periods. Additionally, there seems to be a relation between exposure to early life stress (ELS) and increased reactivity to stress in adulthood, suggesting a 'programming' effect in the circuits involved in the stress response (Lupien *et al*, 2009).

1.1. The stress response

The stress response is mediated by two distinct systems, the sympathetic-adrenomedullary (SAM) and the hypothalamic-pituitary-adrenocortical (HPA) systems (Frankenhaeuser, 1986; Stratakis and Chrousos, 1995). The SAM system is comprised by the

autonomic nervous system and upon stimulation, releases catecholamines, predominantly epinephrine, but also norepinephrine (NE) (Vollmer, 1996). NE and epinephrine increase heart rate, vasodilatation in muscles and constriction of blood vessels in the skin, ensuring blood supply to the brain and muscles, thus playing an important role in the fight/flight response. Even though neither of these two catecholamines are able to cross the blood brain barrier, alongside their peripheral action, the locus coeruleus produces NE in the brain, supporting vigilance and attention and participating in processes that activate the HPA axis (Morilak *et al*, 2005; Gunnar and Quevedo, 2007). Corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) are released by cells in the paraventricular nuclei of the hypothalamus, upon activation of the HPA axis (Figure 1) (Plotsky *et al*, 1993). These hormones act on the anterior pituitary, leading to the release of adrenocorticotropic hormone (ACTH), which interacts with receptors on the cortex of adrenal glands, stimulating the production and release of glucocorticoids (GC; cortisol in humans and corticosterone in rodents) and some mineralocorticoid steroid hormones, into the bloodstream (Plotsky *et al*, 1993; Stratakis and Chrousos, 1995; Checkley, 1996; Gunnar and Quevedo, 2007). Upon release of GCs, these hormones also participate in the termination of the stress response by exerting a negative feedback in the secretion of CRH and ACTH, in the paraventricular nuclei of the hypothalamus and the anterior pituitary, respectively. Also, the GCs feedback inhibition acts upon the medial prefrontal cortex (mPFC) and hippocampus, thus limiting the tissue exposure to GCs (Charmandari *et al*, 2005).

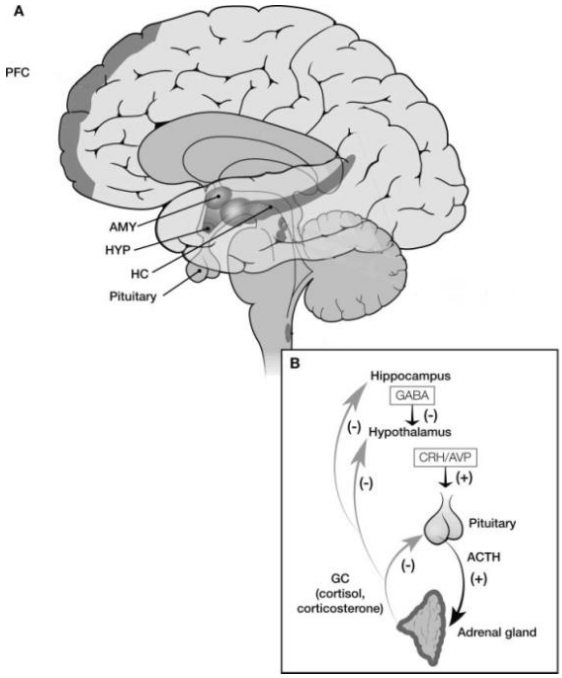

Figure 1. The anatomy of the HPA system and the important structures in its regulation (A). The activation (+) and negative feedback inhibition (-) pathways of the HPA system is also represented (B). Increase in glucocorticoids (GCs) is initiated by the release of corticotropin-releasing hormone/arginine vasopressin (CRH/AVP) from the paraventricular nucleus (PVN) in the hypothalamus. Through GCs, a negative feedback inhibition occurs in the pituitary, hypothalamus (HYP), and hippocampus (HC). ACTH, adrenocorticotropic hormone; AMY, amygdala; GABA, gamma aminobutyric acid; HC, hippocampus; HYP, hypothalamus; PFC, prefrontal cortex. Adapted from Gunnar and Quevedo, 2007.

The Central Nervous System (CNS), through its activity, coordinates both the SAM and HPA systems. For short term periods, balanced activation of these systems tends to support adaptive functioning. However, over chronic activation, the suppressive impact of constant elevated, or even decreased, levels of GCs and frequent SAM responses can have deleterious effects on physical and mental health, affecting behavior as well as growth, metabolism, circulation, reproduction and the inflammatory/immune response, increasing the vulnerability to a variety of disorders, including heart disease, hypertension, type II diabetes, anxiety, depression, post-traumatic stress disorder (PTSD), eating and sleeping disorders and addiction (Chrousos and Gold, 1992; Charmandari *et al*, 2005; Gunnar and Quevedo, 2007; Chrousos, 2009).

1.2. Stress and glucocorticoids action

Glucocorticoids have a variety of effects in target systems in the organism, aiming to increase the availability of energy substrates and allow optimal adaptation to changes in the environment. Under basal conditions, GCs are secreted in a 24h circadian rhythm, with a

morning maximum concentration (circadian peak), that slowly declines throughout the day and night, with an abrupt elevation after the first hours of sleep (Sapolsky *et al*, 2000; Lupien *et al*, 2005). Once released into general circulation, GCs enter the cytoplasm of cells throughout the body and brain, where they bind to two receptor subtypes: the high affinity mineralocorticoid receptor (MR), or type I receptor, and the low affinity glucocorticoid receptor (GR), or type II receptor (De Kloet, 1991). The activation of these receptors is responsible for the activation of transcription of genes with GC-responsive regions (Sapolsky *et al*, 2000).

The presence of an enzyme, 11 Beta-hydroxysteroid dehydrogenase (11 β-HSD) prevents GCs from binding to MRs, by converting cortisol/corticosterone to its inactive form (cortisone/11 dehydrocorticosterone). In the brain, 11 β-HSD is minimally expressed and, despite having an ability to bind to both MR and GR, GCs have higher affinity to MRs (80%-90% of MRs are occupied by GCs) in a basal condition (De Kloet, 1991; Gunnar and Vazquez, 2006). Conversely, GRs are occupied either at the peak of the circadian cycle or upon stimulation of the HPA axis by stress. Considering the distribution pattern of these receptors in the brain, MRs are exclusively present in the limbic system, such as the hippocampus, parahippocampal gyrus, amygdala, entorhinal and insular cortices, whereas GRs are present in the nucleus accumbens (NAcc) and cortical structures, such as the PFC, along with hypothalamus, hippocampus and parahippocampal gyrus (McEwen *et al*, 1986; Diorio *et al*, 1993; Lupien *et al*, 2005).

The effects mediated by GRs are characterized as suppressive, considering that they are necessary for the termination of the HPA stress response and facilitate the recovery of cellular homeostasis (Sapolsky *et al*, 2000). However, when prolonged, these suppressive effects begin to exceed their benefit, ultimately leading to increased vulnerability to several neuropsychiatric disorders.

1.3. Use of glucocorticoids in clinics

Synthetic GCs are widely used in clinics for a broad range of disorders, due to their pleiotropic effects. Betamethasone (BET) and dexamethasone (DEX) are synthetic glucocorticoids commonly administered to pregnant women in case of premature delivery. This type of treatment, reported by Liggins and Howie (1972), has long been validated, since it promotes fetal lung maturation, ensuring the efficacy and safety against disorders, such as respiratory distress syndrome, reducing neonatal morbidity and mortality (Crowley, 1995). Besides the reduction of

respiratory distress syndrome occurrences, treatment with either BET or DEX is also known to reduce cases of intraventricular hemorrhage (Liggins and Howie, 1972) and congenital adrenal hyperplasia, the most frequent being the 21-hydroxylase deficiency (Lunghi *et al*, 2010), preventing genital masculinization in female fetuses.

Despite the obvious beneficial effects of glucocorticoid usage, one cannot overlook the outcome and the long-term consequences that emerge from such hormone treatment, such as low birth weight, both in animal models (Nyirenda *et al,* 1998; Ikegami *et al,* 1997; French *et al,* 1999) and humans (French *et al,* 1999; Newnham and Moss, 2001; Bloom *et al,* 2001), which escalate and are associated with the development of hypertension, insulin resistance, type 2 diabetes and cardiovascular disease (Barker *et al,* 1993; Rich-Edwards *et al,* 1997; Curhan *et al,* 1996a; Curhan *et al,* 1996b).

These synthetic GCs differ from their endogenous equivalent in their chemical structure, pharmacokinetics and target specificity properties, exhibiting thus an intensified affinity to the GR than to the MR (Tegethoff *et al,* 2009) and possess an ability to cross the placenta easily. In normal conditions, fetal GC levels are much lower than maternal ones (Beitens *et al,* 1973; Klemcke, 1995) due to 11 β-HSD activity, that is also expressed in the placenta, and inactivates endogenous GCs. However, DEX and BET are poor substrates for 11 β-HSD, hence resulting in the crossing of the synthetic hormones through the placenta, from the mother to the fetus (Brown *et al,* 1996; Seckl, 2004), activating GR-induced transcription in the later (Welberg and Seckl, 2001). This early high exposure to GC can, potentially and persistently, affect the developing brain, programming it in a way that may predispose the system to disease (Welberg and Seckl, 2001).

1.4. Early life stress and the 'programming' effects of glucocorticoids in the brain

During the early life period, the developing brain is very susceptible and sensitive to a so-called 'programming', i.e., modulation of fetal physiological and metabolic processes by a stimulus or an insult during a critical period of development (Barker, 2001). Increasing evidence revealed a major role played by the intra-uterine environment in the development of several adult diseases. Different early life stress episodes have also been linked to increased risk for the development of personality disorders, attention deficit hyperactivity disorder (ADHD), anxiety,

depression, schizophrenia and addictive disorders (Agid *et al,* 1999; Bernet and Stein, 1999; Weiss *et al,* 1999; Heim and Nemeroff, 2001; Dube *et al,* 2003; Chapman *et al*, 2004).

Exposure to stress or glucocorticoids of a pregnant female or even other types of extreme conditions, such as undernutrition, lead to an increase in maternal GC secretion (Cadet *et al,* 1986; Dean and Matthews, 1999; Maccari *et al,* 2003). Since a part of the GCs cross the placental barrier and reaches the fetus, activity of the fetal HPA axis is increased, affecting brain development (Seckl, 2007). It is important to know that throughout pregnancy and fetal development, GCs are crucial and play an important role in the normal maturation of the developing CNS (Meaney *et al,* 1996) by initiating terminal maturation, remodeling axons and dendrites and cell survival (Meyer, 1983). However, prenatal treatment with GCs is also known to delay maturation of neurons, myelination, glia and vasculature (Hung *et al,* 2001a; Hung *et al*, 2001b). Along with these effects, when *in utero* exposure to GCs is excessive, resulting in the programming of the HPA axis and subsequent impairment of the HPA axis function, different neurobiological pathways can be affected and altered. Additionally, alterations have been noted in the morphology, structure and number of various neuronal areas (Oliveira *et al*, 2006; Leão *et al*, 2007; McArthur *et al,* 2005; Rodrigues *et al,* 2011; Oliveira *et al,* 2012). Several studies revealed that prenatal exposure to stress/GCs leads to neurodegeneration in hippocampus (Stein *et al,* 1997) and anxiety-like behavior due to altered levels of CRH in the amygdala (Welberg *et al*, 2001). Furthermore, a decrease in neuronal numbers in the nucleus accumbens (NAcc) is noted (Leão *et al*, 2007), along with an increase in the number of immature spines. Studies from Oliveira *et al.* (2012) demonstrated an increase in the volume and dendritic length of neurons of the bed nucleus of the stria terminalis (BNST), as well as a decrease in the volume of the basolateral nucleus of the amygdala (BLA) and central nucleus of the amygdala (CeA). Regarding the effects of prenatal exposure to GCs in behavior, individuals developed anxious-like behavior (Oliveira *et al,* 2006; Oliveira *et al,* 2012), depressive-like behavior (Roque *et al,* 2011) and addictive-like behavior (Rodrigues *et al,* 2011).

Altogether these evidences pinpoint the mesocorticolimbic circuit as extremely sensitive to GC programming effects, which may explain the appearance of the above mentioned mood and reward disorders.

1.5. Mesocorticolimbic system and related behaviors

The mesocorticolimbic system, otherwise known as the reward system, is responsible for the processing of the rewards, whether natural, such as sex and food (Kelley and Berridge, 2002) or addictive rewards, like drugs of abuse, and where dopamine (DA) plays a major role. Increasing evidence suggests that acute DA signaling is critical for the motivational reward, since it mediates the attribution of incentive salience to reward-related stimuli, increasing the motivation for the reinforcer (Berridge, 2007). It is important to mention the different dimensions in the reward system, namely the process of learning the occurrence of the reward, the hedonic impact of the reward ('liking') and the incentive salience or the motivation attributed to reward-related stimuli ('wanting') to obtain a certain reward. In this way, when incentive salience is attributed to the reward-related stimulus, the brain perception of the reward is altered into a motivationally potent incentive (Berridge, 2007). Thus, the mesocorticolimbic dopaminergic system becomes a key component in the study of addictive behavior.

The mesocorticolimbic circuit, usually divided in mesolimbic and mesocortical systems, arises from the ventral tegmental area (VTA), which projects to the PFC, controlling executive function and cognition, and the NAcc, amygdala and hippocampus, regulating memory, motivation, reward and addiction (Rodrigues *et al,* 2010).

The VTA neurons can be activated by stress and the dopaminergic projections to, not only the NAcc, but also the medial PFC (mPFC) are also a target for the stress hormone (Abercrombie *et al.,* 1989; McEwen *et al.* 1986). Studies developed by Leão and colleagues (2007) showed a decrease in proliferating cells in the NAcc core and shell and in the VTA that resulted in a reduction of the dopaminergic innervation in this system. Rodrigues *et al.* (2011) showed a decrease on the basal levels of DA in the NAcc and an increase on the mRNA and protein expression levels of dopamine receptor 2 (D2), but not dopamine receptor 1 (D1). A decrease on DA levels, along with an increase only in the mRNA expression level of D2, but not D1, was noted in the amygdala, after prenatal exposure to GCs (Oliveira *et al*, 2012). All of these structural and molecular changes led to the development of behavioral alterations. Animals that were prenatally exposed to GCs revealed to be hyperanxious (Oliveira *et al*, 2006; Oliveira *et al*, 2012), depressive (Roque *et al*, 2011) and are more vulnerable to the development of an addictive-like phenotype (Rodrigues *et al*, 2011). In this context, it is important to mention that there was a correlation between behavioral deficits of animals exposed to prenatal GCs and dopaminergic dysfunction, since that after stabilization of the DA levels (through the use of a DA

precursor), the drug-seeking behavior was reverted and expression levels of D2 were normalized (Rodrigues *et al*, 2011).

Dependent behaviors or addiction are attributed to different brain regions, resulting in a complex behavior. Studies from Leão *et al.* (2007) and Rodrigues *et al.* (2011) uncovered the deleterious effects of prenatal exposure to DEX in the mesolimbic 'rewarding' pathway, particularly in the NAcc, as aforementioned, highlighting new insights in the mechanism that underlies reward-related and addictive behavior.

1.6. Impulsivity and novelty-seeking behavior in substance abuse disorders

Substance abuse is defined by the American Psychiatric Association in the diagnostic and statistical manual of mental disorders (DSM-IV) as a maladaptive pattern of substance use leading to significant impairment, manifested by recurrent substance use that may culminate in several persistent and recurrent problems, such as increasing dosage intake and compulsive drug seeking that persists despite the outcome (1994). Individual genetic/epigenetic and external factors seem to contribute for the development of such disorder, even though the mechanisms can be substantially different. In this manner, it is plausible to assume that some individuals are more vulnerable when initially exposed to addictive drugs (Anthony *et al,* 1994) and prediction of drug intake escalation, followed by compulsive drug seeking and increased propensity to relapse after abstinence has been related to behavioral characteristics such as impulsivity and novelty-seeking (Everitt *et al*, 2008; Verdejo-Garcia and Perez-Garcia, 2007).

Impulsivity is a key characteristic of several psychiatric disorders, including personality disorders, mania, ADHD and drug addiction, as already mentioned, and is a complex multidimensional behavior (Evenden, 1999) that has been defined as an inability to wait, a tendency to act without forethought, insensitivity to consequences and an inability to inhibit inappropriate behaviors (Ainslie, 1975; Barkley, 1997; Barratt and Patton, 1983; Eysenck, 1993; Rachlin and Green, 1972). Thus, this trait can be divided into two categories: behavior that results from deficits in the ability to withhold responding, showing poor inhibitory control (impulsive action); and behavior that does not result from such inhibitory deficits but from an insensitivity to delay of gratification, delay aversion, leading to impulsive decision making, that is represented by increased preference for immediate reward over a large but delayed reward (impulsive choice) (Winstanley *et al,* 2006; Diergaarde *et al,* 2008).

Increasing evidence supports the important role of impulsivity in drug addiction and disturbances in inhibition of behavior that result from prolonged drug intake have been proposed to maintain compulsive drug intake (Jentsh and Taylor, 1999). Various studies reported that high impulsive behavior might predispose individuals to initiate or maintain drug seeking and taking in animal models (Krishnan-Sarin *et al*, 2007; Paulus *et al*, 2006) and humans (Dalley *et al,* 2007; Diergaarde *et al,* 2008; Perry *et al,* 2005). For instance, impulsive choice is increased in smokers as compared with nonsmoking individuals (Bickel *et al,* 1999; Mitchell, 1999) and an association between elevated impulsivity and increased self-administration behavior has been reported for cocaine (Dalley *et al,* 2007; Perry *et al,* 2005). However, the findings for a given drug have not always been consistent; as studies from Ortner *et al.* (2003) showed that alcohol reduced impulsive choice in humans, while Richards *et al.* (1999) found no effect of alcohol and other experiments revealed alcohol-induced impulsive choice in rats (Evenden and Ryan, 1999; Tomie *et al,* 1998). These discrepancies may in some cases be because the drugs have a situation and choice dependent effect (Cardinal, 2006).

Considering that novelty/sensation seeking are constructs useful in predicting human risk-taking behaviors, it is plausible to link these traits with addictive behavior. Novelty is associated with events not previously experienced, as well as new combinations of familiar stimuli (Bevins, 2001). Different studies revealed an association between the sensation/novelty-seeking trait and cocaine abuse or addiction (Franques *et al*, 2000; Franques, 2003; Kreek *et al*, 2005). For that, individuals that are novelty/sensation seekers are also more prone to experience addictive drugs, similar to several risky activities (Zuckerman *et al*, 1990; Horvath and Zuckerman, 1993; Kalichman *et al*, 1994; Wills *et al*, 1994; Jonah, 1997; Sher *et al*, 2000; Zuckerman and Kuhlman, 2000; Woicik *et al*, 2009). Some individuals may present increased novelty-seeking behavior, predisposing them to consume drugs of abuse for the first time or increased impulsive behavior, presenting a higher probability to engage in dangerous behaviors (Belin *et al,* 2010). Regarding spontaneously high impulsive (HI) rodent studies, a significant escalation of intravenous cocaine and nicotine self-administration has been shown (Dalley *et al,* 2007; Diergaarde *et al,* 2008) and, interestingly, no differences in anxious-like behavior nor in the reactivity to novelty were detected (Molander *et al,* 2011). Taking into account these results, it is possible to separate HI animals from the high responder (HR) phenotype described by Piazza and colleagues (1991) that revealed increased exploratory activity and a greater propensity to acquire drug self-administration. In this way, it is possible to differentiate the novelty-seeking

dimension from the impulsive one, despite the general preference of impulsive rats for novelty (Molander *et al,* 2011), since HI rats show persistent responding for cocaine despite punishment (Belin *et al,* 2008), together with an increased propensity to escalate sucrose-seeking behavior and intravenous cocaine and nicotine self-administration (Dalley *et al,* 2007; Diergaarde *et al,* 2008, 2009). This indicates that impulsive rats may be more reactive to positive reinforcement than negative one, highlighting the trait-like impulsivity as a core behavior underlying vulnerability for stimulant addiction (Molander *et al,* 2011). Alterations in novelty-seeking or sensation-seeking behaviors are also associated with a variety of psychiatric disorders including alcoholism and drug addiction (Zuckerman and Neeb 1979; Zuckerman 1990; Wills *et al,* 1994; Woicik *et al,* 2009) and Belin and colleagues (2010) showed a clear distinction between two vulnerable phenotypes predisposing to cocaine addiction: a 'drug use prone' phenotype such as high responder which brings an individual to develop drug use and an 'addiction prone' phenotype, such as high-novelty-preference, which facilitates the shift from sustained to compulsive drug intake and addiction.

Taken together, studies performed seem to point out that both novelty/sensation-seeking and impulsive traits are associated with addictive behavior, not necessarily independently from one and another, thus making imperative a behavioral assessment, in individuals prenatally exposed to GCs and with increased vulnerability to drug-seeking behavior, which we will tackle in the next section.

1.7. Assessment of impulsive behavior

The different aspects of impulsivity can be measured using paradigms that focus on different aspects of the impulsive response, dividing them in tasks that evaluate response inhibition, which involves premature or difficult to suppress actions (impulsive action) and impulsive choice response, that comprises actions that fail to take into account other outcomes and that may be sub-optimal. This type of behavior can be measured in experimental animals using tests that are based on equivalent tasks in humans (Dalley and Roiser, 2012).

Assessment of impulsive choice is usually accomplished by performing a delay discounting (DD) task, where subjects are trained to choose between small immediate rewards and larger but delayed rewards (Cardinal *et al*, 2001). Moreover, abnormally steep delay discounting has been demonstrated in drug addicts, including alcoholics (Petry, 2001; Vuchinich

and Calamas, 1997), cocaine users (Coffey *et al,* 2003; Kirby and Petry, 2004), opiate users (Kirby and Petry, 2004; Kirby *et al*, 1999; Madden *et al*, 1997), and smokers (Bickel *et al,* 1999; Mitchell, 1999; Ohmura *et al*, 2005; Reynolds *et al,* 2004).

Additionally, impulsive action can be evaluated by the 5-Choice Serial Reaction Time Task (5-CSRTT) paradigm, in which subjects are trained to detect the spatial location of a brief visual stimuli presented in one of five recesses in an operant chamber (Robbins, 2002), and, similar to the analogous performance test in humans, it evaluates sustained and selective attention. This form of impulsivity is associated with 'restraint' and defined by the inability of an individual to withhold a strong behavioral tendency (Schachar *et al,* 2007). Furthermore, impulsive action is measured by the number of premature or anticipatory responses made before the onset of the target stimulus and increases with the extension of the pre-stimulus interval.

Correlational studies have been performed in order to associate questionnaire measures of impulsivity and performance on behavioral tests, such as delay discounting (Reynolds *et al*, 2006) and further analysis of the behavioral measures revealed two independent latent variables: one corresponded to impulsive action measures and the other corresponded to impulsive choice measures (delay discounting; risk taking). Moreover, studies that scored individuals as ''high'' impulsive in the questionnaires have been reported to perform worse on decision-making tests (Crean *et al,* 2000; Franken *et al,* 2008).

1.8. Assessment of novelty-seeking behavior

The sensation/novelty-seeking behavior trait can be divided in different dimensions and can be studied in rodents both by high locomotor reactivity to a new inescapable environment, corresponding in different studies to the HR animal phenotype (Dellu *et al*, 1996; Blanchard *et al*, 2009). However, they do not predict the vulnerability to switch from sustained drug use to addiction (Belin *et al,* 2008; Belin *et al,* 2010). Also, it is possible to assess high propensity to visit a new environment in a free-choice procedure, i.e. novelty-induced in a conditioned place preference (CPP) apparatus, which are typically seen in the high novelty preferring (HNP) phenotype (Bardo *et al,* 1996; Cain *et al,* 2005) or even the preference for a novel object, in comparison to a familiar one (Bardo *et al,* 1990; Molander *et al,* 2011). These two behavioral measurements are suggested to be different (Bardo *et al,* 1996; Cain *et al,* 2005) and predict different dimensions of drug reward (Bardo *et al,* 1996). In this way, it has been shown that HR

animals are more vulnerable than low responder (LR) ones in their propensity to acquire drug self-administration (Piazza *et al,* 1990; Piazza *et al,* 2000), whereas HNP animals differ from their low novelty preferring (LNP) littermates in their vulnerability to express place preference for amphetamine (Bardo *et al,* 1996) but not in their propensity to acquire drug self-administration (Klebaur *et al,* 2001). Hence, these different novelty traits may differently contribute to the vulnerability to addiction, revealing a dissociation between the propensity to acquire drug administration and compulsive drug use (Belin *et al,* 2008).

Moreover, correlational studies between novelty and the different forms of impulsive traits have been performed to establish whether a particular form of impulsivity was associated more strongly with novelty-seeking than other forms of impulsivity. It has been recently shown that animals with high reactivity to a novel environment revealed an increase in the approach to food and cocaine-predictive cues compared with low responder rats and were also less impulsive on a delay discounting task (Flagel *et al,* 2010). However, in a measure of impulsive action, they have been shown to be more impulsive (Pattij and Vanderschuren 2008; Winstanley *et al,* 2006). This distinction is important and may indicate multiple predisposing determinants of the propensity to drug self-administration (Molander *et al,* 2011).

Taking into account the possible impact that stress may have in decision making and personality traits of individuals, we decided to evaluate how ELS affects traits associated with drug addiction, such as impulsive and novelty seeking traits and identify the alterations in neuronal activation of several areas involved in these types of behavior.

Chapter 2 – Objectives

2. Objectives

High exposure of GCs, during the prenatal period, has permanent and persistent effects in the CNS, culminating in increased vulnerability to addictive behavior. Yet, little is known about the traits that may underlie this increased propensity to develop drug addiction. In this context, we aimed to evaluate behavior traits that may explain the observed increased vulnerability to drug-seeking behavior in animals that received synthetic GCs *in utero* (iuGC). Our main goals were to:

- Assess the effect of iuGC exposure in novelty-seeking behavior using different paradigms, namely Locomotor Reactivity to a novel environment, Novelty Place Preference (NPP) and Novel Object Recognition (NOR);

- Unravel the potential impact of prenatal exposure to iuGC in impulsive behavior, through 5-Choice Serial Reaction Time Task (5-CSRTT) and Delay Discounting (DD) task;

- Search for alterations in the activation pattern of several brain regions associated with DD, using c-fos staining.

Chapter 3 – Materials and Methods

3. Materials and Methods

3.1. Animals and treatment

Adult female pregnant Wistar han rats (Charles River Laboratories, Barcelona, Spain) were individually housed under controlled conditions (light/dark cycle of 12/12h with lights on at 08:00h and ambient temperature of 22°C±1°C) with food and water *ad libitum*. Subcutaneous injections of dexamethasone (iuGC animals, 1mg/kg, Sigma-Aldrich, St. Louis, MO, USA; n=3/4 pregnant female animals) or vehicle (control; CONT animals; n=3/4 pregnant female animals) were administered on gestation days 18 and 19. Progeny were separated according to gender and prenatal treatment (either CONT or iuGC animals) on postnatal day 21 and handled daily for 5-10min for a week before behavioral testing.

Subjects were male offspring, 3 months old at the start of the experiment, and were only food-deprived to approximately 85% of their free feeding weight until the end of the 5-Choice Serial Reaction Time Task (5-CSRTT) or the Delay Discounting (DD) task procedures, with food made available at the end of the experimental day. Control and iuGC animals used for Novelty reactivity in an Open Field (OF), Novelty Place Preference (NPP) and Novel object recognition (NOR) were selected from one cohort of rats (n=40). Twenty animals were screened in the 5-CSRTT and an additional group of rats (n=20) were subjected to the DD task.

All experiments were performed in accordance with the local regulations (European Union Directive 2010/63/EU) and National Institute of Health (NIH) guidelines on animal care and experimentation.

3.2. Behavioral assessment

3.2.1. 5-Choice Serial Reaction Time Task

3.2.1.1. Apparatus and training

Two identical automated operant 5-CSRTT chambers (25 x 25 x 30cm), housed in ventilated and sound attenuating boxes, were used. Each chamber contained five square holes (2.54 x 2.2 x 2.25cm), each fitted with a photocell beam to detect nose pokes and a stimulus light positioned above, located on a curved rear wall. On the opposite wall, 45mg food pellets (Bio-Serve, Frenchtown, NJ) could be delivered into a magazine by a dispenser. Chamber

illumination was provided by a 100-mA house light located at the center of the roof. Control of the apparatus and data collection was performed by a PC with TSE software.

Rats were trained according to the protocol described by Muir *et al.* (1999). For the first two days, food pellets were made available in the magazine and response apertures were covered with metal caps for 15min. In the next two sessions, the metal caps were removed from apertures and several food pellets were placed in each hole as well as within the magazine. Animals were allowed to explore for 30min. During the fifth session the test schedule was implemented.

3.2.1.2. 5-CSRTT assessment

The test protocol consisted of illumination of the house light and delivery of a single food pellet to the magazine. The trial was initiated by the rat performing a magazine entry to collect this pellet. After a 3-5s inter-trial interval (ITI), the light of one of the holes was illuminated for a short period. The light stimulus was presented randomly and in an equal number of times in each of the five holes during each daily session. Sessions consisted of 100 trials or were terminated after 30min of testing and the target parameters for each session were: stimulus duration, 0.5s; limited hold period, 5s; ITI and time-out period, 5s. Responses or nose pokes performed in this aperture were rewarded with the delivery of a food pellet and a correct response was recorded. If an animal executed additional nose pokes, perseverative responses were recorded and resulted in a 3-5s period of darkness (time-out). Further nose pokes in the apertures during this period restarted the time-out. An incorrect response was recorded when an animal performed a nose poke in a non-illuminated hole during the signal period and failures to respond within the limited hold period were recorded as omissions. In both situations the chamber entered the ITI state and any additional responses in this period restarted the time-out. A response in the food magazine after the delivery of a food pellet, or after the time-out period, initiated the next trial and further entries in the magazine during the ITI or time-out periods were recorded but had no further consequences. Responses in the apertures during the ITI were recorded as anticipatory responses and, as in previous situations, resulted in a time-out period. Also, further responses during this period of darkness restarted the time-out. The end of a test session was signaled by extinguishing all the lights (Figure 2).

For the first session of training, the stimulus duration and limited hold periods were both set at 60s, and the ITI and time-out periods set at 3s. These variables were altered on subsequent trials (stimulus duration (SD): 30s, 10s, 2s, 0.5s) according to the individual performance of each animal, until the target set of task parameters could be instituted. The animals were trained to a criterion of, at least 80% correct responses and <20% omissions within the 30min session time, when target parameters were attained on 2 consecutive days. When animals attained the target set of stimulus duration parameter of 0.5s for 4 consecutive days, a session with a stimulus of 0.25s was performed. However, the majority of the animals did not reach the 0.5s stimulus parameter of the task (only four animals successfully reached this variable, while the rest was still being tested). Several behavioral measures were recorded for assessment of the task: i) Accuracy - Measured as the proportion of responses that were correct (number of correct responses/total number of responses) and expressed as a percentage; ii) Speed - Speed was measured as latencies: the first was the latency to respond correctly (time between the onset of the visual stimulus and the point at which the nose of the animal breaks the photocell beam of the hole and the second was the magazine latency (time between performance of a correct response and the magazine entry to collect the food pellet); iii) Anticipatory responses - Number of responses in the apertures during the ITI state; iv) Perseverative responses - Additional nose pokes in the holes following the initial response in an aperture; v) Omission - Number of trials on which no response was made during the limited hold period. Animals received one session per day, for a total of 50 sessions.

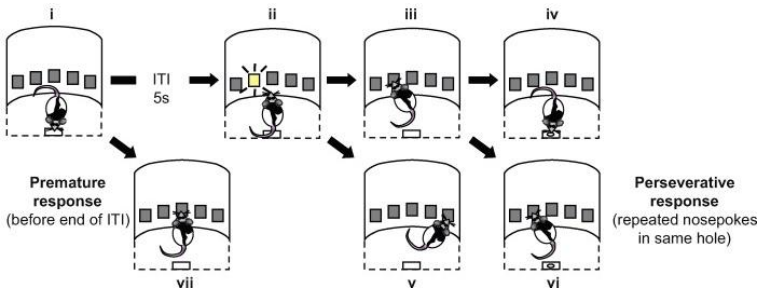

Figure 2. Schematic representation of the 5-Choice Serial Reaction Time task. Animals are trained to respond to a brief stimulus (visual cue) that has duration of 60s, 30s, 10s, 2 s and 0.5s, according to the phase of training, and may appear randomly in one of the 5 apertures. This stimulus is predictive of a food reward, since a correct response towards the stimulus would result in the delivery of a food pellet. Animals may respond prematurely by performing an action before the end of the inter-trial interval (ITI) period (impulsive action) or they may have a perseverative response when performing repeated nose pokes in the same hole. Adapted from Eagle and Baunez, 2010.

3.2.2. Locomotor activity in a novel environment

Locomotor activity was measured using an Open Field (OF) arena (43.2 x 43.2cm) equipped with two horizontal photocell beams situated 1cm from the floor that automatically registered activity (Med-Associates, St. Albans, VT, USA). Animals were placed in the center of the arena, an unfamiliar environment to them, and their ambulatory activity was recorded over a 2h period. The total number of photocell beam breaks was used as indicator of reactivity to the novel environment. Additionally, the number of rearings, time and distances in the central and peripheral areas were recorded.

3.2.3. Novelty place preference

The place preference apparatus consisted of two equally sized compartments (28 × 22 x 21cm) with distinct cues separated by a central neutral grey area (13 × 22 × 21cm), with two opposite entrances that could be closed by sliding doors (Med-Associates, St. Albans, VT, USA). One of the compartments had white walls and a mesh floor and the other had black walls and a grid floor. All compartments were equipped with photocell beams to record locomotor activity, number of visits and time spent in each compartment. Briefly, each animal was placed in the central grey area for 5min and allowed to explore one of the compartments for 30min in order to define the familiar compartment. The familiar compartment was counterbalanced for both groups of animals and animals were habituated for 2 consecutive days. During the test phase (day 3), subjects were placed in the central grey area for 5min, after which the sliding doors were opened to let the animal freely explore both compartments for 15min. The time spent in the new compartment was used as index of novelty preference.

3.2.4. Novel object recognition

The novel object recognition protocol was adapted from Bevins and Besheer (2006). The setup consisted of two rectangular dark chambers (50 x 50 x 100cm) and sessions were recorded using a video camera placed right above the apparatus. Animals were first habituated to the chambers for 10-15min. Each trial was composed by two phases, a sample phase and a choice phase. In the first, two identical to-be-familiarized objects (O1 and O2) were placed in the back left and right corner of the apparatus and each animal, placed facing away from the objects,

was allowed to explore them for a total of 3min. The second choice phase started after a delay of 1h (Short-term memory assessment), with animals maintained in their home cage. In this phase, one of the familiar objects (e.g. O1) was replaced with a novel object (N). The trial ended when animals had explored both objects, for 2min. Animal nose directly contacting the object or directing it towards the object at a distance of ≤2cm was used as an indicator of object exploration. This protocol was repeated for Long-term memory assessment by altering the delay time between phases to 24h.

3.2.5. Delay discounting task

3.2.5.1. Apparatus and training

Two identical standard operant chambers (30.5 x 24 x 21cm; Med-Associates, St. Albans, VT, USA), enclosed in sound-attenuating boxes and equipped with ventilation to mask outside noise, were used. Each operant chamber contained a central food magazine, with two retractable levers with a 2.8W round stimulus light above each lever, positioned on each side of the magazine. A photocell beam was fitted in the magazine to detect head entry and, on the top center of the opposite wall, a single 100-mA house light provided illumination. 45mg food pellets (Bio-Serve, Frenchtown, NJ) were delivered by a pellet dispenser into the magazine. The apparatus was controlled by MedPC-IV software (Med-Associates, St. Albans, VT, USA) running in a PC connected to the chambers and all experimental data were recorded.

Training protocols were adapted from Cardinal *et al.* (2000) and Floresco *et al.* (2008). On the first day, animals were placed in the operant chambers where two to three pellets were already placed in the magazine and on the active lever. The first stage of training consisted of an instrumental task of continuous reinforcement (CRF) in order to obtain a food pellet. Animals were trained under a fixed ratio 1 schedule to respond first on the left lever then the right one (left/right levers counterbalanced between subjects), the criterion being 50 presses in 30min sessions. Once this stage was completed, animals were trained to make a head entry into the magazine to trigger lever presentation for 10s. A training session consisted of 90 trials and began with the chamber in darkness and both levers retracted (defined as inter-trial (ITI) state). Each trial began every 40s and with illumination of the house light. The animal was required to make a head entry within 10s to extend one lever or an omission would be recorded and the chamber returned to darkness. A lever was presented for 10s and if the animal failed to respond, the lever

retracted and the chamber darkened. However, if a response occurred, the lever retracted, a single pellet was delivered immediately into the magazine and the house light turned off after reward retrieval and for the rest of the inter-trial state. The left or right lever was presented once in a random order, with no more than two consecutive presentations of the same lever. Animals were trained to a criterion of 80 or more successful trial per session.

3.2.5.2. Delay discounting assessment

Each session began with the chamber in darkness and levers retracted. A session consisted of 4 blocks of 12 trials and lasted 56min and each trial lasted 70s. Session blocks initiated with two forced choice trials, and began with illumination of the house light. After an initial head entry, one of the two levers was presented randomly. These forced trials were followed by ten free choice trials where both levers were extended. As in training sessions, animals were required to perform a head entry or an omission would be recorded (type 1) and the chamber entered the ITI state (house light off until the beginning of the next trial). One of the levers was designated the immediate lever (Lever 1), the other one the delayed lever (Lever 2) (counterbalanced left/right) and lever presentation lasted 10s (if the animal failed to respond, an omission was recorded (type 2) and the chamber returned to the ITI state). Choice of Lever 1 originated an immediate delivery of a single pellet reward, whereas Lever 2 originated a large delivery of four pellets following a delay. For the first block of trials, a response on either lever led to an immediate reward (0s). The following blocks increased the delay period in 15s intervals, such that in the second block, a response on Lever 2 resulted in large reward delivery after a 15s interval and in the third and fourth block the delay was 30s and 45s, respectively. After a lever press and during the delay, the stimulus light, positioned above Lever 2, was turned on until reward delivery, serving as a cue to signal the duration of the delay. After reward retrieval, the house light was turned off and the chamber entered the ITI state for the remainder of the trial. Animals received one session per day, for a total of 21 sessions (Figure 3A and 3B).

A similar version of the DD test was also performed. Here, the number of blocks of trials was increased to 5, with increasing delays throughout blocks. Again, for the first block of trials, a response on either lever, despite being the delayed or immediate lever, led to a delivery of a reward with no delay time (0s). The next block increased the delay time by a 5s interval and, as mentioned before, a response on Lever 2 caused a large reward delivery after that period. The

delay of the third block was 10s, 15s in the fourth block and, lastly, 30s in the fifth block. Animals received one session per day, for a total of 28 sessions.

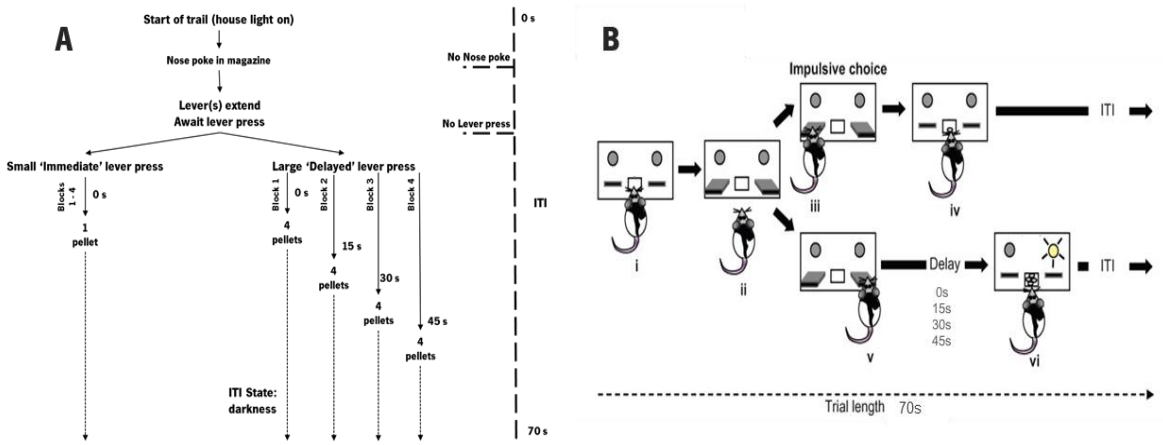

Figure 3. Schematic representations of the Delay Discounting task. **(A)** Outline of the Delay Discounting task, showing the basic structure of individual trials. **(B)** Animals are required to do a nose poke in order to start a trial and, after lever presentation, they have to choose to press a lever, between a small, but immediate reward lever and a large, but with delayed delivery, reward lever. As the session progresses, the delay time in each block increases (block of trials with 0s, 15s, 30s and 45s delay time). After collection of the food reward, the chamber returns to a state of darkness, the inter-trial interval, until the next trial begins (each trail lasts for 70s to ensure that animals that complete trials with no delays do not earn greater numbers of rewards by completing greater numbers of trials). **(B)** Adapted from Eagle and Baunez, 2010.

3.3. Histological procedures

3.3.1. Preparation of tissue for analysis

Nine months old CONT and iuGC subjects were deeply anesthetized with sodium pentobarbital, after 60min of exposure to the operant chambers, with 45mg pellets made available (n=4, for each group), or after performing DD task (n=6, for each group). Afterwards, animals were transcardially perfused, with 0.9% saline solution for about 5min, followed by 4% paraformaldehyde solution for 20min. Brains were carefully removed and immersed in 4% paraformaldehyde solution for 2h at room temperature, before being transferred to a 8% sucrose solution (Panreac, Barcelona, Spain) at 4°C, for a minimum of 48h.

Coronal sections of 50µm were serially cut in a vibrotome (Leica VT 1000S, Nußloch, Germany) and those of interest, selected for further histological processing.

3.3.2. Immunohistochemistry for c-fos detection

Induction of immediate early gene expression, such as c-fos, has long been accepted as a marker of neuronal activation and correlated with the activation of brain areas putatively involved in different actions, following external or internal stimuli (Herrera and Robertson, 1996; Puurunen *et al,* 2001; Lin *et al,* 1996).

In order to detect c-fos expression, slices containing OFC (dorsolateral OFC (dlOFC), lateral OFC (lOFC), ventral OFC (vOFC) and medial OFC (mOFC)) and mPFC (Anterior Cingulate cortex (ACC), Prelimbic cortex (PLC), Infralimbic cortex (ILC)) subregions and amygdala (central nucleus of the amygdala (CeA) and basolateral nucleus of amygdala (BLA)) were selected and submitted to a free floating immunohistochemical procedure adapted from Dragunow *et al.* (1987). Briefly, brain slices were rinsed in phosphate–buffered saline (PBS; 0.1M, pH 7.2) and endogenous peroxidases activity was inhibited by incubating slices with 3.3% hydrogen peroxidase in PBS for 30min. Cellular membranes were permeabilized in a series of PBS-T (0.3% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) in PBS, 0.1M, pH 7.2) washes. After blocking with a solution containing 2.5% fetal bovine serum in PBS-T for 2h, sections were incubated with rabbit anti-c-fos (Ab-5) (4-17) polyclonal antibody (Calbiochem, USA) at 1:2000 dilution in 2% fetal bovine serum and PBS-T, overnight at room temperature. Afterwards, slices were washed in PBS-T, following incubation with a biotinylated swine anti-rabbit IgG secondary antibody (DakoCytomation, Denmark) at 1:200 dilution, for 1h at room temperature. Sections were then rinsed in PBS-T and reacted with avidin-biotin complex solution (Vectorstain Elite, Vector, USA), for 1h, at 1:200 dilution. The bound chromogen was revealed using 50% 3.3-diaminobenzidine (DAB; Sigma-Aldrich, St. Louis, MO, USA) and 0.02% hydrogen peroxide in Trizma base solution and reaction was finalized by a series of rinses in PBS. Finally, slides were mounted, counterstained with diluted hematoxylin and coverslipped with Entellan (Merck, USA) for microscopic observation.

3.3.3. Quantification of c-fos staining

The neuronal activation of the OFC (dlOFC, lOFC, vOFC and mOFC), mPFC (ACC, PLC and ILC) and amygdala (CeA and BLA) were outlined according to the rat brain atlas of Paxinos and Watson (2007) and matched as closely as possible based on landmarks and cytoarchitectural differences. The distance of the brain regions counted from bregma ranged

from: 5.64mm to 3.24mm for vOFC and mOFC; 5.64mm to 4.20mm for mOFC; 5.16mm to 4.68mm for dlOFC; 4.20mm to 2.52mm for ACC; 5.16mm to 2.52mm for PLC; 3.72mm to 2.52mm for ILC; and -1.56mm to -2.64mm for CeA and BLA. The quantification was done by sampling each of the regions selected using counting frames superimposed over the region. The size of the counting frames was 0.0025mm^2 and the percentage sampled with respect to the total area of the brain regions selected was 20%. c-fos positive cells were identified on the basis of a brown reaction product confined to cell nucleus, thus presenting a circular/oval shape with a clear brown staining. Counting of c-fos positive nuclei was performed using Visiopharm Integrator System software (Visiopharm, Copenhagen, Denmark) and a camera (PL- A622, Ontario, Canada) coupled to a motorized microscope (BX51TF, Olympus, Tokyo, Japan). Finally, cell density (c-fos positive nuclei per mm^2) for each brain area was calculated.

3.4. Data analysis

Statistical analysis was conducted using GraphpPad Prism 5 and IBM SPSS Statistics 19. Normality of all data sets was assessed using Kolmogorov-Smirnov test. Since equal variance or normality was assumed (data not shown), parametric tests were used throughout the analysis of the behavioral data. Differences between two groups were compared using Student's t-test or analysis of variance (ANOVA) for comparing more than two groups. Regarding behavior results from locomotor activity, 5-CSRTT and DD task, data was subjected to two-way repeated measures ANOVA. Significant effects produced from analysis were compared via post hoc Bonferroni's t test. For results from the histological analysis, normality of variances was not assumed and nonparametric tests were used (Mann-Whitney for comparing two groups). All data are presented as mean ± standard error (SEM) and, for each CONT and iuGC group, n represents the number of animals used. Differences were considered to be statistically significant when $p \leq 0.05$.

Chapter 4 – Results

4. Results

4.1. Prenatal exposure to GCs does not alter locomotor reactivity to a novel environment

Adult male progeny of dams treated with either 1mg Kg^{-1} of DEX (iuGC) or vehicle (CONT) on gestation days 18 and 19 were used. To evaluate novelty reactivity, the number of ambulatory counts was compared between groups (CONT and iuGC) in an open field apparatus. As shown in Figure 4, both groups decreased the number of ambulatory counts throughout the 120min of testing.

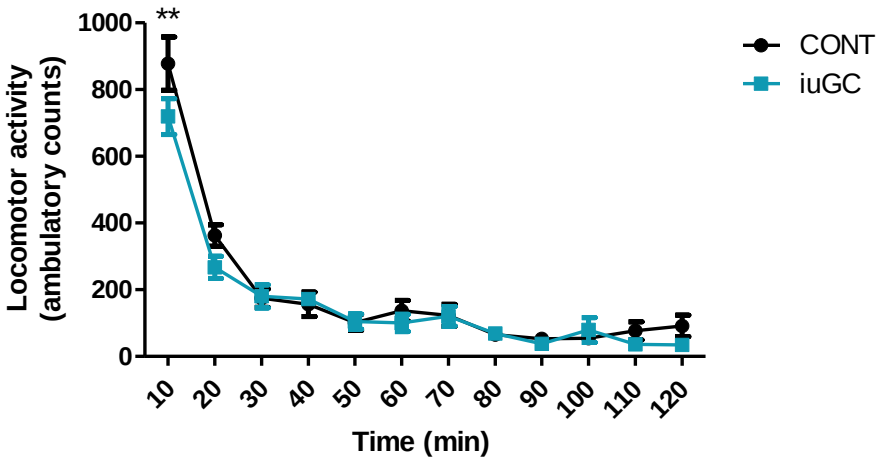

Figure 4. Novelty-induced locomotor reactivity in animals prenatally exposed to GCs. CONT and iuGC subjects did not present major differences in their locomotor response to a novel inescapable environment, with the exception of the first 10min in which iuGC had decreased ambulatory counts. Locomotor activity is represented as the mean of ambulatory counts, performed during the test session. Error bars represent SEM. n=24/group. $^{**}p<0.01$.

Repeated measures analysis revealed no effect of group ($F_{(1,\ 473)} = 2.235$, $p = 0.142$). However, for the first 10min of testing, CONT group showed an increased locomotor activity, or ambulatory counts, in comparison to the iuGC group (Bonferroni corrected t-test: $t = 3.472$, $p < 0.01$).

Additional parameters were analyzed to assess the effect of prenatal exposure to GCs in locomotor reactivity, namely the number of rearings and percentages of distance and time. iuGC animals did not present any differences in the total number of rearings ($t_{(41)} = 1.287$, $p = 0.205$; Figure 5A) nor in the assessment of this parameter along the 120min of the trial ($F_{(1,\ 440)} = 2.483$, $p = 0.123$; Figure 5B).

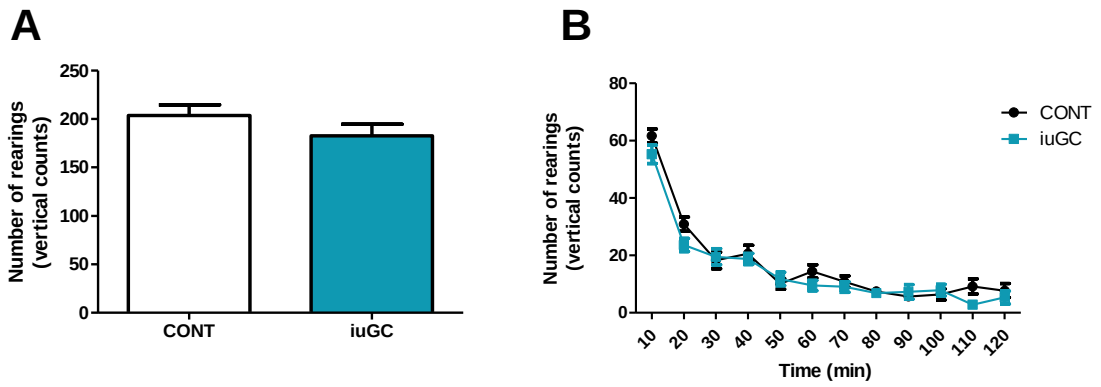

Figure 5. Number of rearings performed during assessment of locomotor activity in an Open Field apparatus. **(A)** Total number of rearings. **(B)** Number of rearings counted during the course of the locomotor reactivity test. No differences were found between groups. Rearings represented as the mean of vertical counts performed during the test session. Error bars represent SEM. n=24/group.

Regarding the percentage of the distance travelled in both central and peripheral areas of the Open Field (OF) arena, both groups displayed an increase in distance in the central area when compared to the peripheral zone ($F_{(3, 79)}$ = 20.91, p < 0.0001; Bonferroni corrected t-test CONT: t = 6.721, p < 0.001; Bonferroni corrected t-test iuGC: t = 4.189, p < 0.001; Figure 6A). Analysis of the percentage of time spent in the central and peripheral areas revealed an effect of GC prenatal treatment ($F_{(3, 79)}$ = 19.70, p < 0.0001; Figure 6B). iuGC animals showed an increase in the time spent in the peripheral area in comparison to the central one (Bonferroni corrected t-test: t = 7.511, p < 0.001; Figure 6B). Additionally, a decrease in the time spent in the central area was found in the iuGC group when compared to the CONT group (Bonferroni corrected t-test: t = 2.822, p < 0.05; Figure 6B) and iuGC animals spent more time in the peripheral area than the CONT group (Bonferroni corrected t-test: t = 2.822, p < 0.05; Figure 6B).

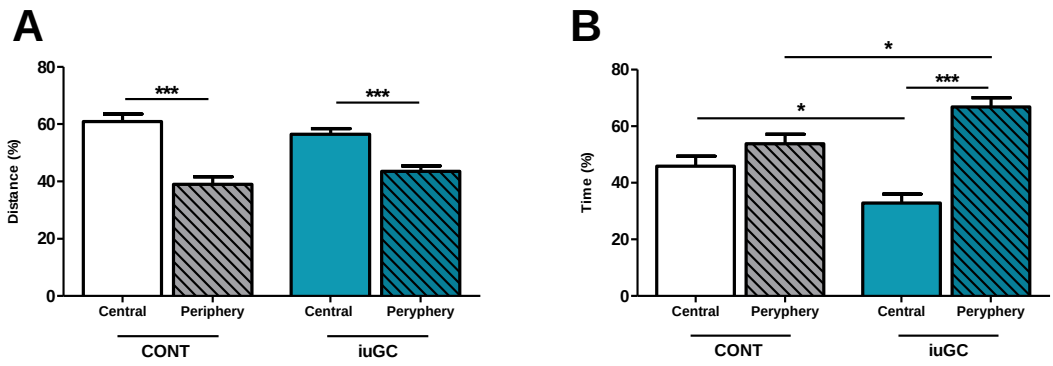

Figure 6. Parameters of locomotion and exploratory activity in animals prenatally exposed to GCs. **(A)** Percentage of distance travelled in central and peripheral areas of the Open Field arena. Both groups display an increase in the distance travelled in the central area. **(B)** Percentage of time spent in central and peripheral areas. No differences were found between groups. iuGC animals spend less time in the central area than CONT animals. Error bars represent SEM. n=24/group.

4.2. Effects of prenatal GC exposure in novelty-seeking behavior

Animals were subjected to Novelty Place Preference (NPP) and Novel Object Recognition (NOR) tests to further assess novelty-seeking behavior. Regarding NPP, ANOVA analysis revealed differences between groups ($F_{(3, 132)} = 5.411$, $p = 0.0015$; Figure 7). CONT animals explored the novel compartment for a significantly longer period of time when compared with the time spent exploring the familiar compartment, which was not observed in the iuGC group (Bonferroni corrected t-test; CONT: $t = 3.640$, $p < 0.01$; iuGC: t-test $t = 1.698$, $p > 0.05$; Figure 7). No significant differences were found between both groups in the total time spent in the novel or the familiar compartment.

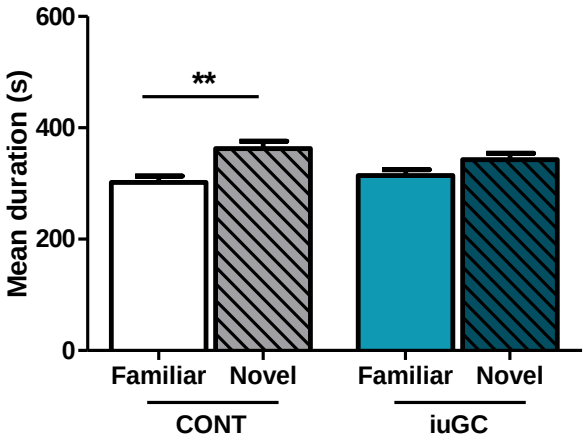

Figure 7. Preference of CONT and iuGC animals for the novel and familiar compartments. CONT animals spent more time exploring the novel compartment in comparison to the familiar one, contrary to iuGC animals that do not present differences, as they spend the same amount of time in both compartments. Bars represent the mean duration of time spent in each compartment ± SEM. n_{CONT}=35; n_{iuGC}=39. ***p*<0.01. (Familiar vs. Novel compartment)

Taking into account the flexibility of the NOR test, animals performed two versions of the test that comprised a delay time of 1h and 24h between phases, allowing an assessment of the condition of the short-term memory and long-term memory, respectively. Concerning the NOR protocol with a 1h delay time, during initial sampling phase, both CONT and iuGC groups spent the same amount of time exploring the object (t_{71} = 0.3536, p = 0.725; Figure 8A). Moreover, both CONT and iuGC animals explored the novel object, during choice phase, for a significantly longer period of time than the familiar object ($F_{(3, 134)}$ = 26.28, p < 0.0001; Bonferroni corrected t-test CONT: t = 8.436, p < 0.001; Bonferroni corrected t-test iuGC: t = 2.722, p < 0.05; Figure 8B), though this was more evident in the control group.

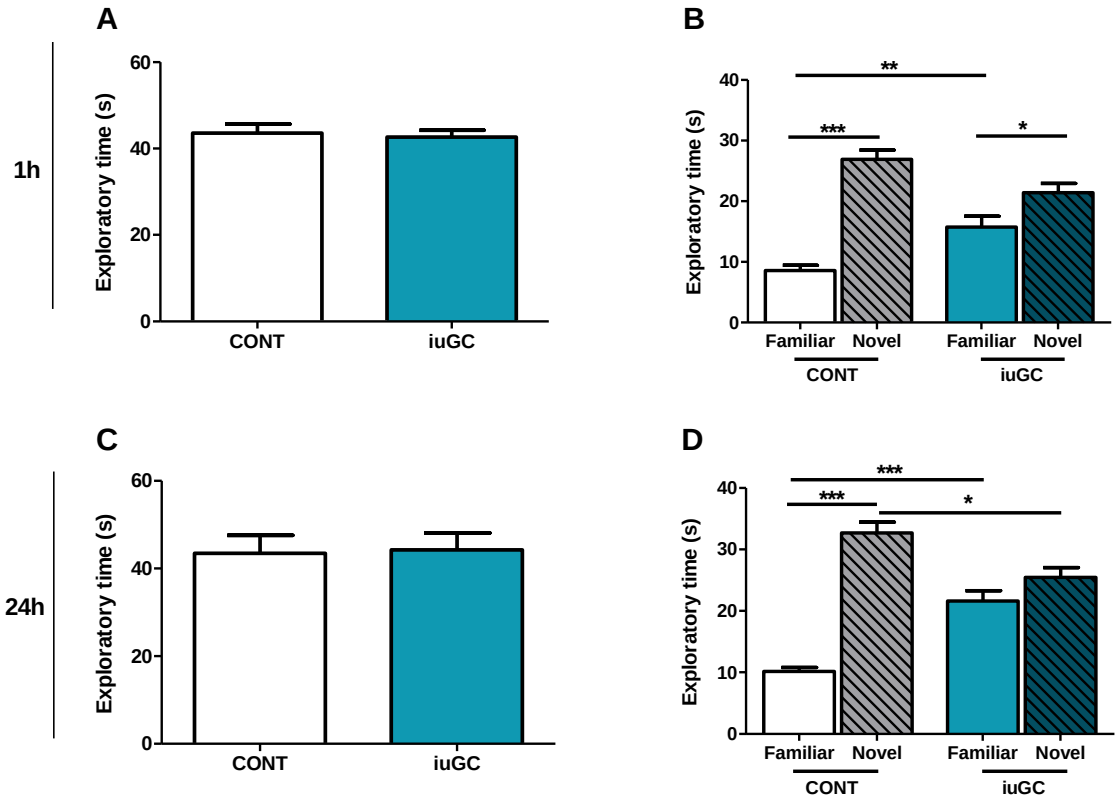

Figure 8. Effect of prenatal exposure to GCs on two versions of a novel object recognition test. **(A)** Total time exploring the object in the sample phase of the NOR test with a 1h delay time between phases. **(B)** Novel object recognition with a 1h delay time between phases. Both CONT and iuGC animals spent more time exploring the novel object, in the choice phase. **(C)** Total time exploring the object in the sample phase of the NOR test with a 24h delay time between phases. **(D)** Novel object recognition with a 24h delay time between phases. In the choice phase, only CONT animals spent more time exploring the novel object, indicating a disruption of NOR in iuGC animals. Bars represent means ± SEM. **(A), (B)** n_{CONT}=35; n_{iuGC}=39; **(C), (D)** n_{CONT}=11; n_{iuGC}=15. *p<0.05, **p<0.01, ***p<0.001. (Familiar vs. Novel object)

As presented in Figure 8C, during the sample phase of the 24h delay version of the NOR test, no differences were observed for both groups (t_{24} = 0.1301, p = 0.898). ANOVA analysis showed a significant difference between groups ($F_{(3, 36)}$ = 33.99, p <0.0001; Figure 8D), in the choice phase, with CONT animals spending significantly longer period of time exploring the novel object than the familiar one (Bonferroni corrected t-test: t = 9.831, p < 0.001; Figure 8D). However, iuGC animals did not differ in their time exploring either object (Bonferroni corrected t-test: t = 1.850, p > 0.05; Figure 8D), indicating a disruption in novel object recognition and, possibly, long-term memory impairment. Also, significant differences between CONT and iuGC in the time exploring the familiar and novel objects was detected, during the choice phase (Bonferroni corrected t-test Familiar: t = 5.253, p < 0.001; Bonferroni corrected t-test Novel: t = 3.303, p < 0.05; Figure 8D).

4.3. Impulsive choice but not impulsive action is altered in animals exposed to prenatal GCs

The characterization of traits, such as drug-seeking behavior, has been associated with impulsiveness, besides novelty-seeking behavior. Considering the complexity of such feature, a 5-Choice Serial Reaction Time Task (5-CSRTT) and a Delay Discounting (DD) protocol were performed, in order to assess the two dimensions of impulsive trait: impulsive action and impulsive choice, respectively.

For the purpose of this thesis, in the 5-CSRTT, we evaluated the anticipatory responses, omissions and accuracy levels, to further assess impulsivity/impulsive action (or the ability to withhold a response) and the level of motivation and attention, respectively.

No detectable differences in the performance of CONT and iuGC animals during training at each stimulus duration (SD; 60s, 30s, 10s, 2s and 0.5s) were found. In Figure 9, it is depicted the data from the last two sessions of task performance of different SDs (60s, 30s, 10s and 2s) and the last four sessions of training of 0.5s SD. At each SD, there was no significant main effect for group in the percentage of accuracy (Table 1; Figure 9A).

Table 1. Summary table of the 5-Choice Serial Reaction Time Task measurements of percentage of accuracy, percentage of omissions and anticipatory responses. Representation of the statistical analysis (comparison between CONT and iuGC animals).

	Statistics		
	CONT x iuGC (F, *p*)		
	Accuracy	**Omissions**	**Anticipatory responses**
60s	$F_{(1,36)}$ 0.149, 0.702	$F_{(1,36)}$ 3.399, 0.074	$F_{(1,36)}$ 0.7766, 0.384
30s	$F_{(1,36)}$ 0.000, 1.000	$F_{(1,36)}$ 15.58, 0.0004***	$F_{(1,36)}$ 0.3567, 0.554
10s	$F_{(1,36)}$ 1.904, 0.176	$F_{(1,36)}$ 1.833, 0.179	$F_{(1,36)}$ 3.492, 0.070
2s	$F_{(1,36)}$ 0.8588, 0.360	$F_{(1,36)}$ 2.052, 0.161	$F_{(1,36)}$ 0.05207, 0.821
0.5s	$F_{(1,72)}$ 2.4747, 0.102	$F_{(1,72)}$ 1.470, 0.229	$F_{(1,72)}$ 0.06909, 0.793

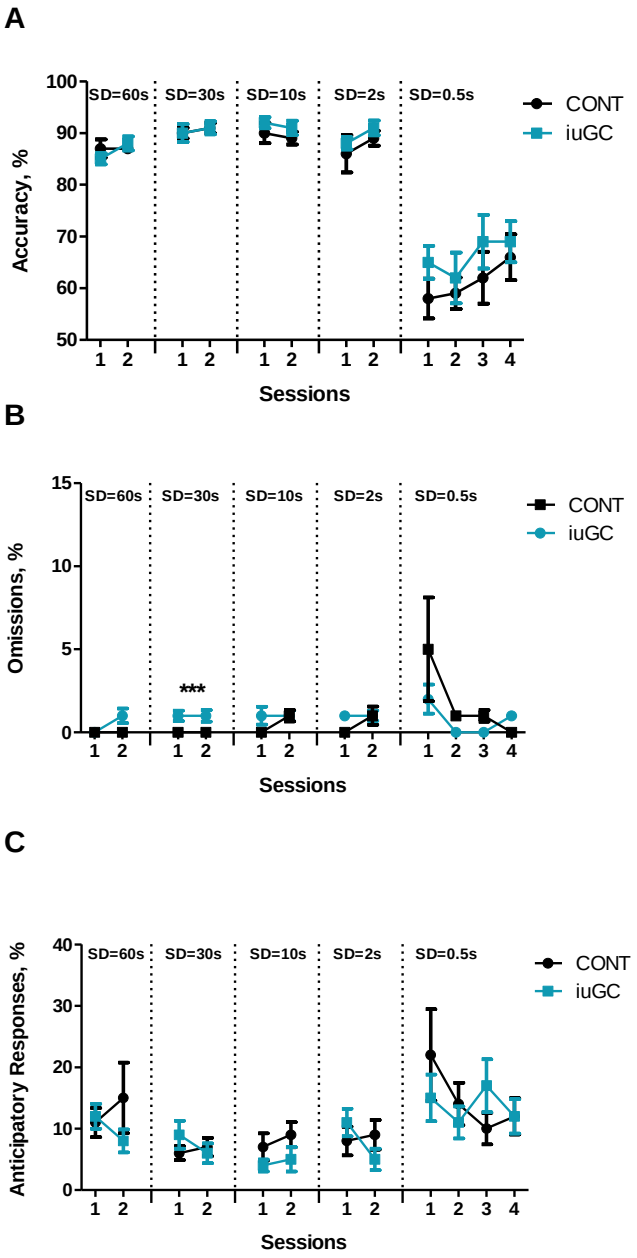

Figure 9. Performance of CONT and iuGC animals in the 5-Choice Serial Reaction Time Task. Shown is the different stages of training with stimulus durations of 60s, 30s, 10s, 2s and 0.5s, for 3 different measurements: **(A)** Percentage of accuracy (number of correct responses/total number of responses); **(B)** Percentage of omissions (number of trials that resulted in no response); **(C)** Percentage of anticipatory responses (number of responses in the apertures during the ITI state). All measurements showed no major differences between groups, except during SD of 30s in **(B)**. Results are shown as means ± SEM. n_{CONT}=10; n_{iuGC}=10. ***p<0.001.

Regarding the percentage of omissions, no significant differences were found within groups (Table 1; Figure 9B). However, this measurement showed a statistically significant effect for training with a 30s SD between both groups (Table 1; Figure 9B). Despite this early difference between groups, as training progressed, that same effect disappeared, leading to a similar

response between groups. The final behavioral parameter measured in the 5-CSRTT was anticipatory or premature responses, where ANOVA analysis revealed no significant differences between both CONT and iuGC animals (Table 1; Figure 9C).

In order to assess impulsive choice, animals performed a delay discounting task. As time progresses, a difference between the CONT and iuGC group emerges (Figure 10). Animals from both groups decreased their preference for a delayed, large, reward in a delay dependent way (Delay: Figure 10A: $F_{(3, 72)} = 10.63$, $p < 0.0001$; Figure 10B: $F_{(3, 72)} = 23.07$, $p < 0.0001$; Figure 10C: $F_{(3, 72)} = 25.41$, $p < 0.0001$; Figure 10D: $F_{(3, 72)} = 41.16$, $p < 0.0001$). No effect of GC treatment on impulsive choice was found in the first 5 sessions (Group: Figure 10A: $F_{(1, 72)} = 1.766$, $p = 0.1881$; Group x Delay: Figure 10A: $F_{(3, 72)} = 0.03458$, $p = 0.9913$). From sessions 6 to 21, the performance of both groups is different. iuGC animals present increased lever presses in the large reward lever, in the no-delay condition (first block of trials) when compared to CONT animals (Group: Figure 10B: $F_{(1, 72)} = 5.082$, $p = 0.0272$; Figure 10C: $F_{(1, 72)} = 2.711$, $p = 0.1040$; Figure 10D: $F_{(1, 72)} = 9.683$, $p = 0.0027$).Further post hoc analysis confirmed the initial difference shown between CONT and iuGC animals, when no delay was present (Figure 10B: Bonferroni corrected t-test: $t = 2.917$, $p < 0.05$; Figure 10D: Bonferroni corrected t-test: $t = 4.309$, $p < 0.001$). Despite initial differences, iuGC animals present a steeper discounting curve when compared to CONT animals, suggesting decreased delay gratification.

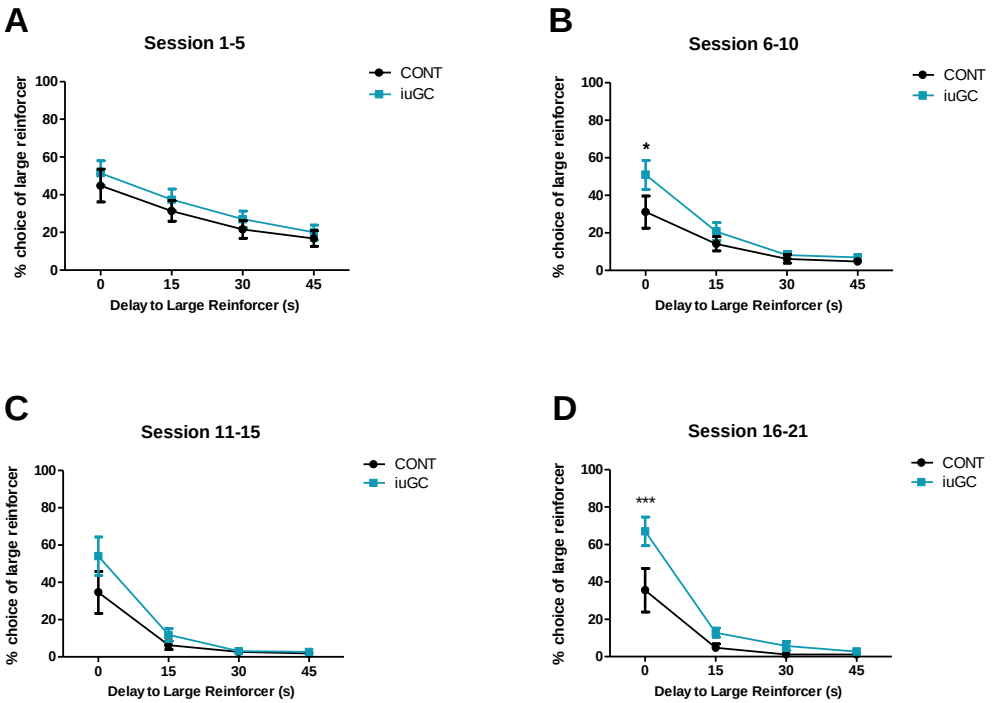

Figure 10. Performance in the delay discounting task of control and iuGC animals. **(A)** Choice percentage of the large reward across delays for CONT and iuGC animals, for the first 5 sessions. No differences found between groups. **(B)** Choice percentage of the large reward across delays in sessions 6-10, where, for the first block of trials (no delay block) iuGC animals had increased preference for the large reward, when compared to CONT animals. **(C)** Sessions 11-15 revealed no differences between groups in the percentage of preference for the large reinforcer. **(D)** Choice percentage of the large reward across delays in sessions 16-21. iuGC animals had increased preference for the large reinforcer in the first block of trials, when compared to CONT. Data is shown as means ± SEM. n_{CONT}=10; n_{iuGC}=10. *p<0.05, ***p<0.001.

In order to further dissect the DD paradigm and the results obtained, a version of the DD task with shorter delays was performed (delay times: 0s, 5s, 10s, 15s and 30s; Figure 11). Results from this delay discounting paradigm showed that, as seen in the previous test, both CONT and iuGC animals delay-dependently decreased their choice for a large, but delayed, reward (Delay: Figure 11A: $F_{(4, 90)}$ = 23.98, p < 0.0001; Figure 11B: $F_{(4, 90)}$ = 25.98, p < 0.0001; Figure 11C: $F_{(4, 90)}$ = 31.03, p < 0.0001; Figure 11D: $F_{(4, 90)}$ = 21.83, p < 0.0001; Figure 11E: $F_{(4, 90)}$ = 33.36, p < 0.0001). Until session 16 and beyond, no significant difference was found between both groups in the percentage of choice for the large delayed reward (Group: Figure 11A: $F_{(1, 90)}$ = 0.06812, p = 0.7947; Figure 11B: $F_{(1, 90)}$ = 0.3765, p = 0.5410; Figure 11C: $F_{(4, 90)}$ = 0.2071, p = 0.6501; Group x Delay: Figure 11A: $F_{(4, 90)}$ = 1.196, p = 0.3183; Figure 11B: $F_{(4, 90)}$ = 1.551, p = 0.1942; Figure 11C: $F_{(4, 90)}$ = 1.863, p = 0.1239), though there is a clear trend for iuGC animals to prefer the large reward lever in the no-delay block.

With further training, iuGC animals displayed an increased preference for the lever associated with a larger reinforcer when compared to the CONT group, in the no-delay condition.

Both groups were different in a delay-dependent manner (Group: Figure 11D: $F_{(1, 90)} = 0.2178$, $p = 0.6419$; Figure 11E: $F_{(1, 90)} = 1.612$, $p = 0.2075$; Group x Delay: Figure 11D: $F_{(4, 90)} = 3.494$, $p = 0.0106$; Figure 11E: $F_{(4, 90)} = 3.394$, $p = 0.0124$). Additional post hoc analysis further supported the difference between both animal groups (Figure 11D: Bonferroni corrected t-test: t = 3.355, $p < 0.01$; Figure 11E: Bonferroni corrected t-test: t = 3.566, $p < 0.01$). Despite the shorter delays applied in this task, iuGC animals revealed a steeper discount for the large reward than CONT animals, immediately after introduction of a delay of 5 s, suggesting a disruption in this type of behavior.

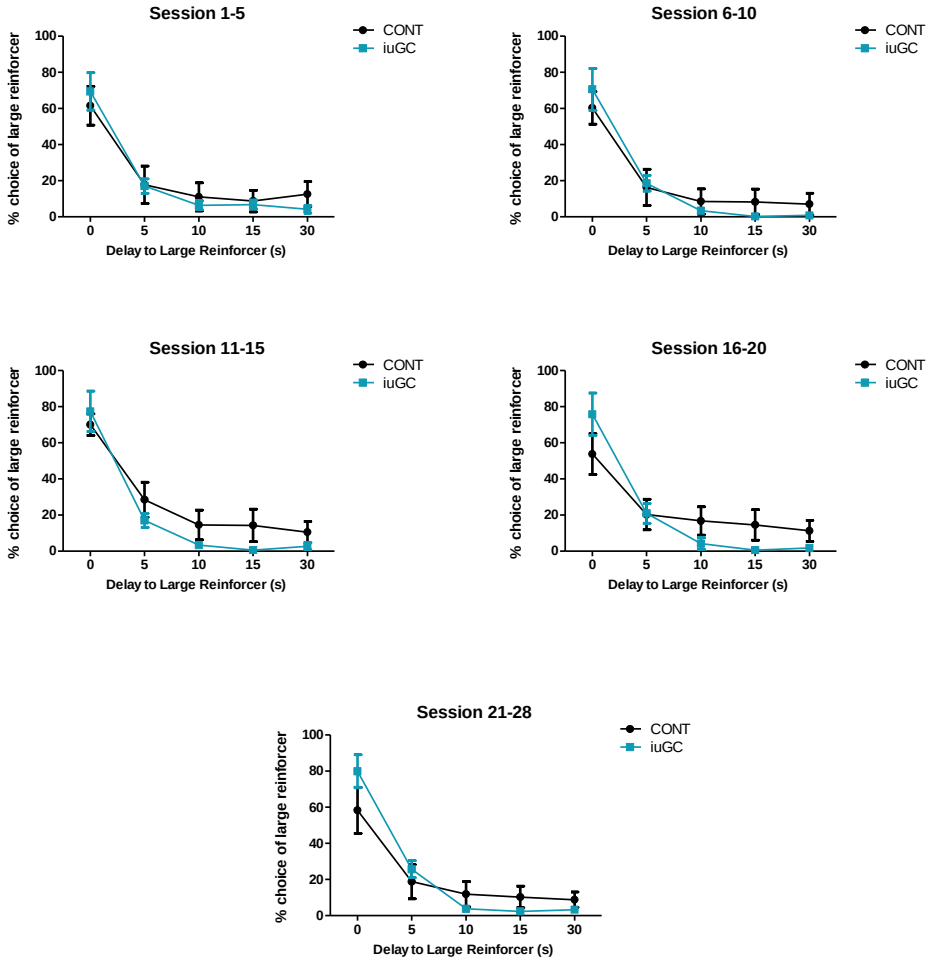

Figure 11. Performance in the delay discounting task of control and iuGC animals, with shorter delay intervals. **(A)** Choice percentage of the large reward across delays for CONT and iuGC animals, for the first 5 sessions. No differences were found between groups. **(B)** Choice percentage of the large reward across delays in sessions 6-10, where, also, no differences were revealed between iuGC animals and CONT animals. **(C)** Sessions 11-15 revealed no differences between groups in the percentage of preference for the large reinforcer. **(D)** Choice percentage of the large reward across delays in sessions 16-20, here, iuGC animals had increased preference for the large reinforcer in the first block of trials, when compared to CONT. **(E)** Sessions 21-28 showed that iuGC animals had increase preference for the large reinforcer, when compared to CONT animals. Results are represented as means ± SEM. $n_{CONT}=10$; $n_{iuGC}=10$. $^{**}p<0.01$.

4.4. Neuronal activation upon exposure to a delay discounting task

Several brain regions have been associated with the DD task, such as the OFC, mPFC and the amygdala (Cardinal, 2006). The expression of the immediate early gene c-fos was evaluated as an indicator of regional activation, after DD performance. Animals were subdivided in each group: part of the animals performed the task as described in the previous chapter (task+) and another sub-group did not perform the task, but were allowed to eat food pellets until they were satiated in the operant chamber (task-). Representative photomicrographs from OFC, mPFC and amygdala are displayed in Figures 12, 14 and 16, respectively. The densities of c-fos positive nuclei (positive cell counts/mm^2) in all areas of interest in the four groups of animals are shown in Figures 13, 15 and 17.

Expression of c-fos was detected throughout the areas of interest of CONT and iuGC animals at 60min following initiation of DD task/placement in operant chamber, after 28 days of training. Animals presented neuronal activation of the 4 sub-regions of OFC (Figures 12 and 13). In the lOFC, animals that performed the task had an increase in c-fos reactivity when compared to the non-performing animals (CONT task- x CONT task+: Mann-Whitney U = 0.000, $p = 0.0159$; iuGC task- x iuGC task+: Mann-Whitney U = 1.000, $p = 0.0190$; Figure 13A).

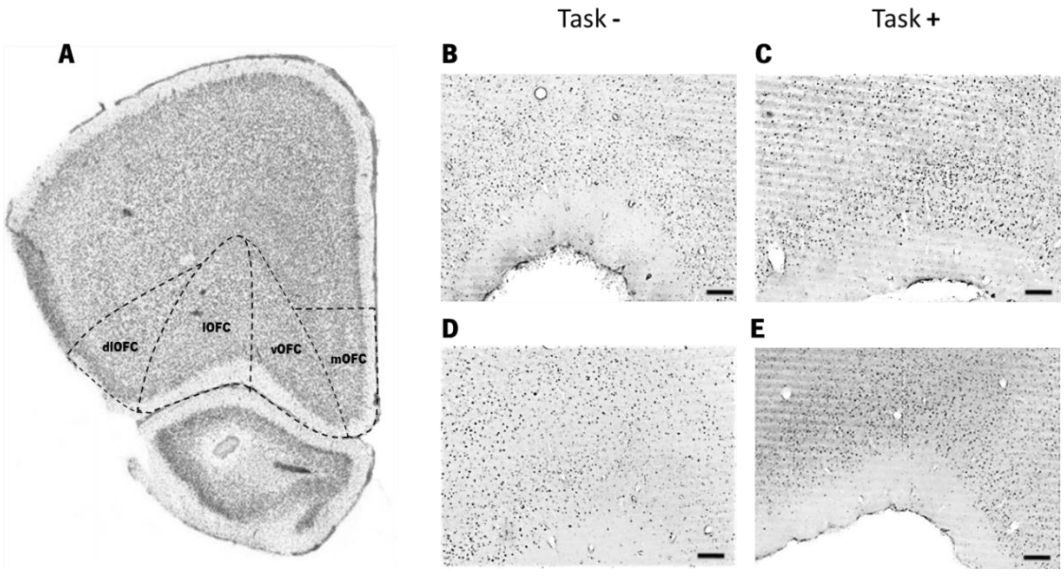

Figure 12. Neuronal activation upon delay discounting task performance in the orbitofrontal cortex. **(A)** Boundaries between different sub-regions of OFC traced over brain slices correspond to the outlined counting regions (Adapted from Paxinos and Watson, 2007). **(B)** Photomicrograph from a representative CONT animal that did not perform the DD task, showing part of the OFC sub-regions and the c-fos expression (dark points). **(C)** Photomicrograph from a representative CONT animal that performed the DD task, showing representative c-fos expression. Photomicrograph from a representative iuGC animal that did not perform the DD task **(D)** or that performed DD task **(E)**. Note the greater number of c-fos positive units in the animals that performed the task. dlOFC - dorsolateral orbitofrontal cortex; lOFC - lateral orbitofrontal cortex; vOFC - ventral orbitofrontal cortex; mOFC - medial orbitofrontal cortex; regions coordinates: 4.20mm from bregma for all OFC sub-regions. Bar = 100 µm.

In the vOFC (Figure 13B), iuGC task+ animals displayed a greater c-fos positive cell density count than iuGC task- animals (Mann-Whitney U = 0.000, p = 0.0238), whereas CONT task- and CONT task+ groups presented no statistically significant differences (Mann-Whitney U = 2.000, p = 0.1429).

On the contrary, statistical analysis of neuronal activation in the dlOFC revealed an increase in cell density of c-fos positive cells in the CONT task+ group, when compared to CONT task- animals (Mann-Whitney U = 1.000, p = 0.0317) and no differences between iuGC task- and iuGC task+ groups (Mann-Whitney U = 5.000, p = 0.2857), as displayed in Figure 11C. However, there is a tendency for an enhanced activation of the dlOFC area in the iuGC task- group than in the CONT task- group (Mann-Whitney U = 1.000, p = 0.0571; Figure 13C).

Regarding the mOFC (Figure 13D), again, iuGC task+ animals showed a greater density in the c-fos positive cell population count than iuGC task- animals (Mann-Whitney U = 0.000, p = 0.0238) and no differences were revealed between both CONT groups (Mann-Whitney U = 4.000, p = 0.2619).

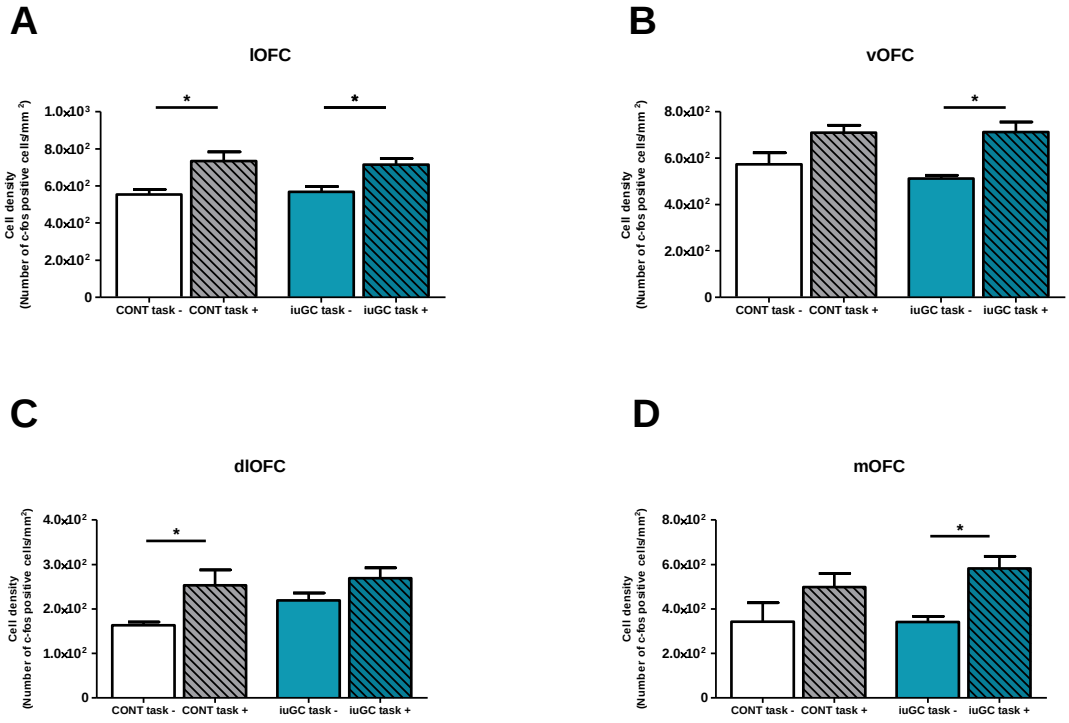

Figure 13. Density of c-fos positive cells counted in OFC sub-regions, upon delay discounting task. **(A)** Both CONT and iuGC task+ animals had an increase in the c-fos positive cell density in the lOFC, when compared to CONT and iuGC task- animals. **(B)** In the vOFC, only iuGC task+ animals showed a significant increase in the density of c-fos positive cells. **(C)** CONT task+ animals show an increase in neuronal activation in the dlOFC. **(D)** In the mOFC, only iuGC task+ animals show a significant increase in neuronal activation. Data is shown as means ± SEM. $n_{CONT\ task\ -}$=4; $n_{CONT\ task\ +}$=6; $n_{iuGC\ task\ -}$=4; $n_{iuGC\ task\ +}$=6. *p<0.05. dlOFC - dorsolateral orbitofrontal cortex; lOFC - lateral orbitofrontal cortex; vOFC - ventral orbitofrontal cortex; mOFC - medial orbitofrontal cortex. CONT/iuGC task- represents animals that did not perform delay discounting task; CONT/iuGC task+ represents animals that did perform delay discounting task.

Neuronal activation in mPFC sub-regions (ACC, PLC and ILC) was also examined (Figures 14 and 15). The ACC sub-region showed greater c-fos positive cell density in the iuGC task+ group, when compared to iuGC task+ animals (Mann-Whitney U = 0.000, p = 0.0286; Figure 15A). This difference was not noted in the CONT sub-groups (Mann-Whitney U = 3.500, p = 0.1383; Figure 15A). Also, a decrease in cell density was noted in the iuGC task- group, comparing to CONT task- animals (Mann-Whitney U = 0.000, p = 0.0294; Figure 15A).

Regarding the results from the PLC (Figure 15B), animals in the CONT task+ group had an increase in c-fos positive cell density, when compared to the CONT task- group (Mann-Whitney U = 0.500, p = 0.0262), whereas both iuGC task- and iuGC task+ groups showed no differences (Mann-Whitney U = 5.000, p = 0.3810).

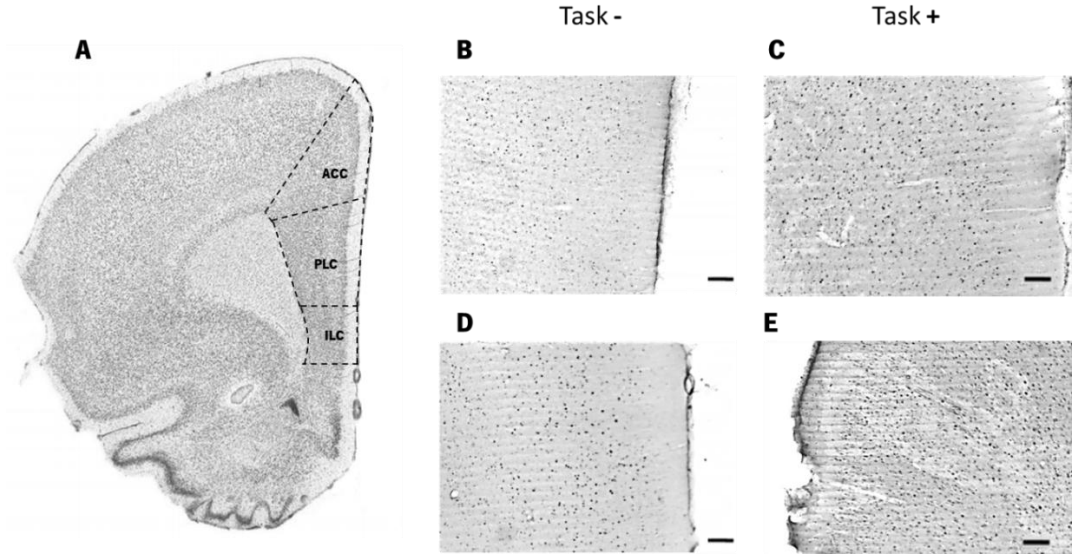

Figure 14. Neuronal activation upon delay discounting task performance in the medial prefrontal cortex. **(A)** Boundaries between different sub-regions of mPFC traced over brain slices correspond to the outlined counting regions (Adapted from Paxinos and Watson, 2007). **(B)** Photomicrograph from a representative CONT animal that did not perform the DD task, showing part of the mPFC sub-regions and the c-fos expression (dark points). **(C)** Photomicrograph from a representative CONT animal that performed the DD task, showing representative c-fos expression. Photomicrograph from a representative iuGC animal that did not perform the DD task **(D)** or that performed DD task **(E)**. Note the greater number of c-fos positive units in the animals that performed the task. ACC - anterior cingulate cortex; PLC - prelimbic cortex; ILC - infralimbic cortex; regions coordinates: 3.24mm from bregma for all mPFC sub-regions. Bar = 100 µm.

As in the previous sub-region, in the ILC, the CONT task+ group had an increase in c-fos positive cell density, when compared to the CONT task- group, (Mann-Whitney U = 0.000, p = 0.0195; Figure 15C), whereas this was not observed with iuGC animals (Mann-Whitney U = 7.000, p = 0.3524; Figure 15C).

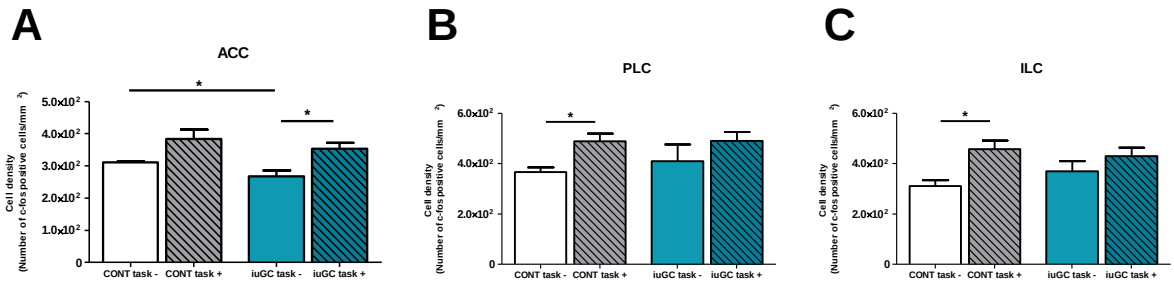

Figure 15. Density of c-fos positive cells in mPFC sub-regions, 60min after delay discounting task. **(A)** iuGC task+ animals had an increase in the c-fos positive cell density in the ACC and CONT task- animals show greater cell density counts that iuGC task- animals. **(B)** In the PLC, only CONT task+ animals show a significant increase in the density of c-fos positive cells. **(C)** CONT task+ animals show an increase in neuronal activation in the ILC. Data is shown as means ± SEM. $n_{CONT\ task\ -}$=4; $n_{CONT\ task\ +}$=6; $n_{iuGC\ task\ -}$=4; $n_{iuGC\ task\ +}$=6. *p<0.05. ACC - anterior cingulate cortex; PLC - prelimbic cortex; ILC - infralimbic cortex. CONT/iuGC task- represents animals that did not perform delay discounting task; CONT/iuGC task+ represents animals that did perform delay discounting task.

Then, the two sub-regions of the amygdala (BLA and CeA) were evaluated (Figures 16 and 17). Density of c-fos positive cells examination was different between CONT task- and CONT task + groups in the BLA (Mann-Whitney U = 0.000, p = 0.0358; Figure 17A). Similarly, iuGC task+ animals presented an increase in neuronal activation, comparing to iuGC task- animals (Mann-Whitney U = 1.000, p = 0.0247; Figure 17A). Also, a higher neuronal activation in this sub-region was noted in the iuGC task- group, when compared to the CONT task- group (Mann-Whitney U = 0.000, p = 0.0498; Figure 17A).

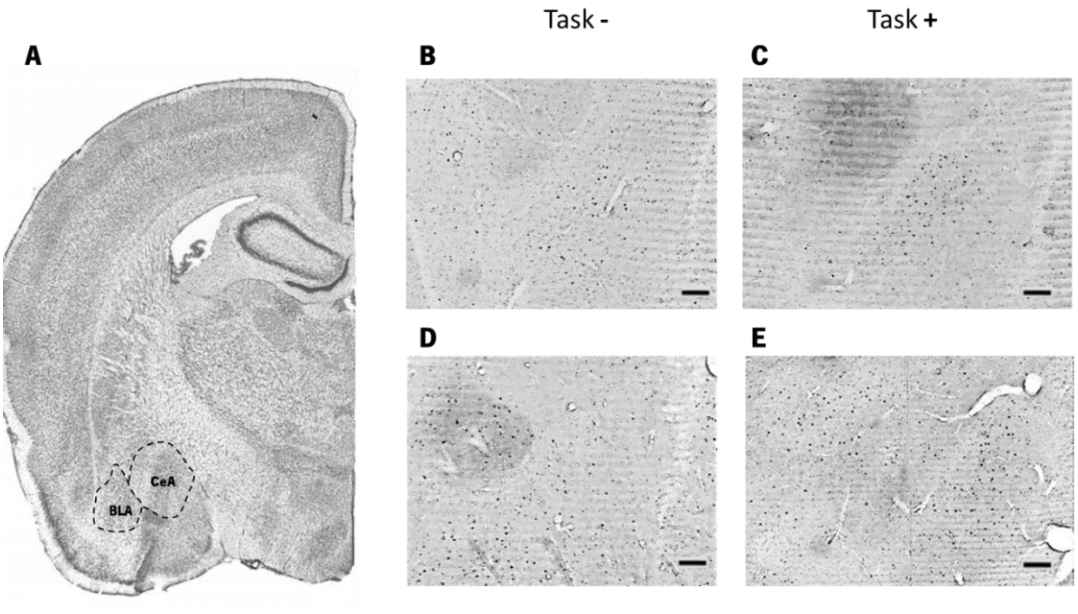

Figure 16. Neuronal activation in the amygdala, coming after delay discounting task performance. **(A)** Boundaries between different sub-regions of the amygdala traced over brain slices correspond to the outlined counting regions (Adapted from Paxinos and Watson, 2007). **(B)** Photomicrograph from a representative CONT animal that did not perform the DD task, showing part of the amygdala sub-regions and the c-fos expression (dark points). **(C)** Photomicrograph from a representative CONT animal that performed the DD task, showing representative c-fos expression. Photomicrograph from a representative iuGC animal that did not perform the DD task **(D)** or that performed DD task **(E)**. Note the greater number of c-fos positive units in the animals that performed the task. BLA – basolateral nucleus of the amygdala; CeA – central nucleus of the amygdala; regions coordinates: -2.28mm from bregma for the two amygdala sub-regions. Bar = 100 μm.

Concerning the density of c-fos positive cells in the CeA, both CONT task+ and iuGC task+ display an increase, when compared to CONT task- and iuGC task- animals, respectively (CONT task- x CONT task+: Mann-Whitney U = 0.000, p = 0.0358; iuGC task- x iuGC task+: Mann-Whitney U = 0.000, p = 0.0139; Figure 17B), suggesting a similar activation between both groups.

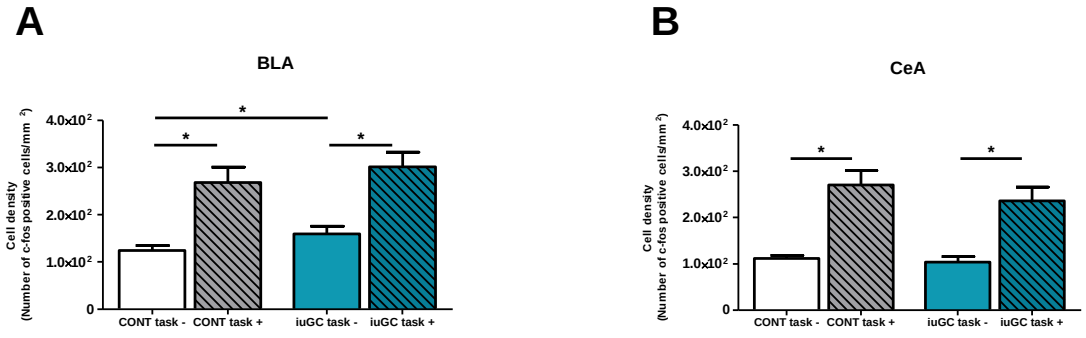

Figure 17. Density of c-fos positive cells counted in amygdala sub-regions, coming after delay discounting task. **(A)** iuGC task+ and CONT task+ animals had an increase in the c-fos positive cell density in the BLA and iuGC task- animals show greater cell density counts that CONT task- animals. **(B)** In the CeA, CONT task+ and iuGC task+ animals show a significant increase in the density of c-fos positive cells. Data is shown as means ± SEM. $n_{CONT\ task\ -}$=4; $n_{CONT\ task\ +}$=6; $n_{iuGC\ task\ -}$=4; $n_{iuGC\ task\ +}$=6. *p<0.05. BLA - basolateral amygdala; CeA - central amygdala. CONT/iuGC task- represents animals that did not perform delay discounting task; CONT/iuGC task+ represents animals that did perform delay discounting task.

Chapter 5 – General Discussion

5. General Discussion

Impact of stress in early development

During prenatal period, the fetus is protected from the effects of elevated GCs due to the action of the enzyme 11 -β-HSD-2, which is highly expressed in the placenta and forms a potent barrier to maternal GCs (Seckl, 2004). However, a small quantity of endogenous GCs is able to cross the barrier and reach the fetus (Brown *et al.,* 1996). This 'leakage' is particularly important since GCs are responsible for neuronal maturation, remodeling of axons and dendrites, as well as cell survival (Meyer, 1983; Meaney *et al*, 1996; McEwen, 2005; Cerqueira *et al.*, 2005; Dias-Ferreira *et al.*, 2009). The balance in the production of GCs allows normal brain development, so prolonged exposure to stress/GCs can alter the neuronal patterns/circuitry of the developing brain. Indeed, studies have shown that offspring of mothers that received DEX treatment during the prenatal period, developed emotional and social problems (Trautman *et al.* 1995). Also, derived from this hormone treatment, the offspring presented low birth weight, altered cardiovascular, endocrine and metabolic functions (French *et al.,* 1999). Nonetheless, the beneficial effects of prenatal GCs are undeniable and these hormones are widely used in cases of risk of preterm delivery, to promote fetal lung maturation.

Our group showed permanent deleterious effects of prenatal exposure to stress/GCs in the periphery and the brain (Oliveira *et al.,* 2006; Leão *et al.,* 2007; Rodrigues *et al.,* 2011; Roque *et al.,* 2011; Oliveira et al., 2012). This animal model of prenatal exposure to DEX showed an impairment of the HPA axis response; behavioral studies demonstrated that these animals developed an anxious-like phenotype and a depressive-like behavior. Furthermore, studies revealed that the mesolimbic dopaminergic system is a target of prenatal GCs action (Leão *et al.,* 2007; Rodrigues *et al.,* 2011), since the VTA, which projects to several areas including the NAcc and PFC, displays a decrease in dopaminergic fibers (Leão *et al.*, 2007). The alterations seen in the VTA and the hypodopaminergic state developed in the NAcc (Leão *et al.*, 2007; Rodrigues *et al.*, 2011) were associated with an increase in the vulnerability towards drug-seeking behavior in the offspring of DEX-treated dams (Rodrigues *et al.*, 2011). These results are in accordance with those showing that dopamine (DA) is crucial for the motivating actions to achieve certain goals, eventually developing drug-seeking behavior (Robinson and Berridge, 2003; Berridge and Robinson, 1998; Palmiter, 2008).

Personality traits and addictive behavior

Drug addiction is a complex behavioral disorder that can be instigated and perpetuated by stressful life events and predisposing personality characteristics, most notably impulsivity and novelty-seeking behavior. Those who become addicted may be more susceptible to the reinforcing effects of drugs or to the learned associations related to drug taking (Koob and Kreek, 2007). Furthermore, vulnerability to addiction can be increased by genetic or physiological influences, enhancing the probability of an individual to shift from experimental to compulsive drug use (Nestler *et al*, 2001; Olmstead, 2006; Piazza and Le Moal, 1996). Together, these vulnerabilities may increase the risk of first-time use as well as maintenance and relapse of drug use.

Increasing evidence has revealed a link between personality traits characterized by behavioral disinhibition, such as sensation/novelty-seeking and impulsivity, and the development of substance use disorders (Conway *et al*, 2003; Iacono *et al*, 2003; Kreek *et al*, 2005; Li, 2000; Limosin *et al*, 2003; Young *et al*, 2000). The association between these personality traits and substance abuse is not surprising, since the mesolimbic dopaminergic system, highly implicated in addictive disorders, may also underlie individual differences in trait impulsivity (Bergh *et al*, 1997; Laine *et al*, 2001; Mann *et al*, 2001; Reist *et al*, 1996; Swann, 2003; Wade *et al*, 2000).

Novelty-seeking behavior has been strongly associated with drug addiction (Koob and Le Moal, 2005; Wills *et al,* 1994; Woicik *et al,* 2009; Zuckerman and Neeb, 1979) and has been defined as the amount of activity displayed in a novel environment with animals that exhibit a high level of exploration, namely high responders in comparison with little exploration termed as low responders animals (Piazza *et al*, 1989). The high responder and low responder phenotypes represent differences in vulnerability to substance abuse and not only individual differences in locomotor activity, where high responder animals self-administer cocaine and amphetamine at higher rates (Piazza *et al*, 1989) and exhibit notable differences in their interaction with the environment, since high responder animals tend to seek novelty, variety, and emotional stimulation (Dellu *et al*, 1996; Kabbaj *et al*, 2000). In this manner, novelty-seeking behavior in the rat resembles sensation-seeking in humans (Dellu *et al*, 1996), a personality trait characterized for voluntary participation in activities involving personal risk and associated with substance abuse and alcoholism (Zuckerman and Neeb, 1979). Several paradigms may be used to the novelty-seeking condition, such as novelty-induced locomotor reactivity, Novelty Place Preference (NPP) and Novel Object Recognition (NOR) tests (Dellu *et al,* 1996; Blanchard *et al*,

2009; Molander *et al,* 2011). Regarding the locomotor reactivity in a novel environment, iuGC animals did not differ from their age-matched CONT animals, apart from the first 10 min of analysis and the higher percentage of time spent in the central area of the arena that further supported results from Oliveira *et al.* (2006).

Differences in novelty responsiveness determine the degree of success in differentiating novel and old objects in an open arena, such that more reactive animals are less likely to recognize objects/places because of the training-induced emotional arousal. Based on the spontaneous and natural tendency of animals to explore novelty, we performed the NPP test, where iuGC animals did not explored the novel environment more than the familiar one, since the amount of time spent in each place was almost the same, which reveals a decrease in novelty preference and/or possibly, indicate some sort of memory impairment or unequal memory processing. Concerning the NOR test, iuGC animals presented an increase in the preference to a novel object as opposed to a familiar one, when the delay between test phases was 1 h. However, as this delay increased (24 h between phases), the offspring of DEX-treated dams did not distinguish the familiar and novel object, as opposed their age-matched CONT animals, further suggesting long-term memory impairment. The NOR paradigm is actually a model of spontaneous-episodic memory and not spontaneous-spatial memory (Ennaceur and Delacour, 1988; Ennaceur *et al*, 1989). The task requires animals to remember previously encountered objects after a delay-dependent decline in memory retention, making this test sensitive to cognition-impairing (Ennaceur and Meliani, 1992; Puma *et al*, 1998; Woolley *et al*, 2003) and cognition-enhancing (Ennaceur *et al*, 1989; Puma *et al*, 1998) situations, also allowing pharmacological manipulations, if needed. Therefore it is important to note that this paradigm shows validity in the assessment of attention and memory, alongside novelty preference (Ballaz *et al,* 2007; Molander *et al,* 2010).

Different endophenotypes have been associated with a predisposition to different stages of the addiction process, such as the high responder phenotype, predisposing to drug use, or others, including the high impulsive and high novelty preference phenotypes, facilitating the shift to compulsive drug intake and addiction (Belin *et al,* 2010). It is important to refer the dissociation between novelty preference and novelty-induced locomotor activity, since either pharmacological (Bardo *et al*, 1990) or molecular (Adriani *et al*, 2009) manipulations of novelty preference have been shown not to impact reactivity to a novel environment. Although novelty preference and locomotor reactivity to novelty are both blocked by microinfusions of

dopaminergic antagonists into the NAcc in rats (Bardo *et al*, 1989; Hooks and Kalivas, 1995), these behavioral tests are underlined by dissociable neurobiological mechanisms, including an important role of dopamine receptor 1 (D1) in the expression of novelty preference (Bardo *et al*., 1993). Several studies revealed important implications for the neurobiology of the endophenotypes underlying the risk for drug addiction. Novelty-seeking high responder animals had increases in the DA release in the NAcc (Hooks *et al*, 1992; Flagel *et al,* 2010), a prolonged corticosterone response to mild novelty stress (Dellu *et al,* 1996) and lower levels of dopamine receptor 2 (D2) mRNA, also in the NAcc (Hooks *et al,* 1994). The high impulsive phenotype showed a reduction in dopamine receptors 2 and 3 (D2/3) in the ventral striatum, with no major differences in DA, when compared with the low impulsive phenotype (Dalley *et al,* 2007; Diergaarde *et al,* 2008). Thus, these studies suggest that impulsivity and novelty-seeking have distinct behavior outcomes and may also be associated with different adaptations within the mesolimbic dopaminenergic system (Molander *et al,* 2011).

Impulsivity is characterized by several distinct features including delay aversion, impaired ability to consider consequences of an action, and behavioral disinhibition (Evenden, 1999). Across the lifespan, individuals may vary their degree of impulsivity and high levels of impulsivity can make an individual more susceptible to several psychiatric disorders, such as drug addiction (Olmstead, 2006), which can directly related with impulsive action and choice. Impulsive action reflects the inhibition of the capacity to withhold a response and impulsive choice refers to delay aversion or the inability to delay gratification. One can separate these types of impulsivity and they can be mutually exclusive (Winstanley *et al*, 2006). High baseline levels of either impulsive action or choice increase the risk of initiating, maintaining and relapsing to drug use in humans and animals (Perry and Carroll, 2008; Perry *et al*, 2005). Different human studies showed that cocaine or heroin abusers prefer smaller, immediate rewards over larger but delayed rewards (both drug and non-drug) and displayed impaired inhibition on tasks measuring impulsive action (Fillmore and Rush, 2002; Kirby and Petry, 2004; Li *et al,* 2006). In animal studies, high impulsive rats display greater drug-seeking behavior than low impulsive animals, suggesting that impulsivity influences drug taking behavior (Belin *et al,* 2008). Furthermore, impulsivity predicts the acquisition, escalation, and reinstatement of cocaine taking-behavior in that (Dalley *et al,* 2007; Perry *et al*, 2005; Perry *et al*, 2008), where high impulsive animals displayed greater drug-taking behavior in comparison to low impulsive rats. Thus, impulsivity appears to be a reliable

endophenotype for stimulant abuse in rodents, consistent with recent findings in human drug abusers (Ersche *et al,* 2010).

Impulsive action was assessed through 5-CSRTT, where animals were tested for the anticipatory responses. Additionally, we checked the accuracy and omissions performed, that allowed to assess the level of attention and motivation. In this behavioral test, iuGC animals did not present differences in impulsive action, since their response was quite similar to CONT animals, as well as their motivation and attention levels. Moreover, an impulsive choice paradigm was used, the delay discounting test, where animals were tested on their preference for a small immediate reward rather than a large, more beneficial, but delayed reward. Here, iuGC animals presented a higher preference for the large reward at the moment where no delays were present; however, once a delay was introduced, these animals decrease their preference instantly. Moreover, when a DD test was performed with shorter periods of delay, a similar response was observed, meaning that with an introduction of a delay, iuGC animals shifted their response to preferably choose the small immediate reward. Although evidence suggests an altered performance in the DD test, it is important to refer that CONT and iuGC animals revealed a difference at the starting point of the last sessions of the DD test, which may influence the outcome of the test and biased our conclusions. Is therefore crucial to make adjustments in the DD protocol in order to achieve at least 80% of choice for the large reinforcer.

It is also important to refer that many studies have looked at the pharmacological effects of addictive drugs on measures of impulsivity including response inhibition. Cocaine administration increases impulsive choice in rats (Paine *et al*, 2003), as does acute morphine administration (Kieres *et al*, 2004) and chronic methamphetamine has been shown to increase impulsive choice in rats (Richards *et al*, 1999). It is, however, important to mention that some results for a given drug have not always been consistent. For example, studies showed that alcohol reduced delay discounting in humans (Ortner *et al*, 2003), while others revealed no effect of alcohol on this measure (Richards *et al,* 1999), and several investigators have found impulsive choice to be induced by alcohol in rats (Evenden & Ryan, 1999; Tomie *et al*, 1998). These discrepancies may in some cases be because the drugs (or the state of addiction) do not have a unitary effect on discounting, but one which depends heavily on the situation and the particular choices involved.

Neural correlates underlying impulsive choice

The neural system in delay discounting performance is a complex and well balanced system comprising regions such as OFC, mPFC and amygdala. Considering the effects of GCs, these brain regions are target areas of their action and may be responsible for alterations in impulsive choice behavior.

The OFC is a brain region that projects to the NAcc and is strongly implicated in the assessment of reward value, since studies revealed that lesions encompassing the OFC induced impulsive choice (Mobini *et al*, 2002). Also, dissociable effects of lesions to OFC sub-regions were found in the rat (Mar *et al,* 2011); mOFC-lesioned rats showed increased preference for the large delay reward, as opposed to the decrease seen in lOFC-lesioned animals. These findings were consistent with human imaging studies and suggest that functions of these sub-regions can contribute differently to the decision-making process. The increase in the neuronal activation of the vOFC and mOFC of iuGC animals may help explain the difference seen in the DD task, since CONT animals did not display any difference in the activation of the areas mentioned, apart from the dlOFC, though this remains to be confirmed.

The mPFC that also projects to the NAcc is involved in reward-related learning (Balleine and Dickinson, 1998; Bechara *et al*, 1999; Richardson and Gratton, 1998; Tzschentke, 2000). ACC dysfunction may not be an important contributor to impulsive choice, despite the involvement of the ACC in reward-related learning (Bussey *et al*, 1997a; Bussey *et al*, 1997b; Cardinal *et al*, 2003; Parkinson *et al*, 2000), as studies that showed that lesions of the ACC had no effect in the choice of delayed reinforcements (Cardinal *et al*, 2001), but ACC seems to be involved in the selection of effortful options (Walton *et al*, 2003; Walton *et al*, 2002). However, ACC lesions can make rats motorically impulsive (leading to impulsive action) and to respond prematurely in situations where they are required to wait (Muir *et al,* 1996). Regarding animals prenatally exposed to GCs, the ACC showed a higher activation of this area upon performance of the DD task, though no differences were found in the 5-CSRTT.

Considering the PLC and ILC sub-regions, iuGC animals did not display any differences in the c-fos positive cell density, comparing task – and task + animals. This reduced activation may be explained by studies performed where lesions in PLC and ILC appeared to lead to a loss of temporal discriminative stimulus control (Cardinal *et al,* 2003) and lesions of the mPFC induced a deficit in timing ability in rats (Dietrich and Allen, 1998), with impaired temporal discrimination

in the peak procedure, an operant task that assesses the ability to time a stimulus (Catania, 1970; Roberts, 1981).

The amygdala is interconnected with the NAcc and OFC. Animals prenatally exposed to GCs revealed a similar neuronal activation in both CeA and BLA. However, this area displayed a higher c-fos positive cell density before exposure to the DD test, in iuGC animals. Some studies comprising lesions in the BLA showed an increase in impulsive choice in a task involving choice between an immediate one-pellet reward and a delayed four-pellet reward (Winstanley *et al*, 2004a; Winstanley *et al*, 2004b) and others demonstrated deficits when reward size is suddenly changed, upon amygdala inactivation (Salinas and McGaugh, 1996; Salinas *et al*, 1993), which has emotional significance and this brain area is well known to be involved in affective representation (Cardinal *et al*, 2002). As mentioned before, iuGC animals present an anxious-like behavior and, altogether, this could help explain the increased cell density seen in the BLA, in a basal situation.

One should also mention the importance of the NAcc in this task. This area responds to anticipated rewards in humans, other primates, chicks, and rats (Bjork *et al*, 2004; Breiter *et al*, 2001; Cromwell and Schultz, 2003; Izawa *et al*, 2005; Knutson *et al*, 2001; Martin and Ono, 2000; Miyazaki *et al*, 1998; Schultz *et al*, 1992; Schultz *et al*, 2000) and is innervated by DA neurons that respond to errors in reward prediction in a manner appropriate for a teaching signal (Schultz, 1998; Schultz and Dickinson, 2000; Schultz *et al*, 1998) and interventional studies showed this area to be a key site for the motivational impact of impending rewards (Robbins and Everitt, 1996; Everitt *et al,* 1999; Parkinson *et al,* 2000; Cardinal *et al,* 2002; Robbins *et al,* 2005). Considering results from Cardinal and colleagues (2001, 2003), lesion studies of the NAcc have shown to produce impulsive choice.

It is of importance to mention complementary measures such as the assessment of DA levels, since studies from Wade *et al.* (2000) showed that D2 antagonists and mixed D1/D2 antagonists induce impulsive choice. Impulsive choice or preference for immediate reinforcement is due to abnormally steep temporal discounting and it was suggested that this is due to a hypofunctional NAcc dopaminergic system (Johansen *et al,* 2002; Sagvolden *et al,* 1998). In this context, the observed NAcc hypodopaminergic state, together with D2 prominent changes in iuGC animals, may explain the changes observed in the DD protocol.

Chapter 6 – Conclusion

6. Conclusion

Increasing evidence from the last few years has shown the permanent effects of prenatal stress that may ultimately lead to the development of several neuropsychiatric disorders in adulthood, such as depression, anxiety, or drug addiction. Herein, we showed that iuGC animals do not differ in their locomotor reactivity to a novel environment, nor to a novel object. However, a long-term memory impairment was revealed with the NOR test. Regarding the trait of impulsivity, these animals did not show alterations in impulsive action, but present differences in impulsive choice, though further tests are required in order to validate these observations. Moreover, iuGC animals present changes in the neuronal activation of OFC and mPFC sub-regions. Altogether, our results seem to suggest that prenatal exposure to GCs program neuronal circuits involved in impulsivity which may may contribute for the enhanced drug-seeking behavior observed in these animals.

Chapter 7 – Future Perspectives

7. Future Perspectives

Further complement of the analysis on traits associated with drug addiction would be achieved by measuring risky behavior. Thus, we believe that the evaluation of this trait using the Minho Gambling Test would be crucial. Furthermore, an additional impulsive task can be performed to better assess impulsive action. In the Go/No-Go task, or Stop-Signal task, a response must be intermittently withheld upon presentation of a cue, measuring impulsive action (Logan and Cowan, 1984; Logan, 1994).

As explained in previous chapters, it would be crucial to perform the DD task using different parameters, in order to allow CONT animals to achieve better performance accuracy.

This would be accompanied with further determination of the neuronal activation patterns in the NAcc, also crucial in this type of behavior. Moreover, it would be interesting to control for the food reward component (naïve group), since we only had a group that was exposed to food pellets, and this may mask some of the c-fos activation results.

It will be important to characterize the levels of DA in these areas, since this neurotransmitter plays a major role in impulsivity. Nonetheless, an interesting approach would be to evaluate the interactive nature of DA and serotonin and their influence in impulsive behavior, since there is a persistent paradox on DA-releasing stimulant medications and their improvement in symptoms of ADHD, while at the same time drugs that increase DA transmission (agonists or levodopa) appear to increase impulsivity, i.e., in the case of medication-induced side effects in Parkinson's Disease (Dalley and Roiser, 2012). Moreover, a possible control and maintenance of future developments of addictive behavior may be achieved in individuals already vulnerable.

Chapter 8 – References

8. References

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