Universidade do Minho
Escola de Medicina

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Neurobiological basis of anxiety and social behaviors

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Trabalho Efetuado sob a orientação de:
Doutora Ana João Gomes Rodrigues
Professor Doutor José Miguel Pêgo

Março de 2017

DECLARAÇÃO DE INTEGRIDADE

Declaro ter atuado com integridade na elaboração da presente tese. Confirmo que em todo o trabalho conducente à sua elaboração não recorri à prática de plágio ou qualquer forma de falsificação de resultados.

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Assinatura:

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Neurobiological basis of anxiety and social behavior

Abstract

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

In this dissertation we have studied some of the neurobiological circuits involved in anxiety and social behaviors by studying a rat model injected with a synthetic GC (dexamethasone – 1mg/kg), at days 18 and 19 of pregnancy, which has been shown to present an anxious behavior and social impairments (iuGC model).

iuGC animals present hyperanxiety, increased fear behavior, and hyper-reactivity to negative stimuli, accompanied with an increase of 22 kHz ultrasonic vocalizations. In parallel, we found that iuGC animals present increased choline acetyltransferase (ChAT) expression in the laterodorsal tegmental nucleus (LDT) and pedunculopontine tegmental nucleus (PPT) in a basal situation. These results were interesting because this pathway is responsible for the initiation of

22 kHz negative vocalizations and is important for the control of emotional arousal. In addition, and in accordance with the amplified response to an adverse stimulus in iuGC animals, we found an increase in ChAT/c-fos expression in the LDT and PPT regions.

We also analyzed the effect of iuGC exposure in the bed nucleus of stria terminalis (BNST), a brain region known to be involved in the modulation of stress response and anxiety behaviors. No major effect in BNST basal or evoked activity was found in iuGC animals in Sprague Dawley background. Importantly, these animals also do not show the prominent anxious behavior observed in Wistar Han strain with iuGC exposure, suggesting that different strains have distinctive susceptibility to GC effects.

Next, we observed the impact of modulating different neuronal populations in social behaviors. First, we used optogenetic tools to selectively activate (or inhibit) LDT neurons during social encounters. Optical activation of LDT-ventral tegmental area (VTA) projections leads to a moderate increase in social interaction. This effect is specific, since no differences were observed in

object interaction. In addition, bidirectional modulation of the LDT- nucleus accumbens (NAc) projections has no effect on same-sex, non-aggressive, social or in object interaction.

Second, because previous work from our team has shown that iuGC exposure alters NAc dopamine receptor D2 (D2R) neurons, we decided to evaluate the role of this neuronal population in social behaviors. Activation of NAc D2R-expressing neurons leads to a modest increase in social interaction, with no differences observed in object interaction. Inhibition of NAc D2R-expressing neurons had no impact in social or object interaction. However, we found that optical activation of NAc D2R-expressing neurons rescues the social impairments observed in the iuGC model.

In conclusion, we have shown that iuGC exposure programs different brain regions, namely the LDT and the NAc, and leads to anxious behavior and social impairments. We further show an important modulatory role of LDT-VTA projections in social behaviors.

Bases neurobiológicas do comportamento ansioso e social

Resumo

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

Nesta dissertação estudamos alguns dos circuitos neurobiológicos envolvidos no comportamento ansioso e social, usando um modelo de ratos exposto a um GC sintético (dexametasona - 1mg/kg), nos dias embrionários 18 e 19, no qual previamente se demonstrou um comportamento ansioso e défices sociais (modelo iuGC).

Os animais iuGC apresentam híper-ansiedade, um aumento do comportamento do medo, e uma hiper-reactividade a um estímulo negativo; estes comportamentos foram acompanhados com um aumento do número vocalizações ultrassónicas negativas emitidas, i.e., de 22 kHz . Em paralelo, os animais iuGC apresentam um aumento na expressão da colina acetil-transferase (ChAT) no núcleo laterodorsal tegmental (LDT) e no núcleo pedúnculo pontino tegmental (PPT), numa situação basal. Estes resultados foram interessantes uma vez que este circuito neuronal é responsável pela iniciação das vocalizações de 22 kHz e é importante no controlo emocional. De acordo com a resposta amplificada a um estímulo adverso dos animais iuGC, encontramos um aumento no número de células ChAT/c-fos positivas nas regiões LDT e no PTT.

Também analisamos o efeito da exposição ao iuGC no núcleo da estria terminal (BNST), uma região cerebral envolvida na modulação da resposta ao stress e no comportamento ansioso. Não encontrámos diferenças na ativada basal ou evocada no BNST nos animais iuGC na estirpe Sprague Dawley. Contudo, observamos que estes animais não apresentam o clássico comportamento ansioso previamente observado na estirpe Wistar Han sujeita a iuGC, sugerindo que diferentes estirpes de rato têm diferentes vulnerabilidades aos efeitos deletérios dos GCs.

De seguida, observamos o impacto da modulação de diferentes populações neuronais no comportamento social. Para isso usamos a técnica de optogenética para seletivamente ativar (e inibir) as projeções LDT- área tegmental ventral (VTA) durante encontros sociais. A ativação ótica

das projeções LDT-VTA produz um aumento moderado da interação social. Este efeito é específico, uma vez que não foram observadas diferenças na interação com um objeto. Adicionalmente, a modulação bidirecional das projeções LDT- núcleo accumbens (NAc) aparenta não ter qualquer efeito na interação social entre animais do mesmo sexo ou na interação com objeto.

Posteriormente, tendo em conta resultados anteriores da nossa equipa que mostraram que exposição ao iuGC altera os neurónios D2 no NAc, decidimos avaliar o papel desta população neuronal no comportamento social. Ativação dos neurónios D2 no NAc conduzem a um aumento moderado da interação social, sem diferenças observadas na interação com um objeto. A inibição dos neurónios que D2 não tem impacto na interação social e com o objeto. A ativação ótica destes neurónios normaliza os défices no comportamento social observados nos animais iuGC.

Em conclusão, demonstramos que exposição a iuGC programa diferentes regiões cerebrais, nomeadamente o LDT e o NAc, e induz comportamento ansioso e défices sociais. Por fim, também demostramos um importante papel modulador das projeções LDT-VTA no comportamento social.

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Abbreviations

1:

11β-HSD2 – 11-Beta hydroxylase 2

A:

aBNST – anterior bed nucleus of the stria terminalis

Ach - acetylcholine

ACTH - Adrenocorticotropic hormone

AMPA – aminomethylphosphonic acid

Amy - Amygdala

AP - anteroposterior

B:

BLA – Basolateral nucleus of amygdala

BNST – Bed nucleus of the stria terminalis

C:

CAH - Congenital adrenal hyperplasia

CeA - Central ncules of amygdala

ChR2 – Channel-rhodopsin

ChAT - choline-acetyltranferase

CONT - control

CPP – conditioned place preference

CRF – Corticotrophin releasing factor

D:

D1-MSN - Dopamine receptor D1 medium spiny neuron

D2-MSN – Dopamine receptor D2 medium spiny neuron

D1R - Dopamine receptor 1

D2R - Dopamine receptor 2

DA - Dopamine

DAergic - Dopaminergic

DEX - Dexamethasone

DV - dorsoventral

E:

EPM - Elevated plus maze

G:

GABA – gamma-aminobutyric acid

GC - Glucocorticoid

GHSR-1A – ghrelin receptor 1A

GR - Glucocorticoid receptor

GRE - Glucocorticoid response element

H:

Hipp - Hippocampus

HPA - Hypothalamic-pituitary-adrenal

HPT – Hypothalamus

I:

ILCx - infralimbic cortex

iuGC - *in utero* glucocorticoid

L:

L/D – light/dark

L-DOPA - Levodopa

LA – lateral nucleus of the amygdala

LDT - Laterodorsal tegmental nucleus

LHb – Lateral habenula

LTD - long-term depression

LTP – long-term potentiation

M:

MeA - medial nucleus of amygdala

ML - mediolateral

mPFC – medial prefrontal cortex

MPOA – medial preoptic area

MR - Mineralocorticoid receptor

MSN – medium spiny neuron

N:

NAc – nucleus accumbens

NEC - Necrotizing enterocolitis

O:

OF: open field

P:

PAG – periaqueductal gray area

PB – parabrachial nucleus

PFC - Prefrontal cortex

PND – postnatal day

PPT - pedunculopontine tegmental nucleus

PSTHs – peristimulus time histograms

PTSD – Posttraumatic stress disorder

PVN - Paraventricular nucleus

R:

RDS - Respiratory distress syndrome

RT – room temperature

S:

SEM – standard error of the mean

SD – standard deviation

SN - Substantia nigra

T:

TH - Tyrosine hydroxylase

U:

USV - Ultrasound vocalization

V:

vBNST – ventral bed nucleus of the stria terminalis

VTA - Ventral tegmental area

Thesis Planning

The present thesis is organized in four chapters. Chapter 1 is the Introduction, Chapter 2 and 3 present the experimental work and Chapter 4 is the general discussion of the work.

In Chapter 1, an introduction to the theme of this dissertation is presented. We describe the stress response and the regulation of the hypothalamic–pituitary–adrenal axis. Next, we provide evidence of the impact of in utero exposure to synthetic glucocorticoids on the developing brain. Finally, we summarize the neuronal circuitries involved in anxiety, fear and social behaviors.

In Chapter 2, we investigate the neurobiology of anxiety behavior. In Chapter 2.1, "Glucocorticoid programing of the mesopontine cholinergic system", published in Frontiers in Endocrinology (2014), we provide evidence that in utero glucocorticoid (iuGC) exposure induces anxiety, increased fear behavior, and hyper-reactivity to negative stimuli. iuGC animals present increased choline acetyltransferase expression in the laterodorsal tegmental nucleus (LDT) and pedunculopontine tegmental nucleus (PPT). Moreover, when exposed to an adverse stimulus, iuGC animals present changes in the activation pattern of cholinergic cells in the LDT and PPT regions.

In Chapter 2.2, "Impact of in utero glucocorticoid exposure on the BNST neuronal activity", we used an in vivo electrophysiology approach, to study the effects of iuGC exposure in the activity of the bed nucleus of the stria terminalis (BNST). For that purpose, we analysed BNST response to an electric stimulation of two upstream regions, the infralimbic cortex and central nucleus of the amygdala. In addition, we evaluated the effect of iuGC exposure on the neuronal activity of a downstream region, the ventral tegmental area (VTA). In brief, iuGC exposure did not induce any major effect on the BNST network.

In Chapter 3, we investigate the neuronal circuities of social behavior. In Chapter 3.1, "Impact of optogenetic modulation of LDT-NAc and LDT-VTA pathways in social behaviours", we show that optogenetic activation of LDT-VTA projections slightly increased same sex non-aggressive social behavior. On the contrary, bidirectional modulation of the LDT-nucleus accumbens (NAc) projections did not produce any effect on social behavior.

In Chapter 3.2, “The role of dopamine receptor 2-expressing neurons in social behavior”, we show that NAc dopamine receptor D2 (D2R) expressing neuron stimulation appears to modulate social behaviors. In control animals, activation of D2 neurons slightly increases social interaction, and in iuGC animal, this activation is able to rescue the social deficits of these animals.

Chapter 4 comprises the general discussion of the present thesis. We discuss the main findings, and we critically debate the difficulties and problems encountered during the experimental work, and briefly propose some future experiments.

CHAPTER 1

Introduction

Introduction

1.1 Stress & HPA axis

The ability of the organism to adapt to daily stressful experiences is an important factor to its survival. Stress is commonly defined as a challenge to the organism's homeostasis. These daily stressful situations, such as physical insults, changes in the environment or emotional challenges, elicit a body reaction, which include physiological (e.g. metabolic, hormonal, etc.) and behavioral (e.g. fight or flight reaction) changes. A cascade of events leads to the activation of a series of systems, namely the hypothalamic–pituitary–adrenal (HPA) axis and the central limbic stress-loop, in order to achieve such response that are required for the return of the organism to its homeostatic balance (Avishai-Eliner et al., 2002; Miller and O'Callaghan, 2002).

Upon a 'systemic or interoceptive' stressor, the stress response starts with the activation of the paraventricular nucleus of the hypothalamus (PVN) by visceral afferents, which releases the corticotropin releasing factor (CRF) to the anterior pituitary gland. Here, it stimulates the release of the adrenocorticotropic hormone (ACTH) into the bloodstream. In turn, the ACTH induces the synthesis and release of corticosteroids from the adrenal cortex.

Corticosteroids are divided in glucocorticoids (GCs) and mineralocorticoid. Glucocorticoids binds to two types of receptors: high affinity mineralocorticoid receptor (MR) and low affinity GC receptor (GR). MR are occupied at basal levels, while the GR are occupied when the level of GCs is high in the bloodstream. Ligand-binding results in the translocation to the nucleus. In the interior of the nucleus this ligand-binding binds to the glucocorticoid response elements (GRE), where it regulates (positive or negatively) the expression of target genes (Juruena et al., 2004; de Kloet et al., 2005).

The cessation of HPA axis stress response is tightly regulated by a glucocorticoid negative-feedback, in order to prevent the deleterious effects of both hyper and hypo-cortisolemia (Stephens and Wand, 2012).

If the stressor is 'processive or exteroceptive', involves the activation of the stress-loop pathway. In this case, limbic afferents projections activate the PVN, that include the prefrontal cortex (PFC), hippocampus (Hipp), amygdala (Amy) and the bed nucleus of the stria terminalis (BNST). Interestingly, the BNST also receives the stressor input from upstream regions of the brain involved in cognitive processing, such as the PFC and the hypothalamus (HPT), and in

'emotional' information processing, such as the Amy. Thus, 'cognitive' or 'emotional' stress signals converge on the BNST, which acts as a relay station between upstream regions and the HPA axis (Avishai-Eliner et al., 2002; Herman et al., 1996, 2005).

An effective coping response implies that is effectively triggered when needed and afterwards successfully terminated (de Kloet et al., 2005). Indeed, if the stress response is prolonged in time or even inadequate, it provokes multiple and several changes on brain function and behavior, that can lead to the development of several disorders, such as, anxiety, addition or leads to impairments on social behaviors (Borges et al., 2013; Oliveira et al., 2006; Pego et al., 2008; Pêgo et al., 2009; Rodrigues et al., 2012; Sandi and Haller, 2015, 2015; Sinha, 2008).

1.2 The role of glucocorticoids during development

Apart from mediating some of the physiological effects of stress, glucocorticoids play an important role in cell survival and proliferation (Abrahám et al., 2001; Ganguli et al., 2002; Li et al., 1998; Saffar et al., 2011). Moreover, in the fetus, GCs are crucial for the maturation of various fetal tissues including the liver, lung, gut, skeletal muscle and adipose tissue (Turkay et al., 2012). Indeed, late pregnancy is characterized by a rise in cortisol levels, which matches the increased maturity of fetal organs (Smith and Shearman, 1974).

The fetus is usually protected from the maternal GCs by the placental 11β-hydroxylase 2 (11β -HSD2) enzyme, which has the capacity to convert biologically active cortisol to inactive cortisone (Speirs et al., 2004; Sun et al., 1997). However, in the cases of extreme stressful situations, part of maternal GCs can cross the placenta. Moreover, synthetic GSs are not metabolized by 11β -HSD2, facilitating the passage through the placenta (White et al., 1997) to the fetus. Endogenous GCs bind both GR and MR, while synthetic GCs bind predominantly to the GR as the MR has a low affinity for synthetic GCs. In addition, commonly used synthetic GC, such as dexamethasone (DEX), have 25 times more potency than endogenous cortisol (Folkman and Ingber, 1987).This is particularly relevant because in clinics, synthetic GC treatment (betamethasone or DEX) are used in preterm labour, to promote fetal lung maturation. This treatment is known to reduce the incidence of respiratory distress syndrome (RDS), intraventricular hemorrhage, necrotizing enterocolitis (NEC), respiratory support need, intensive care admissions and systemic infections in the first 48 hours of life and leads to neonatal mortality improvement (Miracle et al., 2008). Much more rarely, it is used during antenatal

period in the foetuses at risk of congenital adrenal hyperplasia (CAH) (New et al., 2001). Although this treatment greatly improves survival, there is evidence that it can pose a risk to the developing brain.

In fact, there is some evidence that elevated levels of GC early in life can induce effects in physical and mental health. Indeed, childhood trauma increases the risk of development of conduct disorders, personality disorders, major depression, schizophrenia, anxiety and addictive disorders (Agid et al., 1999; Bernet and Stein, 1999; Dube et al., 2003; Heim and Nemeroff, 2001; Young et al., 1997).

Moreover, children exposed prenatally to GC present a reduction in the birth weight, and at age of 3, significant alterations in behavior (Newnham, 2001). Additionally, fetal exposure to synthetic glucocorticoids has detrimental effects on birth outcome, childhood cognition and long-term behavior (Crowley, 1995; Miracle et al., 2008; Seckl, 2004).

1.3 iuGC – *in utero* glucocorticoid exposed animal model

Many stress/GC-exposure models have been used to understand the impact of GC exposure during the neurodevelopmental periods. In our laboratory has an animal model which consists in subcutaneous injections of DEX (1mg/kg), on gestation day 18 and 19 – iuGC animals. The adult offspring of this pregnant rats present an obvious anxious phenotype (Oliveira et al., 2006), depression-like-behavior (Roque et al., 2011), enhanced drug-seeking behavior (Rodrigues et al., 2012) and deficits in social behavior (Borges et al., 2013); on the other hand, these animals do not present cognitive impairments (Oliveira et al., 2006).

At the morphological level, GC treatment induces a reduction in cell proliferation in the VTA, reduces the number of TH-positive cells in the VTA, leading to decrease dopaminergic (DAergic) innervation to the NAc (Leao et al., 2007). Moreover, it was observed a reduction on the NAc volume, with significant changes in spine density and neuronal morphology. Furthermore, it was observed decrease volume of basolateral nucleus (BLA) and central nucleus (CeA) of the amygdala but not in the lateral nucleus (LA) division, together with a significant dendritic atrophy of CeA neurons when compared to controls (Oliveira et al., 2012). Additionally, these animals present an increase in BNST total volume. This effect was due to increased volume of the anteromedial division of the BNST, with no major differences in the anterolateral division and posterior division of the BNST. In the anteromedial BNST division, the iuGC

treatment leads to an increase in the total dendritic length, however, no effect was observed in spine densities, even when a separate analysis of mature and immature forms (Oliveira et al., 2012).

At a molecular level, iuGC animals present a decrease in dopamine levels both in the NAc and Amy, which accompanied by an increase in the expression of dopamine receptor 2 (D2), without affecting the expression of dopamine receptors (Oliveira et al., 2012; Rodrigues et al., 2012). In the BNST, no changes were observed in the dopamine levels and dopamine receptors expression (Oliveira et al., 2012).

In summary, prenatal administration of synthetic GCs leads to prominent alterations at a morphologic and molecular level, which can contribute for the observed behavioural alterations of these animals.

1.4 Anxiety and fear behavior

Although anxiety and fear behavior share common physical and psychological manifestations, they are in fact two independent entities that have specific definitions. Anxiety is characterized by a sensation of discomfort and apprehension in response to unconditioned diffuse cues and in the absence of an immediate threat (Koch, 1999; LeDoux, 2000). Fear is an emotional reaction triggered by an immediate threat (Davis et al., 2010); while responses start abruptly, it also end when the threat is removed. Fear is considered to be a primary protective reaction to potentially harmful challenges, which contributes significantly to the survival of the individual.

Occasional anxiety makes part of our daily life. Anxiety is commonly experienced by healthy individuals, is a natural response to stressful stimuli, whereas pathologic anxiety is prolonged in time and/or disproportional in magnitude. It causes poor quality of life, increases mortality and is associated with significant costs to society. Anxiety disorders develop when symptoms occur without any recognizable stimulus or when the stimulus does not justify such a reaction. These disorders are considered one of the more common psychiatric disorders existent in our society. Despite their high prevalence rates, they are often underrecognized and undertreated.

Anxiety disorders include phobias, panic disorder, social anxiety disorder, posttraumatic stress disorder (PTSD) and generalized anxiety disorder. Exposure to stressful life events in childhood and adulthood is considered to be a key triggering factor for these disorders.

Anxiety/fear behavior can be studied in rodents using specific behavioral paradigms such as the elevated plus maze (EPM), the goal standard paradigm to study anxiety behavior (Carobrez and Bertoglio, 2005). In addition to EPM, other tests are used to evaluate anxiety: open field, operant conflict, light-dark box, shock probe avoidance tests (Davis, 1992; Engin and Treit, 2007). The USVs are also an additional measure of conditioned fear in conjunction with freezing (and also anxiety) (Frick et al., 2004; Koo, 2004; Lee, 2004; Lee et al., 2001). Indeed, the 22 kHz USVs is supposed to reflect an aversive behavior state and it is useful in the study of the neural mechanisms of anxiety and fear states (Knapp and Pohorecky, 1995).

The limbic system, which plays an essential role in the modulation of emotional behavior and endocrine responses to stressful events, is believed to be a crucial target and executor of (mal)adaptive responses to stress.

1.4.1 Brain areas involved in anxiety and fear behavior

Anxiety disorders seem to be caused by the interaction of biopsychosocial factors, including genetic vulnerability, but also by adverse life experiences (Fernandes and Osorio, 2015). Therefore, some of the neurological circuits, which are involved in the regulation of the stress response, coincide with the neurocircuitry of anxiety and fear state, namely the extended Amy, which includes the CeA and medial (MeA) nuclei of the Amy and the BNST.

Importantly, the BNST and the CeA are intricately connected (Dong and Swanson, 2006; Dong et al., 2001; Sun and Cassell, 1993; Veinante and Freund-Mercier, 2003) suggesting that their interactions modulate behavioral output, but also present a similar connectivity. Both structures receive excitatory inputs (Dong et al., 2001; Sun and Cassell, 1993) from the BLA, are reciprocally connected, and their brainstem afferents overlap extensively (Dong and Swanson, 2004, 2006). Additionally, the CeA and the BNST also present a similar neuropeptide expression profile and morphology (McDonald, 1983; Roberts et al., 1982; Woodhams et al., 1983). Additionally, the anterior BNST receives GABAergic projections from the CeA, while the posterior region receives projections from the MeA.

However, although both regions share similar connectivity, there are clear differences regarding their biological function. For example, humans with generalized anxiety disorder (GAD) present a decrease in Amy activity and increased activity in the BNST (Yassa et al., 2012). In accordance, in individuals with anxiety, the BNST showed greater overall recruitment and exaggerated tracking of threat proximity (Somerville et al., 2010). In animal studies, stressors and anxiogenic pharmacological agents, such as yohimbine, m-chlorophenylpiperazine, caffeine, isoproterenol (a β-adrenoreceptor agonist) and CRF, increase the level of expression of immediate early gene c-fos in the BNST (Naka et al., 2013; Sahuque et al., 2006; Singewald et al., 2003), further suggesting that increased activity of the BNST is associated with an enhanced state of anxiety. Conversely, injections of glutamate antagonists in the BNST are anxiolytic (Walker and Davis, 1997).

Previously, it was suggested that whereas the BNST is associated with anxiety, Amy is more related to fear (Davis et al., 2010), although this is an oversimplification of the behavioral findings. For example, in a recent optogenetic study, it was shown that activation of the BLA augments anxiety, while activation of the projection from the BLA to the CeA decreased anxiety (Tye et al., 2011).

Briefly, studies show that the CeA contributes to both immediate ("phasic") and longer-lasting ("sustained") responses to threat. The medial division of CeA mediate the phasic responses. Whereas, projections from the lateral division of the CeA to the lateral division of the BNST modulate the responses to more persistent kinds of threats. Indeed, following the onset of a threat (between 4 and 60s) the lateral division of the BNST is rapidly involved. Afterwards, this BNST division inhibit the medial division of the CeA, allowing the transition from phasic to sustained response to threat (Davis et al., 2010). More information about the role of amygdala in anxiety is described elsewhere (reviewed in (Davis, 1992; Davis et al., 2010)).

On the other hand, recent studies suggested that, indeed, both regions, BNST and CeA, play a role in assembling states of fear and anxiety in response to exposure to learned and unlearned threats (Gungor and Pare, 2016; Shackman and Fox, 2016). In fact, both are crucial in regulating sustained responses to diffusely threatening contexts (Duvarci et al., 2009; Jennings et al., 2013; Kim et al., 2013; Moreira et al., 2007; Zimmerman and Maren, 2011; Zimmerman et al., 2007). Furthermore, studies have highlighted that CeA is also important in triggering phasic responses to acute threats (Ciocchi et al., 2010; Li et al., 2013; Wilensky et al., 2006),

and BNST in assembling states of fear and anxiety in response to relatively brief threat cues (Kiyokawa et al., 2015). Moreover, both regions contribute to the overgeneralization of fear and anxiety observed to Pavlovian safety cues (Ciocchi et al., 2010; Duvarci et al., 2009), and learning-dependent plasticity within the CeA is essential for the acquisition of Pavlovian fear conditioning (Ciocchi et al., 2010). In summary, this data suggests that the CeA and the BNST both regulate sustained defensive responses elicited by continued exposure to threatening cues and contexts.

Despite clear evidence that overactivation of the BNST is associated with increased anxiety, the picture is far more complex than initially anticipated, because within the BNST, different nuclei may have opposing roles in anxiety modulation. For example, electrical stimulation of the anterolateral region produces many of the endocrine, cardiovascular and respiratory responses that are normally elicited by anxiogenic stimuli (Casada and Dafny, 1991). Additionally, we have shown that BNST nuclei have distinct activation patterns in response to anxiogenic stimuli: whereas we observe an overactivation of the anterior and dorsomedial nuclei, the posterior division presents a reduction in activation (Ventura-Silva et al., 2012). Supporting this view, Kim et al. showed that decreases in activity in the anterodorsal BNST are anxiogenic, while inactivation of the oval nucleus of the BNST is anxiolytic (Kim et al., 2013).

BNST projections to different nuclei may also play distinct roles in anxiety, adding extra complexity to the circuitry. For example, activation of the anterodorsal BNST-lateral hypothalamus projection is anxiolytic, whereas anterodorsal BNST-parabrachial nucleus (PB) projection decreases respiratory rate (a marker of anxiety), but has no effect in the behavior observed in the open field and elevated plus maze (Kim et al., 2013).

The behavioral outcome modulated by the BNST is also dependent on the balance between different neurotransmitters being recruited in the presence of a stimulus. In response to a foot-shock, the glutamatergic BNST-VTA neurons present an increase in their activity, contrary to GABAergic BNST-VTA neurons (Jennings et al., 2013).

As mentioned before, negative emotional states are associated with emission of 22 kHz vocalizations, which can be induced by cholinergic activation of limbic areas of medial diencephalon and forebrain. Production of these calls is dependent on the mesopontine cholinergic pathway the main sources being the laterodorsal tegmental nucleus (LDT) and pedunculopontine tegmental nucleus (PPT). The negative states accompanied with emission of

vocalizations are initiated by the ascending mesolimbic cholinergic system, as a specific negative arousal system. This pathway originates in the LDT and sends projections to the basal forebrain and limbic areas, such as the VTA and NAc (Brudzynski, 2001, 2014; Brudzynski and Barnabi, 1996; Cornwall et al., 1990; Dautan et al., 2014). The receiving structures of this system, which produced robust responses, included anteromedial hypothalamus, medial preoptic area (MPOA), BNST, and lateral septum (Bihari et al., 2003; Brudzynski, 1994). Indeed, direct pharmacological stimulation of the cholinergic pathways leads to the emission of 22 kHz aversive calls, which also induces a negative state, defined, by Brudynzksi team, as a response to "external or internal stimuli and/or situations (complex stimuli) that present threat or danger to the organism, cause or can cause physical damage or impairment, biological or sociobiological destabilization, and disruption of physiological functions and balances" (Brudzynski, 2007). Moreover, activation of the ascending mesolimbic cholinergic system also initiates a somatic, autonomic, and endocrine activation (Brudzynski, 1994, 2014; Decsi and Karmos-Várszegi, 1969; Várszegi and Decsi, 1967). Based on these evidences, it may be established that 22 kHz calls emission is a reliable predictor of increased cholinergic activity in the brain.

Regarding the PPT, innervates predominantly the substantia nigra (SN), with a minor projection from its posterior part also going to the VTA (Oakman et al., 1995, 1999; Semba and Fibiger, 1992; Steininger et al., 1992). This brain region is involved in sleep/waking cycle (Van Dort et al., 2015) and more recently, was found to be involved in anxiety behaviors (Homs-Ormo et al., 2003; Walker and Winn, 2007).

1.5 Social behavior(s)

The quantity and quality of social interactions are fundamental to the health and well-being of humans and social animal models such as rodents. Increasing evidence suggests that social support in humans, and affiliative behaviors in animals, can lead to a positive impact on health and decrease mortality from many different causes (DeVries et al., 2003). Impairments in social behaviors can take many ways, it can produce deficiency in sociability, increase in aggression and absence or insufficient parental care. Therefore, it is important to understand the mechanisms and circuitries that regulate social behaviors.

Only recently, social dimension has been recognized to be affected by a maladaptive stress response (Kennedy and Adolphs, 2012; Sandi and Haller, 2015), though social

impairment is commonly present in most psychiatric and neurological disorders (Kennedy and Adolphs, 2012). Despite this recent increase in social neuroscience research, most studies have focused on socio-sexual behaviors, however, little is known about neural circuitries regulating adult same-sex, non-aggressive social interaction.

Social behavior paradigms used with rodents assess different aspect of sociality, which are: 1) the subjective feeling of pleasure, this means the hedonic impact or 'liking', 2) approach behavior towards or the willingness to work for social play, this means the incentive motivation, 3) associative learning and memory, i.e. cognitive aspects of social play. The most common social behavior paradigms in rodents available are:

Social interaction test – Measures direct forms of social interaction of each animal, which include, social exploration (sniffing, licking or grooming any part of the body of the test partner, including the anogenital area), pouncing (play solicitations - the soliciting rat attempt to nose or rub the nape of neck of the partner), following and pinning (acceptance - when the animal that is pounced fully rotates to its dorsal surface);

Resident-intruder test – Measure the aggressiveness of the resident towards the intruder (offensive threats, bites and defensive behaviors);

Social competition test – Allow to investigate social hierarchy and other social dimensions;

Three-chamber sociability test – The apparatus is composed by 3 compartments. The 2 opposite comportments contain each one an enclosure (which allow to see and smell its content). One of the enclosure has a conspecific, while the other is empty. It is measured the percentage of time that the test animal spends exploring each compartment;

Social memory test or Social recognition test – The test is performed in the same apparatus as the test above. Each enclosure has an animal – one familiar another unfamiliar. The test compares the time that the test animals spend exploring the unfamiliar versus familiar animal.

Social conditioned place preference (CPP) – It has been used to demonstrate the pleasurable aspects of (playful) social interactions. One particular compartment is coupled with the pleasurable properties of social play. When the animal is allowed to freely move in this

apparatus and to choose, these cues will elicit approach behavior, so the animal will spend more time (or not) in that environment.

Lever-pressing – In the operant conditioning paradigm for social play reward, rodents are trained to lever press for brief episodes of social behavior. This operant conditioning can be used to assess motivational aspects of social play behavior.

Natural rewards are linked with behaviors (such as feeding, drinking and social behaviors) that are essential for survival, consider to be pleasurable and can act as natural reinforces. In fact, it has been shown that social interactions has reinforcing properties in primates and rodents (Bardo and Bevins, 2000; Calcagnetti and Schechter, 1992; Falk, 1958; Humphreys and Einon, 1981; Ikemoto and Panksepp, 1992; Normansell and Panksepp, 1990; Schechter and Calcagnetti, 1993; Trezza et al., 2011; Tzschentke, 1998, 2007; Vanderschuren et al., 2016). During social play, rats emit high frequency, 50 kHz USVs, and their increase was correlated with the amount of play solicitation and with magnitude of play-induced conditional place preference (Burgdorf et al., 2008; Knutson et al., 1998). Moreover, 50 kHz calls are also emitted during other actions correlated with positive value, such as, tickling, social exploratory activity, mating behavior, amphetamine ingestion and expression of unconditioned and conditioned reward states (Brudzynski and Pniak, 2002; Burgdorf et al., 2001, 2008; Knutson et al., 1999; Mällo et al., 2007; McGinnis and Vakulenko, 2003; Panksepp and Burgdorf, 2003). This data reinforce the knowledge that 50 kHz USVs emission during a positive reinforce serve as indicative of their pleasurable emotions.

1.5.1 Brain areas involved in social behaviors

The conception of a "social brain" emerged after studies, using brain imaging, have shown areas that are activated by social cognition tasks, which mostly overlap with "social brain network" observed in animals (Insel and Fernald, 2004; Kas et al., 2014; White et al., 2014; Yu et al., 2014). These studies have shown the involvement of the following areas: in social recognition - fusiform area, superior temporal gyrus and accessory olfactory bulb, in social motivation - the ventral tegmental area (VTA), nucleus accumbens (NAc) and ventral pallidum, in aggression - periaqueductal gray area (PAG), and in execution of social behaviors - HPT, brainstem motor and autonomic pathways.

Lesions to the ventromedial HPT (vHPT) decreased social solicitation without decreasing overall play fighting in juvenile rats (Beatty and Costello, 1983), while lesions on the dorsomedial and parafascicular area of the thalamus decreased pinning (Siviy and Panksepp, 1985). Interestingly, lesions only in the parafascicular area of the thalamus decreased solicitations (Siviy and Panksepp, 1985).

Lesions also confirmed that the Amy is involved in juvenile social play. Studies have shown that juvenile (postnatal day (PND) 21) electrolytic and neonatal (PND 7) excitotoxic lesions reduced social play behaviour in male rats (Daenen et al., 2002; Meaney et al., 1981; Wolterink et al., 2001). Interestingly, the Meaney team did not observed this reduction in female rats, suggesting that sex differences in the anatomy and physiology of the Amy during development have a differential influence on the social play (Meaney et al., 1981).

With optogenetic tools, activation of the BLA-vHipp and BLA-mPFC pathway bidirectionally modulate social behavior, since activation and inhibition of this projections, decrease and increase adult social interaction, respectively (Felix-Ortiz and Tye, 2014; Felix-Ortiz et al., 2016). Indeed, changes in Amy-PFC connections are frequently found in individuals with abnormal social behaviors. Most of these individuals have reduced function on the frontal regions (Glenn and Raine, 2013).

1.5.2 Social behavior and the mesolimbic system

In humans, the striatum is relevant to social information processing, including the processing of social factors that influence how we value experiences, learn from them, and make decisions (Bhanji and Delgado, 2014). Neuronal activity increases in striatum when an individual views or thinks about people with whom they hold an intimate relationship (Fisher et al., 2006; Hughes and Beer, 2012), attractive faces (Aharon et al., 2001; Spreckelmeyer et al., 2009) or people that have a higher social rank when compared to people of low social rank (Zink et al., 2008). Moreover, adolescents like adults, display striatum responses to positive social feedback (being liked) (Davey et al., 2009; Gunther Moor et al., 2010)

In rodents, neonatal treatment with 6-hydroxydopamine in the striatum markedly disrupted the sequential structures of social play, although these rats, continue to exhibit most play behaviors. However, they were more likely to use defensive tactics that shortened the playful contact between partners; and when contacting the partner, they were more likely to switch to

other behaviors, such as allogrooming and sexual mounting, rather than continue with the play sequence (Pellis et al., 1993).

Striatum is a relatively homogeneous structure in terms of cytoarchitecture, and is anatomically and functionally heterogeneous (Voorn et al., 2004; Yin et al., 2008; Zahm, 2000). Striatum is most widely divided in dorsal and ventral part, often named to as caudate-putamen and NAc, respectively. The dorsal division is frequently subdivided into a medial and a lateral portion, and the NAc into core and shell areas.

Indeed, social play induced increase c-fos expression in both dorsal striatum and nucleus accumbens core and shell (Gordon et al., 2003; van Kerkhof et al., 2013b). Pharmacological inactivation of the NAc core with baclofen/muscimol increased social play duration, where pharmacological inactivation of the dorsomedial striatum with an AMPA receptor antagonist enhanced social play (van Kerkhof et al., 2013a).

However, optogenetic enhancement of phasic activation of tyrosine-hydroxylase (TH) VTA-NAc projections increases adult social interaction, but not novel-object interaction. Indeed, the optogenetic activation and inhibition only of the VTA region leads to an increase and decrease of adult social interaction, respectively (Gunaydin et al., 2014). Upregulation of phasic firing of dopamine neurons from the VTA to the NAc were implicated in the development of social avoidance (reduced in social exploration) after repeated exposure to social defeat (Chaudhury et al., 2012; Russo and Nestler, 2013).

In the mesolimbic system, dopamine primarily encodes the incentive value (wanting) of a specific reward. Since social behavior is considered to be natural reward, it is expected that dopamine has the capability to modulate social behaviors. Indeed, treatment with non-selective dopamine receptor antagonists, dopamine D1 receptor antagonist and D2 receptor antagonist inhibit social play (Beatty et al., 1984; Holloway and Thor, 1985; Humphreys and Einon, 1981; Niesink and Van Ree, 1989; Siviy et al., 1996; Trezza and Vanderschuren, 2009).

Treatment with selective dopamine D1 and D2 receptor agonists suppressed social play behaviour (Siviy et al., 1996). However, treatment with a non-selective dopamine receptor agonist induce an increase (Beatty et al., 1984; Vanderschuren et al., 2008) as well an decrease in social play behaviour (Niesink and Van Ree, 1989).

Furthermore, stress-related social subordination has been linked with decreased dopamine transporter binding and increase in D2 receptor binding (Lucas et al., 2004). Moreover, D2 receptors are also associated with aggression. A variant in the gene encoding D2 receptor was connected with social dysfunction in untreated veterans of the Vietnam War with post-traumatic stress disorder (Lawford et al., 2006).

When assessing for hedonic proprieties of social paly, using the CPP apparatus, injections of dopamine antagonist (alpha-flupenthixol) had no effect on social play-induced CPP (Vanderschuren et al., 2016).

One of the main areas responsible for the formation of motivational processes and incentive salience is the NAc (Berridge, 2007; Floresco, 2015; Salamone and Correa, 2012). Infusion, in the NAc, of amphetamine and dopamine receptor agonist was shown to increase social play, in a dopamine D1 and D2 receptor-dependent manner (Manduca et al., 2016). In addition, decreased excitatory synaptic input to D1 receptor from the NAc was associated with the development of social avoidance following repeated exposure to social defeat (Francis et al., 2015). Moreover, infusion of a non-selective dopamine receptor antagonist, in the NAc, was found to inhibit the increase social predictable by the systemic injections of opioid receptor agonist and anandamide hydrolysis inhibitor (Manduca et al., 2016).

Furthermore, as mentioned before, 50 kHz calls are correlated with increases in positive states, and it is initiated by the ascending mesolimbic dopaminergic system, as a specific positive arousal system. Indeed, activation of the ascending dopaminergic system, which originates from the VTA and terminates in the NAc and other basal forebrain structures, induce an increase in locomotor activity, exploration, and an increase in the number of 50 kHz vocalizations (Brudzynski, 2007; Burgdorf et al., 2001; Thompson et al., 2006). Systemic or intra-injection of dopamine agonists, induced emission of number of 50 kHz vocalizations (Barker et al., 2010; Brudzynski, 2007; Brudzynski et al., 2012; Burgdorf et al., 2001, 2007, 2011; Ma et al., 2010; Simola et al., 2012; Thompson et al., 2006; Williams and Undieh, 2010). Based on this evidence, it may be established that 50 kHz calls emission is a reliable predictor of increase of dopaminergic activity in the brain. Interestingly, both VTA and NAc receive projections from the LDT, a region important for the modulation of 22 kHz calls (Cornwall et al., 1990; Dautan et al., 2014). Recent studies have shown that optogenetic stimulation of LDT neurons that project to the VTA enhances conditioned place preference (Lammel et al., 2012) and operant responding in

rodents (Steidl and Veverka, 2015). Yet, the impact of LDT-VTA stimulation in social behaviors remains completely undisclosed. Regarding, the LDT-NAc projections nothing is known about the functional relevance of these projections.

In summary, these studies highlight the important role of dopamine in modulation the motivation for social interaction, by pinpointing the nucleus accumbens as is critical site of action.

Aims

Accumulating evidence suggests that in utero exposure to high levels of GCs increase the propensity to develop a multiplicity of psychiatric disorders in adulthood, such as anxiety and may lead to impairments on social behavior.

In this dissertation, we aim to characterize in detail the anxious and social behaviors of adult animals exposed to GCs at gestation days 18 and 19, and to further dissect the neuronal pathways that regulate these behaviors.

The main objectives of this thesis were:

1) Assess the effect of in utero exposure to DEX (synthetic glucocorticoid; iuGC model) on anxious behaviour, and find some neurobiological correlates;

2) Assess the impact of iuGC exposure in the BNST neuronal network;

3) Evaluate the impact of optogenetic stimulation of the LDT-VTA and LDT-NAc projections on social behaviors, in naïve animals;

4) Evaluate the effects of the optogenetic modulation of NAc dopamine 2 receptor-expressing neurons in social behaviors, in naïve and in iuGC-exposed animals.

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CHAPTER 2

Prenatal glucocorticoid exposure and anxiety: behavioral and neurophysiological correlates

CHAPTER 2.1

Borges S, Coimbra B, Soares-Cunha C, Ventura-Silva AP, Pinto L, Carvalho MM, Pêgo JM, Rodrigues AJ and Sousa N

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Glucocorticoid programing of the mesopontine cholinergic system

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Stress perception, response, adaptation, and coping strategies are individually distinct, and the sequel of stress and/or glucocorticoids (GCs) is also distinct between subjects. In the last years, it has become clear that early life stress is a powerful modulator of neuroendocrine stress-responsive circuits, programing intrinsic susceptibility to stress, and potentiating the appearance of stress-related disorders such as depression, anxiety, and addiction. Herein we were interested in understanding how early life experiences reset the normal processing of negative stimuli, leading to emotional dysfunction. Animals prenatally exposed to GCs (*in utero* glucocorticoid exposure, iuGC) present hyperanxiety, increased fear behavior, and hyper-reactivity to negative stimuli. In parallel, we found a remarkable increase in the number of aversive 22 kHz ultrasonic vocalizations in response to an aversive cue. Considering the suggested role of the mesopontine tegmentum cholinergic pathway, arising from the laterodorsal tegmental nucleus (LDT) and pedunculopontine tegmental nucleus (PPT), in the initiation of 22 kHz vocalizations and hypothetically in the control of emotional arousal and tone, we decided to evaluate the condition of this circuit in iuGC animals. Notably, in a basal situation, iuGC animals present increased choline acetyltransferase (ChAT) expression in the LDT and PPT, but not in other cholinergic nuclei, namely in the nucleus basalis of Meynert. In addition, and in accordance with the amplified response to an adverse stimulus of iuGC animals, we found marked changes in the cholinergic activation pattern of LDT and PPT regions. Altogether, our results suggest a specific cholinergic pathway programing by prenatal GC, and hint that this may be of relevance in setting individual stress vulnerability threshold.

Keywords: glucocorticoids, stress, acetylcholine, anxiety, fear, pedunculopontine tegmental nucleus, laterodorsal tegmental nucleus, ultrasonic vocalizations

INTRODUCTION

Exposure to stressful events or synthetic glucocorticoids (GCs), such as dexamethasone, early in life, are a risk factors for the development of different neuropsychiatric disorders in adulthood, namely depression and anxiety (1). Such effects are partially mediated by de-regulation of the hypothalamic-pituitary-adrenal (HPA) axis, leading to altered GC secretion (2, 3), which can induce long-term molecular and functional changes in GC-sensitive nuclei. Importantly, several recent studies showed that high levels of GCs have a deleterious effect in the developing brain, inducing prominent neurochemical, structural, and molecular changes in several brain regions (4–8) culminating in the development of anxious and depressive-like traits (9–13).

GC programing effects in emotional behavior are far from being completely understood at a mechanistic level, but it has been shown that GCs modulate on the long-term the activity of particular neural pathways. In this perspective it is interesting to refer that early life stress/GC exert a powerful effect in the developing dopaminergic neurons, especially at the mesolimbic level (7). For example, we have shown that animals prenatally exposed to GC present marked hypodopaminergia and D2 epigenetic/expression changes, responsible for their anhedonia and motivational deficits, since administration of a dopamine precursor, L-DOPA, fully reverted the molecular and behavioral impairments (14, 15). Obviously, this dopaminergic de-regulation may also occur indirectly through modulation of upstream neurotransmitter systems. One promising candidate is the ascending cholinergic pathway, comprising projections from acetylcholine (ACh)-rich nuclei within the pons, particularly the laterodorsal (LDT) and pedunculopontine tegmental nuclei (PPT) to the ventral tegmental area (VTA) (16–18). In support of this, it was already shown that stress/GC strongly elicit cholinergic activity of these regions (19), and that they, in turn, critically affect basal and phasic activity of VTA neurons (20–23). Additionally, GC can putatively bind to GC-responsive elements of cholinergic players and control their expression, namely choline acetyltransferase (ChAT) (24) and acetylcholine esterase (AChE) (25), two enzymes of the cholinergic pathway. Also relevant is to note that the ascending cholinergic system is implicated in the control of the stress response by modulating hypothalamic pituitary adrenal (HPA) axis function (26–28) and in mediating the anxiogenic effects of stress (29–31).

Surprisingly, very few studies have focused on the impact of stress/GC exposure in the cholinergic system. Considering the lack of evidence, herein, we focused on the impact of prenatal exposure to GCs in the ascending cholinergic system, and the relevance of such changes in stress-related anxious and fear behaviors.

MATERIALS AND METHODS

ANIMALS AND TREATMENTS

All manipulations were conducted in accordance with local regulations on animal care and experimentation (European Union Directive 2010/63/EU). Pregnant Wistar Han rats were subcutaneously injected with the synthetic GC dexamethasone at 1 mg kg^{-1} (*in utero* glucocorticoid exposure, iuGC animals), or with vehicle (CONT; control animals), on days 18 and 19 of gestation.

Dexamethasone dosage was selected based on our previous studies showing that this regimen effectively impairs HPA axis activity in a long-term basis (12). From a clinical perspective, guidelines on prenatal corticotherapy (32, 33) recommend single course administration (0.3–0.5 mg/kg), however, multiple courses of GCs are often administered (34), despite the lack of evidence of increased therapeutic efficacy. Nevertheless, we must consider the difficulty in the transposition of human doses to rodents due to ADME species specificity.

At weaning day, male offspring were house-paired randomly, according with prenatal treatment, under standard laboratory conditions: artificial 12 h light/dark cycle (lights on from 08:00 a.m. to 08:00 p.m.); room temperature 22°C; food and water were provided *ad libitum*. Animals derived from at least three different litters were used for all the experimental procedures.

BEHAVIORAL TESTS

All tests were performed during the day period, except the confined cage and fear-conditioning protocols that were conducted during the night period (08:30 p.m. to 03:00 a.m.). All behavioral equipment was cleaned between animals (ethanol 10%) in order to remove any olfactory cues.

OPEN FIELD

The open field (OF) test was conducted in an arena (43.2 cm × 43.2 cm) with transparent acrylic walls and white floor (Med Associates Inc., St. Albans, VT, USA). Rats were placed in the center of the arena and movement was monitored over a period of 10 min with the aid of two 16-beam infrared arrays. Total distance traveled was used as an indicator of locomotor activity.

ELEVATED PLUS MAZE

The elevated plus maze (EPM) test was carried out under bright white light. Animals were placed individually for 5 min in the center of a black polypropylene plus-shaped platform elevated 72.4 cm above the floor. The apparatus consisted of two open arms (50.8 cm × 10.2 cm) and two closed arms (50.8 cm × 10.2 cm × 40.6 cm) (MedAssociates Inc., St. Albans, VT, USA). The number of entries into each arm and the time spent therein were recorded.

LIGHT/DARK BOX TEST

The light/dark box (L/D) test was performed inside the OF arena (43.2 cm × 43.2 cm) (MedAssociates Inc., St. Albans, VT, USA). A dark compartment was attached to one side with an opening facing the center of the arena. Animals were individually placed in the center of the illuminated part. The distance traveled and time spent in each compartment was recorded in a single trial of 10 min.

CONFINED CAGE TEST

The confined cage test was performed in a non-restrictive Plexiglas cylinder (inner diameter 8.8 cm, length 22.2 cm), mounted on a Plexiglas platform and placed in a ventilated, sound-attenuated chamber (SR-LAB, San Diego Instruments, San Diego, CA, USA). A stainless steel grid was placed inside the cylinder, through which an electric current could be passed (shock chamber). A microphone and a video camera were placed inside the sound-attenuated chamber. The protocol was performed in two consecutive days, in which the animals were placed inside the shock chamber for 3 min. The ultrasonic vocalizations (USVs) and the percentage of total freezing time were measured.

FEAR-CONDITIONING PARADIGM

The fear-conditioning test was performed in a non-restrictive Plexiglas cylinder (inner diameter 8.8 cm, length 22.2 cm), mounted on a Plexiglas platform and placed in a ventilated, sound-attenuated chamber (SR-LAB, San Diego Instruments, San Diego, CA, USA). The protocol was performed in three consecutive days (35). On the first day (habituation), each animal was placed in the shock chamber for 11 min. On the second day (conditioning), each subject was positioned inside the shock chamber for 3 min (no light, no shock). Afterwards, animals were exposed to six lights/shock pairings (0.4 ± 0.1 mA), with an inter-stimulus-interval (ISI) of 60 s. The shock was given for 500 ms, immediately after the cue light was turned off. On the following day (test day), after an initial phase of 3 min without light, animals were presented with a 20 s cue light for six times, but no shock was given (ISI of 60 s). During all procedures, USVs and freezing behavior were recorded.

USVs ANALYSIS

An ultrasound microphone (CM16/CMPA, Avisoft Bioacoustics, Berlin, Germany) sensitive to frequencies of 10–200 kHz, placed 15 cm above the floor, was used in all experiments. The microphone was connected via an Avisoft UltrasoundGate 416H (Avisoft Bioacoustics) to a personal computer; USVs were recorded using the Avisoft-Recorder (version 5.1.04) with the following settings: sampling rate: 250,000; format: 16 bit. For acoustical analysis, recordings were transferred to Avisoft SASLab Pro (version 5.1.22, Avisoft Bioacoustics). This program was used in order to produce spectrograms of USVs by conducting a fast Fourier transformation (256 FFT-length, 100% frame, Hamming window filter, 50% time window overlap). These spectrograms had a frequency resolution ∼1.2 kHz and a temporal resolution ∼0.4 ms.

Twenty-two kilohertz call detection was provided by an automated threshold-based algorithm (threshold: −40 dB) and a hold time mechanism (hold time: 20 ms). A lower-cut-off-frequency of 18 kHz was used to reduce background noise.

Calls were also inspected manually to ensure that, when necessary, USVs not detected automatically could be subsequently included in the automatic parameter analysis.

IMMUNOHISTOCHEMISTRY

For immunohistochemistry (IHC), 11 rats were sacrificed by pentobarbital (Eutasil, Sanofi) anesthesia and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde (pH 7.4 in 0.1 M phosphate buffer). Brains were removed and post-fixed for 48 h in 4% paraformaldehyde and then rinsed and stored in 30% of sucrose until sectioning. Brains were sectioned coronally, at a thickness of 50 μm, on a vibratome (VT1000S, Leica, Germany) and stored in cryoprotectant solution at −20°C until use.

Briefly, free-floating sections were pre-treated with 3% H_2O_2 in PBS, thoroughly rinsed in PBS, blocked with 2.5% fetal bovine serum (FBS) in PBS-Triton 0.3% for 2 h at room temperature, and then incubated overnight at 4°C with primary antibody goat anti-ChAT (Millipore, MA, USA; 1:1000). Afterwards, sections were washed, incubated with the secondary biotinylated anti-goat (Vector Lab., USA; 1:200) for 1 h, and processed with an avidin-biotin complex solution (ABC-Elite Vectastain reagent; Vector Lab, USA). Detection was done using 0.5 mg/ml 3,3′-diaminobenzidine. Sections were washed and mounted on glass slides, air-dried, counterstained with hematoxylin, and cover slipped with Entellan-New (Merck, Darmstadt, Germany).

IMMUNOFLUORESCENCE

Ninety minutes after completion of the fear-conditioning paradigm, 12 animals were deeply anesthetized with pentobarbital (Eutasil, Sanofi) and were transcardially perfused with 0.9% saline followed by 4% paraformaldehyde. Brains were removed and post-fixed in 4% paraformaldehyde. Coronal vibratome sections (50 μm) were first incubated with rabbit anti-c-fos primary antibody (1:1000; Ab-5, Calbiochem, USA), followed by incubation with goat anti-ChAT primary antibody (1:1000; anti-ChAT, Millipore, MA, USA). Secondary fluorescent antibodies were: Dylight 488-conjugated donkey anti-rabbit IgG (1:500, BioLegend), and Alexa Fluor 568-conjugated donkey anti-goat IgG (1:500, Invitrogen), respectively. Finally, all sections were stained with 4′,6-diamidino-2-phenylindole (DAPI; 1 mg/ml). For each animal, c-fos-positive cells within the PPT and LDT were analyzed after double staining with cholinergic (ChAT) marker and cell counts were performed by confocal microscopy (Olympus FluoViewTMFV1000, Hamburg, Germany). Estimation of cell density was obtained by crossing cell number values with the corresponding areas, determined using an Olympus BX51 optical microscope and the StereoInvestigator software (Microbrightfield, VT, USA).

STEREOLOGICAL PROCEDURES

Eleven animals were perfused transcardially with 4% paraformaldehyde, under deep pentobarbital (Eutasil, Sanofi) anesthesia. Brains were included in glycolmethacrylate (Tecnovit 7100, Heraeus Kulzer, Wehrheim, Germany) and sectioned on a microtome as described in detail elsewhere (36). Every other 30 μm thick coronal section was collected on a gelatinized slide, stained with Giemsa, mounted with Entellan-New (Merck, Darmstadt, Germany), and cover slipped. Stereological procedures were performed by a blind observer.

Laterodorsal tegmental and PPT regions were outlined according to the atlas of Paxinos and Watson (37) and based on noticeable cytoarchitectural differences, namely density of cells and size of the perikarya.

Volume and neuronal number estimations were performed using StereoInvestigator software (Microbrightfield, VT, USA) and a camera attached to a motorized microscope (Axioplan 2, Carl Zeiss, Germany). The Cavalieri's principle was used to assess volume. Briefly, every second section was used and the cross-sectional area was estimated by point counting (final magnification 112×). We used a test point system in which the interpoint distance, at the tissue level, was 150 μm for LDT and 200 μm for PPT. The volume of the region of interest was calculated from the number of points within its boundaries and the distance between sampled sections.

Average cell numbers were estimated using the optical fractionator method (38). Briefly, a grid of virtual 3D-boxes (LDT: 30 μm × 30 μm × 20 μm; PPT: 40 μm × 40 μm × 20 μm) equally spaced (using the same grid spacing as for volume estimations) was superimposed on every second section of the lamina of interest and cells within boxes were counted. Coefficients of error (CE) were automatically computed, according to the formulas of Gundersen et al. (39) for cell numbers and Gundersen et al. (40) for volume estimations. Glial cells were not included in the estimations, and the discrimination between neuronal and glial cell body profiles was based on the criteria described by Ling et al. (41) and Peinado et al. (42).

STATISTICAL ANALYSIS

Statistical analysis was performed in GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). Statistical analysis between two groups was made using Student's *t*-test or Mann–Whitney tests. Two-way analysis of variance (ANOVA) was used when appropriate. Bonferroni's *post hoc* multiple comparison test was used for group differences determination. Non-parametric analysis (Mann–Whitney test) was used when normality of data was not assumed. Results are presented as mean ± SEM. Statistical significance was accepted for $p \leq 0.05$.

RESULTS

IN UTERO GLUCOCORTICOID EXPOSURE IMPAIRS EMOTIONAL BEHAVIOR

Animals were exposed to a battery of behavioral tests that consisted of paradigms studying spontaneous exploratory behavior (OF), tasks of innate anxiety (EPM, L/D test, confined cage), and reactivity to adverse stimulus (version of fear-conditioning paradigm). Since USVs can give information on the emotional status of the animal, we decided to further complement the behavioral characterization by measuring USVs in these different paradigms.

In the OF, iuGC animals presented a decrease in the number of ambulatory counts (**Figure 1A**, $t = 2.197$, $p = 0.037$) and total distance traveled (**Figure 1B**, $t = 3.002$, $p = 0.006$) when compared with control animals. In addition, iuGC animals exhibited a decrease in the percentage of time spent in the center of the arena (**Figure 1C**, $t = 2.416$, $p = 0.023$).

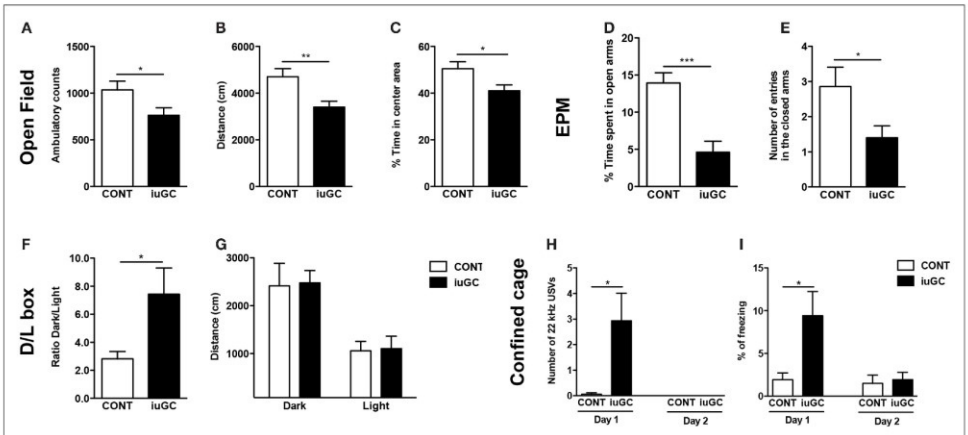

FIGURE 1 | *In utero* glucocorticoid exposure induces an anxious-like behavior. iuGC group presented a decrease in ambulatory counts **(A)**, total distance traveled **(B)**, and percentage of time in the center of the arena **(C)**. In the EPM, iuGC animals exhibited a decrease in the percentage of time spent **(D)** and number of entries **(E)** in the open arms of the maze, when compared with control group. **(F)** In the L/D test, iuGC presented an increase in the ratio dark/light, with no differences on the distance traveled in both compartments **(G)**. In the confined cage, iuGC group presented an increase in the number of 22 kHz USVs **(H)** and freezing behavior **(I)**. Upon habituation, iuGC animals no longer presented this anxious behavior (day 2). CONT, control animals; iuGC, *in utero* GC exposed animals; USVs, ultrasonic vocalizations. $*p < 0.05$, $**p < 0.001$, and $***p < 0.0001$; **(A–C)** $n = 16$; **(D,E)** $n = 11$–13; **(F,G)** $n = 10$, **(H,I)** $n = 8$–16.

In the EPM, iuGC animals spent significantly less time in the open arms (**Figure 1D**, $t = 2.947$, $p = 0.009$) and presented a reduction in the number of open arms entries (**Figure 1E**, $t = 2.375$, $p = 0.031$), when compared with control animals. No differences were found in the time and entries in the closed arms (data not shown; time: $t = 0.479$, $p = 0.637$; entries: $t = 0.872$, $p = 0.392$).

In the L/D test, iuGC animals presented an increase in the ratio dark/light (**Figure 1F**, $t = 2.765$, $p = 0.014$) and no differences in distance traveled in both compartments (**Figure 1G**, dark: $t = 0.117$, $p = 0.909$; light: $t = 0.137$, $p = 0.893$).

In the confined cage, while control animals rarely emitted aversive 22 kHz USVs, iuGC animals vocalized throughout the exposure (**Figure 1H**; $U = 81$, $p = 0.016$). Similarly, iuGC group presented increased freezing behavior (**Figure 1I**; $t = 2.846$, $p = 0.013$). Upon habituation to the cage (2 days of exposure), iuGC animals no longer presented this anxious-like response.

To investigate reactivity to an adverse stimulus, we performed a variation of the classical fear-conditioning paradigm. Animals were conditioned to a cue (light) predicting painful electric shocks. After cage habituation, animals were given six pairs of cue-shock. No emission of 22 kHz USVs was observed in the baseline phase (0–3 min), but upon light/shock pairings, as expected, both groups significantly emitted more negative USVs. iuGC animals emitted negative USVs to a greater extent than control animals (**Figure 2A**, $t = 1.562$, $p = 0.130$) and also presented an increase in freezing behavior time (**Figure 2B**, $t = 2.355$, $p = 0.034$). After this conditioning session, in the next day, animals were exposed to the cue, but no shock was given. In the initial period, both groups emitted more 22 kHz USVs than the day before ($F_{1,58} = 4.10$, $p = 0.047$); cue exposure elicited more negative USVs in both groups, but again, iuGC animals were over-reactive (**Figure 2C**, $t = 2.804$, $p = 0.011$). Analysis of freezing behavior further confirmed the phenotype of iuGC group (**Figure 2D**, $t = 2.087$, $p = 0.049$). Plotting the number of negative USVs along time further confirms that iuGC animals were over-reactive to the cue predicting the adverse stimulus, since iuGC group emitted more context-induced 22 kHz vocalizations than control group, especially in the first 60 s (**Figure 2E**, $F_{1,131} = 104.42$, $p < 0.0001$). Upon the first cue exposure (ON period), both groups increased the number of USVs with no major differences between them. However, upon second cue exposure, iuGC animals emitted more negative USVs than control group and remained over-reactive throughout. The pattern of 22 kHz USVs emission in both groups is interesting since immediately after the light is turned off, both groups emit more negative vocalizations, indicative of the consolidated association of the cue with the electric shock.

iuGC EXPOSURE INDUCES PROMINENT CHOLINERGIC ALTERATIONS

Pharmacological studies suggest that the ascending cholinergic tegmental system is responsible for the initiation and production of negative vocalizations in rodents (43–45). In addition, cholinergic signaling is highly responsive to stress/GC (46) and is important for the manifestation of aversive behaviors (47). Considering these findings, we decided to further explore the impact of iuGC exposure in the cholinergic circuitry. To do so,

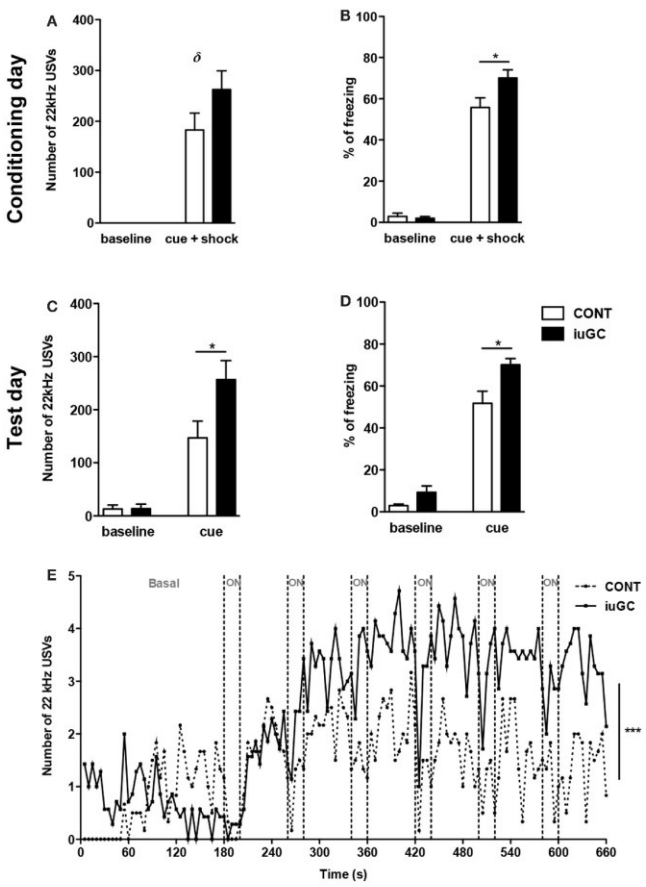

FIGURE 2 | Prenatal exposure to GC leads to amplified response to adverse stimulus. **(A)** Number of 22 kHz USVs on the conditioning day (cue + shock) of the fear-conditioning paradigm. iuGC group emitted more negative calls than control animals in the light/shock period. **(B)** Similarly, iuGC group displayed increased percentage of freezing behavior in comparison with control animals. On the test day, animals were exposed six times to cue but no shock was given. iuGC presented an increase in the number of USVs **(C)** and in freezing time **(D)**. **(E)** iuGC animals are hyper-reactive throughout time, since they emit more context-induced 22 kHz vocalizations than control group, especially in the first 60 s. Upon first cue exposure (ON period), both groups increased the number of USVs with no major differences between them. However, upon second cue exposure, iuGC animals emitted more negative USVs than controls and remained over-reactive during time. CONT, control animals; iuGC, *in utero* GC exposed animals; USVs, ultrasonic vocalizations. $*p < 0.05$, $**p < 0.001$, $***p < 0.0001$, δ: trend, $p = 0.130$; **(A–E)** $n = 8$–16.

we have performed IHC against ChAT, the key enzyme in ACh synthesis. The number of ChAT-positive cells in the LDT was significantly higher in iuGC animals when compared to control group (**Figures 3A,B**; 49% increase; $U = 3$, $p = 0.026$). The PPT, a LDT adjacent region, also presented substantial increase in the number of ChAT-positive cells (**Figures 3C,D**; $U = 2$, $p = 0.024$). On the contrary, the nucleus basalis of Meynert did not show any significant alteration in the number of cholinergic positive cells (**Figures 3E,F**; $U = 8$, $p = 0.315$). Other regions such as the NAc (core and shell), rich in cholinergic interneurons, also did not show any differences in the number of ChAT-positive cells (**Figures 3G,H**; core: $U = 13$, $p = 0.927$; shell: $U = 6$, $p = 0.164$).

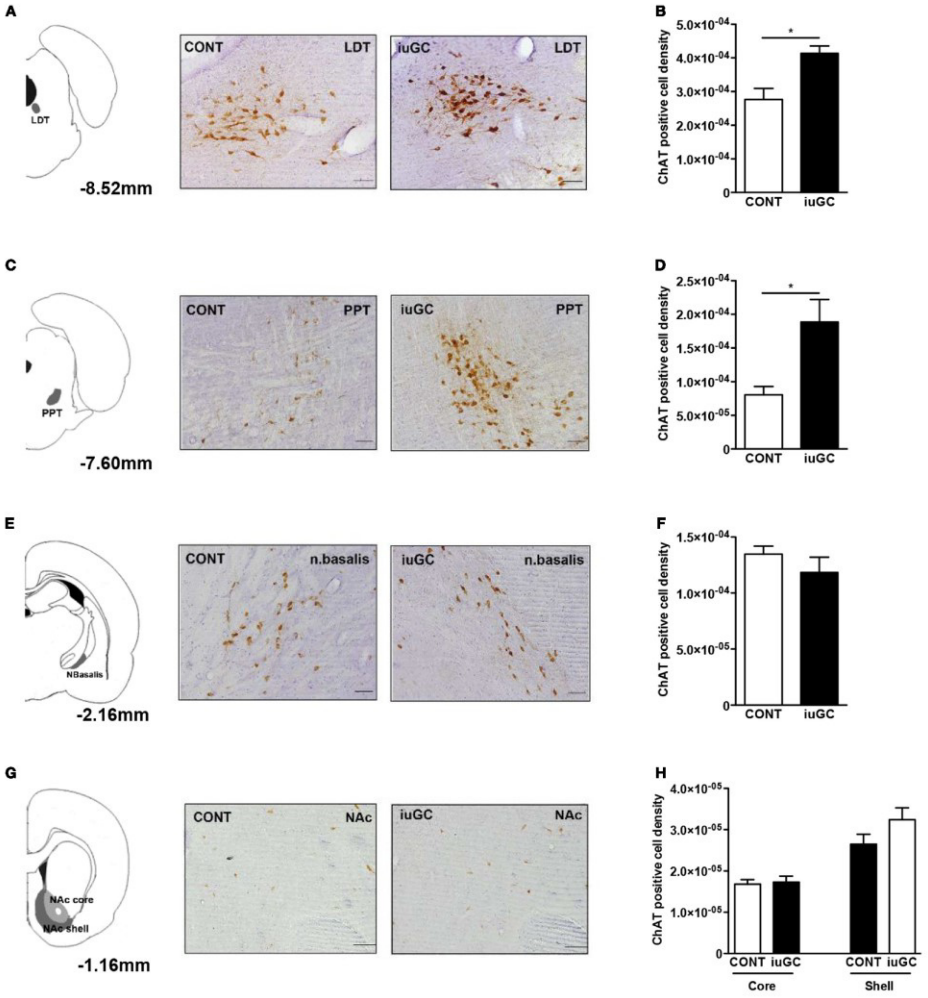

FIGURE 3 | Mesopontine cholinergic de-regulation in iuGC animals. **(A)** LDT ChAT immunohistochemistry revealed that iuGC animals presented increased ChAT expression **(B)**. **(C,D)** Similarly, iuGC animals present increased ChAT staining in the PPT region. Conversely, no differences were found in ChAT expression in the nucleus basalis of Meynert **(E,F)** or in the nucleus accumbens core or shell **(G,H)**. Representative images of coronal brain sections; numbers represent distance in mm posterior to bregma. CONT, control animals; iuGC, *in utero* GC exposed animals; LDT, laterodorsal tegmental nucleus; NAc, nucleus accumbens; PPT, pedunculopontine tegmental nucleus. $*p < 0.05$, $**p < 0.001$, $***p < 0.0001$; **(A)** $n = 4$–7. Scale bars: 50 μm.

Because iuGC can induce relevant structural changes, we measured the volume and number of cells in the LDT and PPT nuclei. No statistical differences regarding the volume of LDT were found between control and iuGC animals (**Figures 4A–C**; volume: $U = 5$, $p = 0.486$; cell numbers: $U = 3$, $p = 0.200$). In the PPT, no significant differences in volume and cell numbers were found

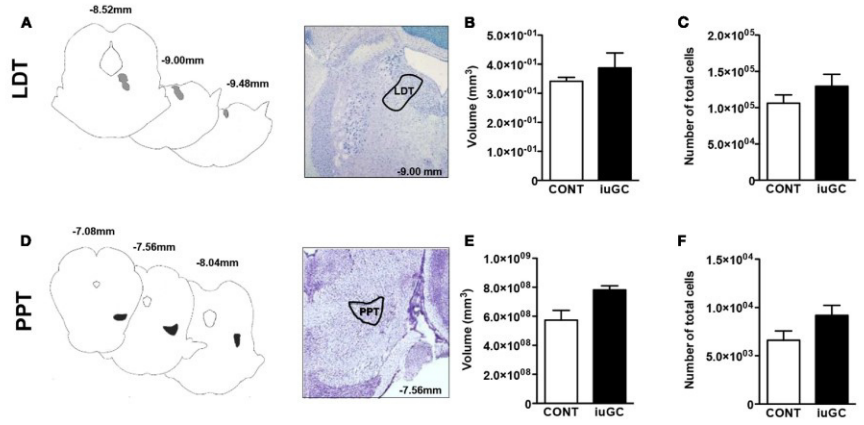

FIGURE 4 | Stereological data of LDT and PPT regions. No major differences were observed in the volumes and cell numbers of LDT **(A–C)** and PPT regions **(D–F)** of iuGC animals when compared to controls. Representative images of coronal brain sections; numbers represent distance in millimeters posterior to bregma. CONT, control animals; iuGC, *in utero* GC exposed animals; LDT, laterodorsal tegmental nucleus; PPT, pedunculopontine tegmental nucleus. **(B,C)** $n = 5$–6; **(E,F)** $n = 4$.

(**Figures 4D–F**; volume: $U = 2$, $p = 0.114$; cell numbers: $U = 2$, $p = 0.229$).

To assess if the observed increase in ChAT-positive cells in iuGC group was translated into augmented ACh release, we measured the levels of ACh in cholinoceptive regions. A trend for increased ACh levels in the hypothalamus and the amygdala was found (data not shown).

CHOLINERGIC NEURONS IN THE LDT AND PPT ARE DIFFERENTIALLY ACTIVATED UPON ADVERSE STIMULUS IN iuGC ANIMALS

To better determine the impact of iuGC in cholinergic neurons, and the relevance of such changes in reaction to adverse stimuli, we evaluated neuronal activation patterns using c-fos labeling in combination with ChAT after the fear-conditioning protocol.

Briefly, animals were subjected to the modified fear-conditioning protocol, and sacrificed 90 min after stimuli on the test day. As depicted in **Figure 5**, after an adverse stimulus, iuGC animals presented a significant increase (85%) in the number of c-fos-positive cells in the LDT (**Figures 5A,B**; $U = 0$, $p = 0.008$). The number of ChAT-positive cells was also substantially augmented (64%) in iuGC animals when compared to control animals (**Figure 5C**; $U = 0$, $p = 0.014$).

Regarding the PPT, after the adverse stimuli, iuGC animals present 2.5 times more c-fos-positive cells than control animals (**Figures 5E,F**; $U = 0$, $p = 0.002$). Similarly, the number of ChAT-positive cells was also increased in iuGC animals (**Figure 5G**; $U = 0$, $p = 0.002$).

To determine if the observed increase in c-fos-positive cells was due to enhanced cholinergic activation, we also quantified the number of c-fos/ChAT-positive cells after fear conditioning. Remarkably, iuGC animals presented a substantial increase (186%) in the number of c-fos/ChAT-positive cells in the LDT in comparison to control animals (**Figure 5D**; $U = 4$, $p = 0.029$). Similarly, in the PPT, we observed that iuGC animals display a four-time increase in the number of double c-fos/ChAT-positive cells (**Figure 5H**; 3.358 vs. 0.801%, $U = 4.5$, $p = 0.030$).

DISCUSSION

Confirming previous findings (12, 14, 48), we observed that iuGC animals present an anxious phenotype. In the EPM, iuGC animals presented a decrease in the time and entries in the open arms when compared with control animals, in accordance with the L/D test, where they spent less time in the anxiogenic compartment. Moreover, iuGC animals emitted more 22 kHz negative calls and presented enhanced freezing behavior in the confined cage paradigm. These differences were eliminated by habituation. This is in accordance with previous work showing that rats that are highly anxious tend to vocalize more often and present augmented freezing time during aversive stimuli than rats that display low anxiety-like trait (35). In the fear-conditioning paradigm, iuGC animals emitted more negative calls and enhanced freezing behavior on the conditioning day. This suggests an emotional over-reactivity of iuGC animals to adverse stimulus. In further support of this idea, iuGC animals emitted more negative calls than control subjects in response to the cue predicting the harmful stimulus and during ISI, suggesting an over-reactive response. Altogether, our data confirms that iuGC exposure leads to anxious behavior and exacerbated response to stressful events in adulthood, a finding also observed in other stress models (49–51). Similarly, a rat line that displays signs of extreme trait anxiety also presents increased stress vulnerability and reactivity (52).

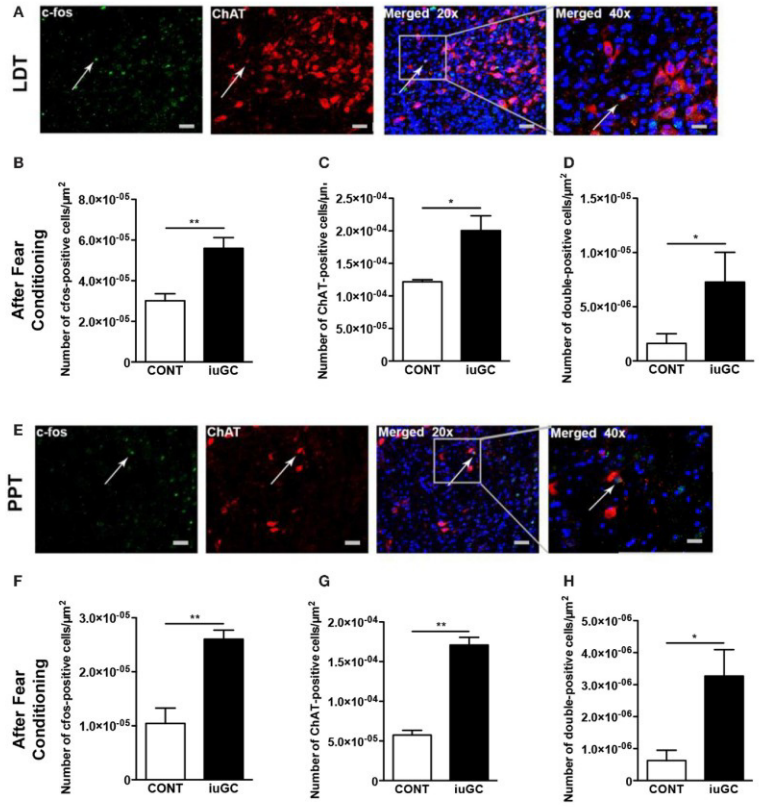

FIGURE 5 | Differential cholinergic activation of the LDT and PPT regions in iuGC animals after an adverse stimulus. **(A)** Representative images of LDT ChAT (red labeling) and c-fos (green labeling) immunostaining in animals exposed to the fear-conditioning test. **(B)** Increased c-fos activation in iuGC animals. **(C)** ChAT expression was increased in iuGC animals in comparison to controls. **(D)** The number of c-fos/ChAT-positive cells was substantially increased in the LDT of iuGC animals. **(E)** Representative images of PPT ChAT and c-fos immunostaining in animals exposed to the fear-conditioning test. **(F)** Increased c-fos staining in iuGC animals. **(G)** ChAT expression was augmented in iuGC animals. **(H)** The number of c-fos/ChAT-positive cells is significantly increased in the PPT of iuGC group. CONT, control animals; iuGC, *in utero* GC exposed animals; LDT, laterodorsal tegmental nucleus; PPT, pedunculopontine tegmental nucleus. $*p < 0.05$, $**p < 0.001$; **(A–H)** $n = 6$. Scale bars: 50 µm.

One remarkable finding was the increased emission of negative calls in iuGC animals, hinting differential activity of the mesopontine cholinergic circuitry, that mainly comprises projections from the LDT region (43, 44, 53, 54). In accordance, we found increased ChAT expression in the LDT of iuGC animals in a basal situation, suggesting overproduction of ACh. Upon exposure to a cue predicting an adverse stimulus, there was a marked increase in the number of c-fos/ChAT-positive cells, potentially explaining iuGC behavioral response. Indeed it was shown that LDT activation induces a complex state of defensiveness, critical for alarm responses to dangerous stimuli (44, 45). Our results are in accordance with previous work, in which they have shown that pharmacological stimulation of the LDT lead to an increase in the emission of 22 kHz USVs (44). In addition, the increased LDT cholinergic signaling could also be implicated in the impaired negative HPA axis feedback of iuGC animals (12) since LDT-arising ACh enhances ACTH and CRF release (28, 55, 56). On the other hand, GC also modulate cholinergic signaling (19), suggesting a reciprocal ACh-GC control that may culminate in a vicious loop.

Alike the LDT, iuGC animals presented increased PPT cholinergic signaling both in a basal situation and after exposure to the cue predicting an adverse stimulus. Apart from the classical role of PPT in sleep and arousal, lesion studies suggest a role in anxiety modulation, although both anxiogenic and anxiolytic effects were found depending on the degree and type of lesion. Excitotoxic and electrolytic PPT lesions induce an anxiogenic-like status (57, 58), contrary to one study that suggests a slight anxiolytic effect (59). On the other hand, ibotenic lesions seem to reduce anxious-like phenotype (60). Apart from distinct technical procedures, it has been suggested that opposing results may arise from damage in different sub-regions within the PPT (pars compacta vs. dissipata) or in surrounding regions, namely the cuneiform nucleus (59, 61).

This programing effect of iuGC exposure is not so surprising considering the importance of GC receptors for the maturation of medial septal and hippocampal cholinergic neurons (62, 63), yet, more studies need to be performed to understand how GC exert long-lasting functional and molecular changes in these neurons.

In utero glucocorticoid exposure-induced alterations in the mesopontine cholinergic pathway may go beyond a direct effect of cholinergic inputs on behavioral output. For instance, LDT-dependent cholinergic activation of VTA evokes dopamine release in the NAc (20, 21, 64) driving motivational and reward behaviors (65, 66). However, these findings are somewhat contradictory to the VTA-accumbal hypodopaminergia and anhedonia observed in iuGC animals (6, 14, 15). One possible explanation is that the sustained augmented LDT-VTA ACh signaling could desensitize or alter the expression/epigenetic status of the nicotinic/muscarinic receptors as a compensatory mechanism. Indeed, GC or acute stress can induce prominent ACh release in specific brain regions (46, 67, 68), and transiently change the expression levels of different cholinergic players through c-fos binding to promoter regions of target genes in order to maintain the homeostasis (19). Additional studies focusing on the regulation of the expression of different cholinergic players by GC will be critical to better comprehend our findings.

In summary, our results show for the first time that prenatal GC exposure programs the mesopontine cholinergic pathway, leading to cholinergic hyperactivation of both the LDT and PPT, which in turn can underlie the anxious behavior and enhanced stress reactivity observed in these animals.

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CHAPTER 2.2

Borges S, Ventura-Silva AP, Sousa N, Georges F, Pêgo JM and Rodrigues AJ

Impact of *in utero* glucocorticoid exposure on the BNST neuronal activity

Impact of *in utero* glucocorticoid exposure on the BNST neuronal activity

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Abstract

In utero exposure to synthetic glucocorticoids (iuGC) induces morphological and structural changes in several brain regions, which can lead to behavioral and neuroendocrine deficits.

One of the brain regions targeted by stress and glucocorticoid exposure is the bed nucleus of the stria terminalis (BNST), which receives and send multiple inputs, being an important modulator of stress response and anxious behavior. The anterior BNST (aBNST) division receives inputs from limbic structures, including the infralimbic cortex (ILCx) and the central nucleus of the amygdala (CeA), and it also sends projections to reward-related areas (such as the ventral tegmental area (VTA)) and stress-related nuclei (including the paraventricular nucleus (PVN) of the hypothalamus).

Due to its important role in the modulation of behaviors altered by iuGC, here we studied the effect of iuGC on the BNST network. Specifically, using an *in vivo* electrophysiology approach in anesthetized rats, we studied the effect of electric stimulation in upstream regions, the ILCx and CeA, on the neuronal activity of the BNST, in control and iuGC animals.

In addition, we also evaluated the effect of iuGC exposure on the neuronal activity of a downstream region, the VTA.

Our results show that iuGC exposure does not induce any major effect on the BNST network.

Introduction

In the recent years, the bed nucleus of the stria terminalis (BNST), has been shown to play a key role in the development of stress-related psychiatric diseases, such as, anxiety and addiction (Avery et al., 2016; Lebow and Chen, 2016). The BNST has a privileged position to instigate allostatic changes in the organism through its dense projections with the paraventricular nucleus (PVN) of the hypothalamus, the knot of hypothalamic–pituitary–adrenal (HPA) axis (Dong and Swanson, 2006), which regulates the corticosteroid response. The BNST has also been documented to be a significant target of stress (Davis et al., 2010; McElligott and Winder, 2009). In addition, the anterior BNST division (aBNST) positively regulate the HPA axis (Ulrich-Lai and Herman, 2009).

The BNST receive projections from upstream limbic areas, among them, two of the most important inputs to the aBNST come from the medial prefrontal cortex (mPFC), especially, the infralimbic cortex (ILCx); and from the amygdala, in particular, the central nucleus of the amygdala (CeA) (Dong et al., 2001; Massi et al., 2008; Vertes, 2004). The ILCx exerts an excitatory influence, through glutamatergic projections to the aBNST, while the CeA is rich in GABAergic neurons that show co-expression of GABA and several neuropeptides (Marchant et al., 2007; Massi et al., 2008). Both upstream regions are important for the development of anxiety behaviors (Bi et al., 2013; Jinks and McGregor, 1997; Kalin, 2004; Ventura-Silva et al., 2013).

Also recent studies have focus on the projection of the ventral BNST (vBNST) to a downstream region, the ventral tegmental area (VTA). Stimulation of the vBNST by local microinfusion of glutamate increased the firing and bursting activity of VTA dopamine (DA) neurons. Whereas, microinfusion of GABA decreased bursting of VTA DA neurons without altering their firing rate. Retrograde and anterograde labeling and antidromic activation of vBNST neurons by VTA stimulation confirmed a direct projection from the vBNST to the VTA (Georges and Aston-Jones, 2002). Moreover, the two existent subpopulations of vBNST–VTA projections have opposing roles. In response to a foot-shock, the glutamatergic BNST-VTA neurons present an increase in their activity, contrary to GABAergic BNST-VTA neurons (Jennings et al., 2013). *In vivo* photostimulation of BNST glutamatergic projections resulted in aversive and anxiogenic behavioral phenotypes, in opposition, activation of BNST GABAergic projections produced rewarding and anxiolytic phenotypes (Jennings et al., 2013).

Despite several studies focusing on the BNST, and the effect of acute and chronic stress on this brain region, little is known about the impact of exposure to elevated levels of glucocorticoids (GCs) during specific periods of gestation. Previous work from our lab has shown that prenatal exposure to synthetic GCs induces the development of anxiety-like behavior in rodents. Additionally, it was observed that this exposure also induces a long-lasting hypertrophy in the BNST (Oliveira et al., 2012), eventually explaining the HPA axis impairment and hyperanxious phenotype observed in these animals.

The aim of this project was to investigate the effect of prenatal exposure to GCs in the BNST network. Hence, we performed an extensive electrophysiological characterization of the neuronal activity of BNST neurons upon ILCx or CeA stimulation in a model of *in utero* GCs (iuGC) exposure. Additionally, we also analysed the basal neuronal activity of a downstream region, the VTA.

Material and Methods

Animals

For electrophysiology recordings, pregnant Sprague Dawley rats were individually housed, under standard laboratory conditions (22°-23°C, 50–55% relative humidity, 12 h light/dark cycle with lights on at 07:00); food and water *ad libitum*. Subcutaneous injections of dexamethasone at 1 mg/kg (iuGC animals) or with the vehicle - sesame oil (control animals – CONT), were administered at 18 and 19 days of gestation. On postnatal day 21 (PND21), male offspring was housed (six per cage), according with prenatal treatment. Electrophysiological recordings were performed when the animals were 2 months old. All protocols were approved by the Bordeaux University Animal Care and Use Committee (N°50120205-A) on animal care and experimentation.

A different group, with 2 months, was used for behavioral assessment. Same prenatal treatment, as previously mentioned, was applied to pregnant rats. On PND21, progeny was weaned according to prenatal treatment and gender (two per cage). All protocols were approved by the Ethics Committee of the Life and Health Sciences Research Institute.

All procedures were conducted in accordance with the European directive 2010-63-EU.

Surgery

Animals were submitted to stereotaxic surgery for the placement of the stimulating and recording electrodes, following anatomical coordinates (Paxinos and Watson, 2007). Surgeries were performed under isoflurane anaesthesia as previously described (Georges and Aston-Jones, 2002); body temperature was maintained at approximately 37°C with a homeothermic heating blanket (DC temperature controller, FHC). Incision was made through the skin and connective tissues. Holes were drilled in the skull and stimulating and recording electrodes were placed in the following coordinates: ILCx: - 3.0 mm anteroposterior (AP), + 0.5 mm lateral mediolateral (ML), - 4.5 mm dorsoventral (DV); CeA: - 2.2 mm AP, + 4.2 mm ML, - 7.0 mm DV; VTA: - 5.8 mm AP, + 0.6 mm ML, - 7.5 to - 8. 5 mm DV and aBNST: - 0.5 mm AP, + 1.2 mm ML, - 6 to - 7.5 mm DV from bregma. A reference electrode was fixed in the muscle.

Electrical Stimulation

A bipolar concentric electrode (Phymep) was inserted in the ILCx or the CeA brain region. The stimulation was administered using a square pulse stimulator (CED micro 1401 interface, SPIKE 2 software, Cambridge Electronic Desing) and a stimulus isolator (DS3, Digitimer). After isolation of a single neuron, before stimulation, spontaneous activity was recorded to establish baseline activity for at least 100 s. Then, single pulses were delivered to the specific brain region every 2 s, at least 100 trials of 0.5 Hz with 0.5 ms of duration with intensity from 0.2-1 mA.

We also performed a plasticity protocol where baseline was recorded for 10 min (2 x 100 pulses; 0.5 Hz) and tetanic stimulation was administered (1 min; 10 Hz) at the same intensity used for baseline (0.2–1 mA). Only one cell per animal was recorded for neuroplastic responses. Recordings were continued for at least 40 min following tetanic stimulation, during this period 100 pulses of 0.5 Hz stimulation were performed. Long term potentiation/long term depression (LTP/LTD) was quantified by comparing the mean percentage of response magnitude over the 35 to 40 min post-tetanus period with the mean percentage of response magnitude during the baseline period.

In vivo anesthetized electrophysiology recordings

Extracellular neural activity in the aBNST and in the VTA was recorded using a glass micropipette (aBNST: tip diameter, 1–2 μm; 10–15 MΩ; VTA: tip diameter, 2–3 μm; 4–6 MΩ), filled with a 2% pontamine sky blue solution in 0.5 M sodium acetate. Recordings were amplified and filtered by the Axoclamp (Molecular Devices) (low-pass filter at 300 Hz and high-pass filter at 0.5 kHz). Spikes of single neuron were discriminated, and digital pulses were led to a computer for online data collection using a laboratory interface and software (CED micro 1401 interface, SPIKE 2 software; Cambridge Electronic Design).

Histology and microscopy

At the end of each electrophysiological experiment, placements of stimulating and recording electrode tips within the ILCx, CeA, VTA or aBNST were verified with histological examination of brain tissue. The glass micropipette placement was marked with an iontophoretic deposit of pontamine sky blue dye (20 μA, 30 min). To mark electrical stimulation site, 50 μA was passed through the stimulation electrode for 90 s.

Animals were sacrificed under isoflurane anaesthesia and brains were collected and snap frozen in isopentane at -80°C. Coronal sections (30 μm) were cut in a cryostat (Leica), mounted and stained with neutral red for histological determination of the localization of recording and stimulation sites.

Electrophysiological data analysis

For electrophysiology data analysis, during electric stimulation of ILCx or CeA, it was generated in the aBNST a peristimulus time histograms (PSTHs; 5 ms bin width) of neuronal activity for each neuron recorded. PSTHs were analyzed to determine excitatory and inhibitory epochs. Briefly, the mean and standard deviation (SD) of counts per bin were determined for a baseline period, definite as the 500 ms epoch previous stimulation. The onset of excitation was defined as the first of five bins whose mean value exceeded mean baseline activity by 2 SD, and response offset was determined as the time at which activity had returned to be consistently within 2 SD of baseline. Response magnitudes for excitation were calculated with the following equation: (counts in excitatory epoch) - (mean counts per baseline bin x number of bins in excitatory epoch). Inhibition was defined as an epoch of at least 15 bins in which the mean count per bin was at least 35% less than that during baseline. Response magnitude for inhibition were calculated with the following equation: (counts in inhibitory epoch) - (mean counts per baseline bin x number of bins in inhibitory epoch).

Two parameters of the VTA recordings were recorded: the spontaneous firing rate and the proportions of spikes that occurred in burst. The onset of a burst was defined as the occurrence of at least two spikes with an interspike interval less than 80 ms (Grace and Bunney, 1983). The population activity is calculated by total number of dopaminergic (DAergic) cells divided by number of tracks. Moreover, the percentage of spikes in bursts was calculated by dividing the number of spikes occurring in bursts by the total number of spikes occurring in the same period of time.

Behavioral tests - Anxiety behavior test

All tests were performed during the day period and all behavioral equipment was cleaned between animals (ethanol 10%), in order to remove any olfactory cues.

Light/dark box test

The light/dark box (L/D) test was performed inside the open field (OF) arena (43.2 cm × 43.2 cm; model ENV-515; MedAssociates Inc.). A dark compartment was attached to one side with an opening facing the center of the arena. Animals were individually placed in the center of the illuminated part. The distance travelled and time spent in each compartment was recorded in a single trial of 10 min.

Open field

Animals were individually tested for 5 min each, in an OF arena that had transparent acrylic walls and a white floor (43.2cm×43.2cm; model ENV-515; MedAssociates Inc.). Each subject was initially placed in the center of the arena and horizontal activity and instant position were registered, using a system of two 16-beam infrared arrays connected to a computer. Total distance was used as indicator of locomotor activity. Time in the predefined central and number of ambulatory counts were recorded.

Statistical analysis

Data is presented as mean ± standard error of the mean (SEM). Statistical analysis was performed using Graphpad Prism 6.01 software. Data was verified for Gaussian distribution. Two group comparisons were achieved by using Student t-test. When no Gaussian distribution was assumed, a nonparametric test (Mann-Whitney U-test) was used. For multiple comparison, statistical significance was assessed, as appropriate, by one- or two-way analysis of variance (ANOVA). Bonferroni's *post-hoc* multiple comparisons were used for group differences determination. Statistical significance was set at $p<0.05$.

Results

BNST neuronal activity in *in utero* glucocorticoid-exposed (iuGC) animals

First, the neuronal activity of the anterior division of the BNST in CONT and in iuGC rats was recorded (Sprague Dawley strain), as shown in figure 1. No differences were found in the spontaneous firing rate between CONT and iuGC groups, although there was a trend for decreased activity in iuGC animals (Fig.1B, U=946, p=0.296).

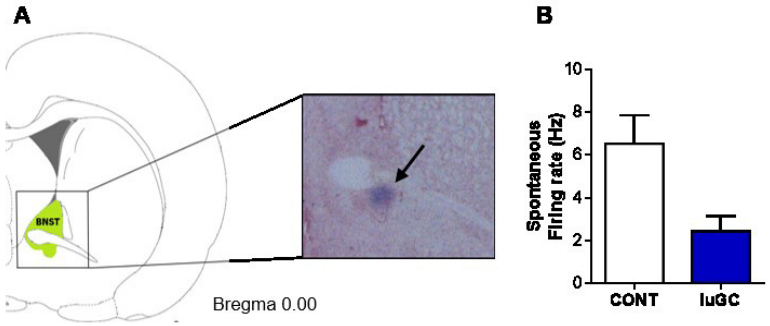

Figure 1. The effect of iuGC exposure on BNST neuronal activity. (A) Histological control of anterior BNST of pipette recordings sites. Bregma 0.00. **(B)** Spontaneous firing rate of CONT and iuGC animals on the anterior BNST. No differences were found between groups.

BNST, Bed nucleus of the stria terminalis; CONT, Control; iuGC, *in utero* glucocorticoid; Total number of cells: n_{CONT}= 65, n_{iuGC}=35; Data presented as mean ± SEM.

Then the activity of these neurons in response to upstream stimulation of the ILCx at low and high intensity was registered (100 pulses; 0.5 Hz; Intensity: Low – 0.2-0.5 mA; High – 1 mA). No differences were found between groups, regarding the magnitude and duration of the response (Fig.2C, Response Magnitude: $F_{(1,149)}$=0.949, p=0.332; $CONT_{Low}$ vs $iuGC_{Low}$ p=0.371; $CONT_{High}$ vs $iuGC_{High}$ p>0.999; Fig.2D, Duration: $F_{(1,144)}$=3.563, p=0.677; $CONT_{Low}$ vs $iuGC_{Low}$ p>0.999; $CONT_{High}$ vs $iuGC_{High}$ p>0.999).

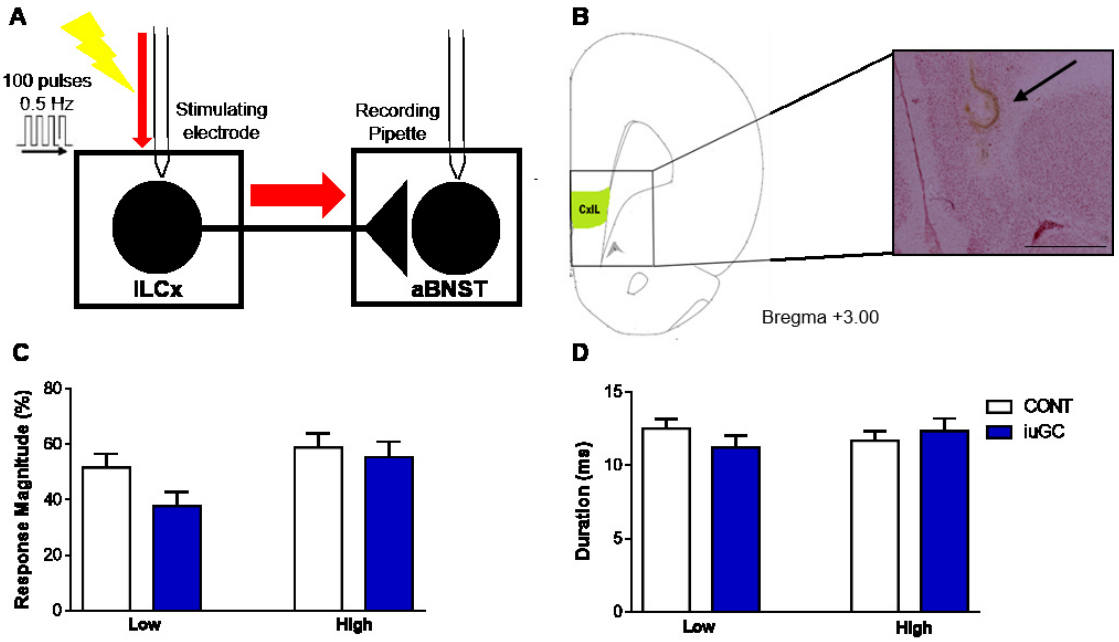

Figure 2. Excitatory response in the aBNST upon ILCx stimulation. (A) Stimulation and recording protocol. **(B)** Histological control of ILCx of stimulation sites. Bregma 3.00. Scale bar: 1mm. **(C)** Percentage of response magnitude and **(D)** duration of aBNST neurons upon an ILCx stimulation. No differences were observed between groups.

aBNST, anterior Bed nucleus of the stria terminalis; ILCx, Infralimbic cortex; CONT, Control; iuGC, *in utero* glucocorticoid; Total number of cells: Response Magnitude - Low - n_{CONT}= 51, n_{iuGC}=32; High - n_{CONT}= 42, n_{iuGC}=28; Duration - Low - n_{CONT}= 48, n_{iuGC}=33; High - n_{CONT}= 39, n_{iuGC}=28; Intensity: Low – 0.2 and 0.5 mA; High – 1mA; Data presented as mean ± SEM.

In addition, we determined if the application of a 1 min *in vivo* electric activation of the ILCx at a physiologically relevant frequency (10 Hz) (Jackson et al., 2001), triggered neuroplastic changes in aBNST neurons. There were no differences through time and between groups (Fig.3A, Time: $F_{(9,98)}$=1.631, p=0.12; Group: $F_{(9,98)}$=0.426, p=0.53). The 10 Hz ILCx stimulation lead to no alteration in ILCx evoked spike probability in the aBNST (Fig.3B, Time: $F_{(1,18)}$=2.043 , p=0.17; Group: $F_{(1,18)}$=0.373, p=0.549; $CONT_{Before}$ vs CTR_{After} p>0.999; $iuGC_{Before}$ vs $iuGC_{After}$ p>0.999; CTR_{Before} vs $iuGC_{Before}$ p>0.999; CTR_{After} vs $iuGC_{After}$ p>0.999).

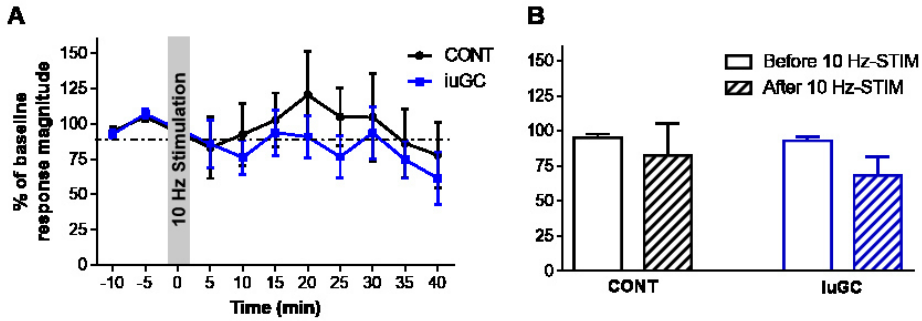

Figure 3. The effect of 10 Hz stimulation in the ILCx onto aBNST neurons. (A) Kinetic (left) and **(B)** quantification (right) of the mean percentage of change in ILCx-evoked spike probability, normalized to the baseline, after a 10 Hz stimulation at t=0 in CONT and iuGC animals. No difference was observed between groups.

aBNST, anterior Bed nucleus of the stria terminalis; ILCx, Infralimbic cortex; CONT, Control; iuGC, *in utero* glucocorticoid; Total number of cells: n_{CONT}= 5, n_{iuGC}=6; Data presented as mean ± SEM.

In addition, CeA was stimulated and BNST-evoked activity was evaluated. Electrical stimulation (100 pulses; 0.5Hz) of the CeA lead to a similar response in the aBNST neuronal activity in CONT and iuGC animals (Fig.4C, Response Magnitude: $F_{(1,40)}$=1.763, p=0.192; $CONT_{Low}$ vs $iuGC_{Low}$ p>0.999; $CONT_{High}$ vs $iuGC_{High}$ p>0.999; Fig.4D, Duration: $F_{(1,38)}$=0.537, p=0.468; $CONT_{Low}$ vs $iuGC_{Low}$ p>0.999; $CONT_{High}$ vs $iuGC_{High}$ p>0.999).

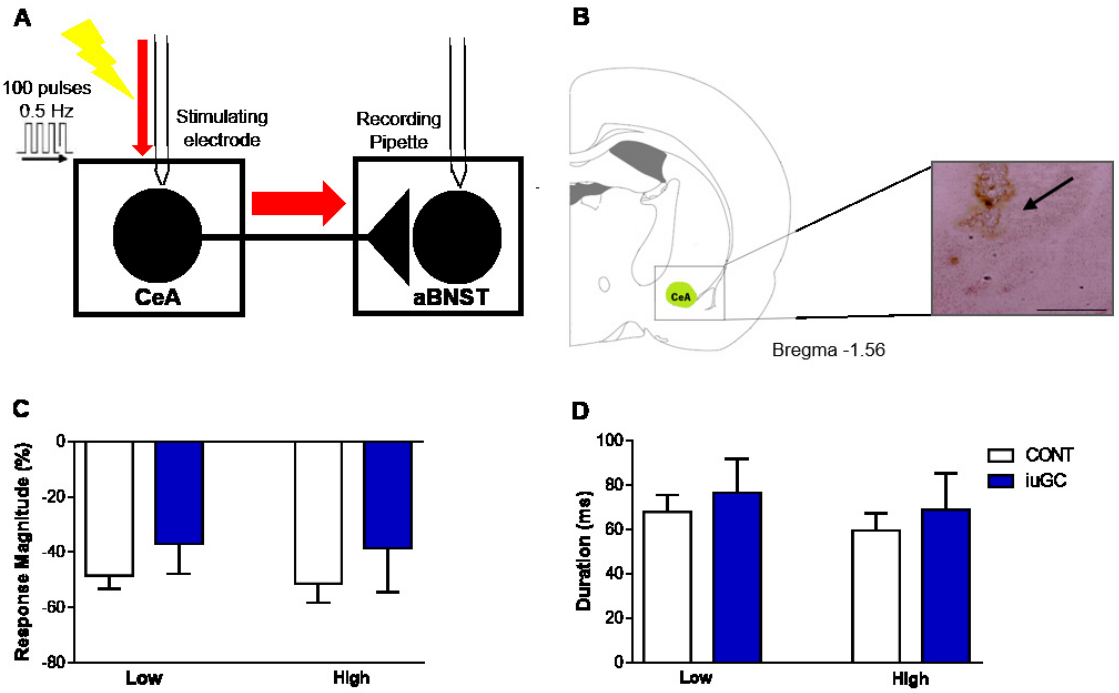

Figure 3. Inhibitory response upon an CeA stimulation. (A) Stimulation and recording protocol. **(B)** Histological control of CeA of stimulation sites. Bregma -1.56. Scale bar: 1mm. **(C)** Percentage of response magnitude and **(D)** duration of aBNST neurons upon a CeA stimulation. No differences were observed between groups.

Central nucleus of the amygdala, CeA; anterior Bed nucleus of the stria terminalis, aBNST; control, CONT; *in utero* glucocorticoid, iuGC; Total number of cells: Response Magnitude - Low - n_{CONT}= 20, n_{iuGC}=6; High - n_{CONT}= 12, n_{iuGC}=4; Duration - Low - n_{CONT}= 0, n_{iuGC}= 6; High - n_{CONT}= 12, n_{iuGC}= 4; Intensity: Low – 0.2 and 0.5 mA; High – 1mA; Data presented as mean ± SEM.

VTA neuronal activity in iuGC animals

Next, we performed recordings in the VTA in basal conditions as it has been previously shown that iuGC animals present significant reduction in tyrosine hydroxylase (TH) neurons in this region (Leao et al., 2007). VTA DA neurons were distinguished according to electrophysiological characteristics previously established: (1) firing frequency (less than 10 Hz) and (2) action potential width (greater than 1.5 ms) (Ungless and Grace, 2012).

Unexpectedly, iuGC exposure did not alter neither the population neuronal activity (Fig.5B, U=12, p>0.999) nor the firing rate of DAergic neurons in the VTA (Fig.5C, U=506.5, p=0.224). Regarding the proprieties of DAergic spikes in burst in the VTA, no differences were found in the burst rate (Fig.5D, U=488.5, p=0.285) and in the percentage of spikes in bursts (Fig.5E, U=511, p=0.428).

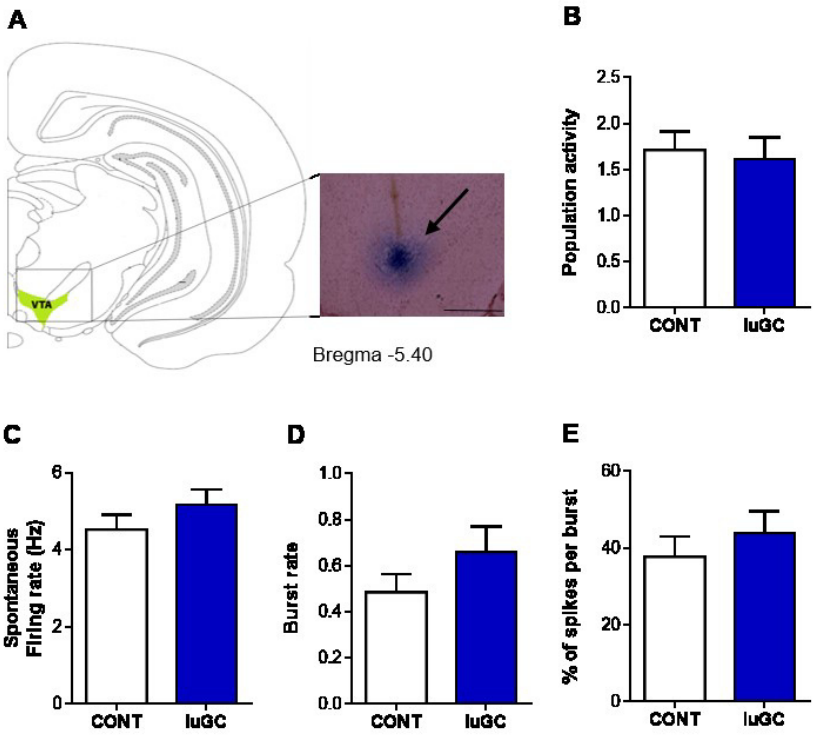

Figure 4. Effect of iuGC in VTA DA neurons basal activity. (A) Histological control of VTA of recording sites. Bregma -5.40. Scale bar: 1mm. **(B)** Population activity, **(C)** spontaneous firing rate, **(D)** burst rate and **(E)** percentage of spike per burst in CONT and iuGC animals. No differences were found between groups.

VTA, Ventral tegmental area; CONT, Control; iuGC, *in utero* glucocorticoid; Total number of animals: Population activity - n_{CONT}= 6, n_{iuGC}=4; Total number of cells: Spontaneous firing rate - n_{CONT}= 37, n_{iuGC}= 33; Burst rate - n_{CONT}= 36, n_{iuGC}= 32; % of spikes per burst: n_{CONT}= 36, n_{iuGC}= 33; Data presented as mean ± SEM.

Anxiety behavior of *in utero* glucocorticoids exposed (iuGC) animals

Previous work showed that iuGC induced significant anxious behavior in Wistar Han rats (Oliveira et al., 2006), thus we decided to evaluate the anxious behavior of iuGC Sprague Dawley animals. Animals were tested in the L/D test and the OF. In the L/D test, there were no differences regarding the ratio dark/light nor the distance travelled by each group (Fig.6A, Ratio Dark/light: U=24, p=0.291; Fig.6B, Distance: Group - $F_{(1,40)}$=0.379, p=0.541; $CONT_{Dark}$ vs $iuGC_{Dark}$ p>0.999; $CONT_{Light}$ vs $iuGC_{Light}$ p>0.999).

In the OF, iuGC animals presented no differences in the number of ambulatory counts (Fig.6C, t_{20}=0.55, p=0.588), total distance travelled (Fig.6D, t_{20}=0.587, p=0.564) and time spent in the center of the arena (Fig.6E, t_{20}=0.387, p=0.703).

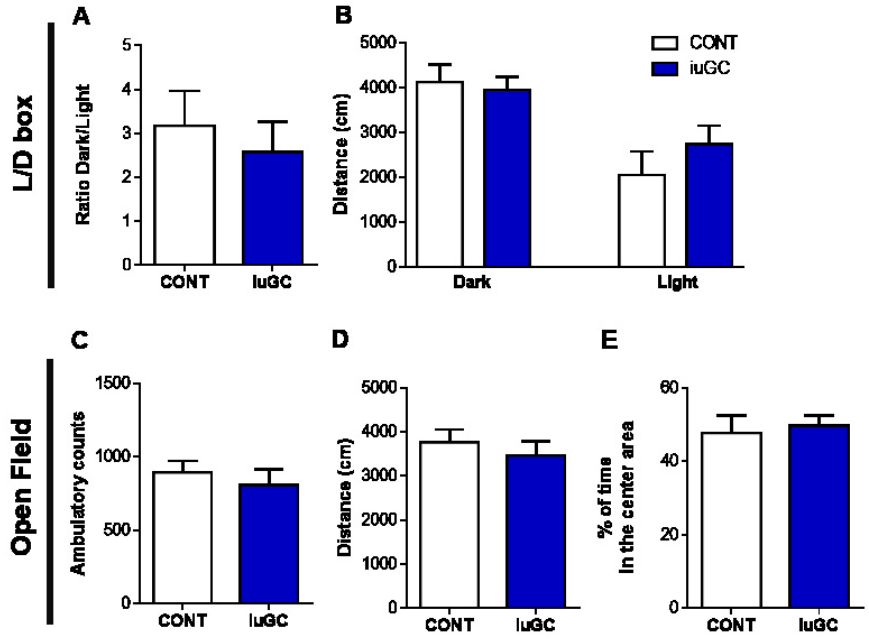

Figure 5. Behavioral results. (A) Ratio of Dark/Light time and **(B)** distance travelled in the light/dark box test by CONT and iuGC Sprague Dawley animals. No differences were found between groups. **(C)** Regarding the open filed, no differences were found in ambulatory counts, **(D)** distance travelled and **(E)** time in the center of the arena.

CONT, Control; iuGC, *in utero* glucocorticoid; L/D, light/dark; Total number of animals: n_{CONT}= 6-8, n_{iuGC}=12-14; Data presented as mean ± SEM.

Discussion

One of the factors that leads to the development of anxiety is the exposure to stressful events. Importantly, it has been shown that the BNST acts as relay station between these anxiety-related areas and downstream regions, such as the PVN, the node of the HPA axis, which regulates the stress response.

The aim of this work was to evaluate the consequences of iuGC exposure on the BNST network, which was particularly interesting to evaluate considering our previous findings showing that i) chronic adult stress affects BNST neuronal activity (data not published), ii) iuGC induces an increase of the BNST volume (Oliveira et al., 2012), iii) iuGC induces a long-lasting anxious phenotype (Oliveira et al., 2006). As expected, the stimulation of the ILCx and CeA induced an excitation and inhibition response in aBNST, concordant with glutamatergic and GABAergic signalling, respectively. However, we did not find any significant differences between CONT and iuGC animals.

Acute and chronic stress leads to synaptic plasticity of cortical excitatory synapses (Diamond et al., 2007). Additionally, electrical stimulation of the mPFC at frequencies that mimic the physiological activation of this region in cognitive behavioral contexts (Jackson et al., 2001) trigger neuroplastic changes in the aBNST neurons (Glangetas et al., 2013). This 10 Hz tetanic stimulation lead to LTD of the aBNST neurons. Indeed, it has been observed that acute stress reverses the LTD to LTP (Glangetas et al., 2013). Once again, no differences were observed between groups, in the aBNST neuronal activity, when this stimulation was applied in the ILCx.

It has been previously reported that BNST projects to and exerts a strong excitatory influence on the firing of DA neurons within the VTA (Georges and Aston-Jones, 2001, 2002). Indeed, it has been shown that iuGC exposure also affects the VTA brain region. Offspring of pregnant rat injected with synthetic glucocorticoids presented a reduction of TH in the VTA-nucleus accumbens (NAc) projections (Leao et al., 2007). However, nothing is known about the electrophysiological proprieties of this region in this animal model. No differences were observed in firing rate and burst firing of DAergic neurons from the VTA between groups. Once again, this was unexpected, since it has been shown that acute restrain stress, repeated and combined restrain stress significantly increase VTA DA neuron population activity and burst firing (Valenti et al., 2011).

The above mentioned results were surprising considering the BNST increased volume and the known effects of stress in the BNST. Yet, one important point to consider was the fact that our previous results were performed in Wistar Han animals, which may have different vulnerability to anxious behaviors (Oliveira et al., 2006). Therefore, we have evaluated if the anxious phenotype was also present in the iuGC Sprague Dawley strain. Interestingly, iuGC exposure does not induce a hyperanxious state in Sprague Dawley animals, as evaluated by the L/D and OF test. This may be due to genetic differences or different susceptibilities between strains. Previous works has shown that prenatal exposure to synthetic glucocorticoids leads to anxiety-like behavior in Sprague Dawley strain (Hossain et al., 2008; Nagano et al., 2008), however, these authors injected synthetic GC from a longer period (from prenatal day 14 until parturition). Moreover, while some rat strains differ in their susceptibility to stressors (for example, greater responsiveness in Flinders Sensitive Line or Wistar-Kyoto strains), the literature indicate that chronic mild stress does not lead to differential stress responsiveness/susceptibility between the Wistar Han and Sprague Dawley strains (Willner, 2016). Nevertheless, it remains to be determined if GC exposure during criticial periods of development may have differential effect between the two strains.

Albeit these outcomes, we still think that the BNST plays an essential role in the development of the hyperanxious in iuGC animals and would be important to perform a similar electrophysiological study in Wistar Han animals.

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CHAPTER 3

Optogenetic modulation of social behavior

CHAPTER 3.1

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Impact of optogenetic modulation of LDT-NAc and LDT-VTA pathways in social behaviors

Impact of optogenetic modulation of LDT-NAc and LDT-VTA pathways in social behaviors

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Abstract

Social behavior is a complex and intrinsic behavior in most mammals. Pharmacological studies have identified key neurotransmitters involved in social behavior, such as oxytocin, however, not much is known about the neural circuits involved in social interaction.

Some recent evidence suggests that dopaminergic projections from the ventral tegmental area (VTA) to the nucleus accumbens (NAc) are important modulators of social behavior. Importantly, VTA activity is tightly modulated by the cholinergic laterodorsal tegmental nucleus (LDT), which is also known to send direct projections to the NAc, though the functional relevance of such projections remains undisclosed.

Thus, we decided to use optogenetics to selectively activate/inhibit LDT-VTA or LDT-NAc direct projections and evaluate their role in same sex adult social interaction.

Excitation of LDT-VTA projections induces a moderate increase in social interaction time, but did not change interaction with an object. Regarding LDT-NAc projections, neither inhibition nor excitation of these terminals had an effect on social interaction.

Introduction

Social behaviors are complex and vary substantially between individuals. Impairments in social interaction are a common hallmark of several psychiatric disorders, such as anxiety, schizophrenia and autism. Despite pharmacological studies have highlighted oxytocin and later, dopamine (DA), as crucial neurotransmitters in mediating social behaviors (Manduca et al., 2016; Peñagarikano et al., 2015), there is still no map for the neuronal circuits involved in this type of behaviors.

Previous studies have suggested the involvement of the mesolimbic system, as also known as the reward pathway, in social behaviors. This pathway mainly comprises dopaminergic (DAergic) projections from the ventral tegmental area (VTA) to the nucleus accumbens (NAc), and has been implicated in processing emotionally salient stimuli of both positive and negative valence (Schultz, 1998; Tan et al., 2012; van Zessen et al., 2012; Wise and Rompre, 1989). Functional studies demonstrated that the VTA is important for regulating reproductive behavior and parental behavior (Dulac et al., 2014; Hansen et al., 1991; Sirinathsinghji et al., 1986). In addition, it was shown that increasing brain DA levels by systemic levodopa (L-DOPA) administration, was able to rescue social deficits in an animal model that presents VTA-NAc hypodopaminergia (Borges et al., 2013). Furthermore, DA augmentation also increases social interaction in control animals (Borges et al., 2013; Manduca et al., 2016). In agreement with these pharmacological studies, recent optogenetic studies have shown that activation or inhibition of VTA tyrosine hydroxylase (TH) neurons leads to an increase or decrease of same sex social interaction, respectively (Gunaydin et al., 2014). In addition to the NAc, the VTA also sends projections to the medial prefrontal cortex (mPFC), and the mPFC also seems to be recruited by VTA stimulation. However, optogenetic stimulation of VTA-mPFC projections has no effect on social behaviors (Gunaydin et al., 2014).

Two major inputs of the VTA are comprised by the laterodorsal tegmental nucleus (LDT) and the lateral habenula (LHb). Optogenetic stimulation of distinct subpopulations of VTA DA neurons, which project to different target structures, by the LDT or LHb projections, trigger reward- or aversion-associated behaviors, respectively (Lammel et al., 2012).

The role of LDT in mediating positive stimuli has for long been proposed since this brain region tightly modulates VTA DAergic activity (Omelchenko and Sesack, 2005a, 2006), with a likely additional contribution of glutamatergic projections (Cornwall et al., 1990; Lammel et al.,

2012; Oakman et al., 1999). This input is vital for the activity of DAergic cells in the VTA, facilitating DA-related behaviors involved in reward signalling or encoding error prediction signals (Lodge and Grace, 2006). Moreover, recent studies have shown that optogenetic stimulation of LDT neurons that project to the VTA enhances conditioned place preference (Lammel et al., 2012) and operant responding in rodents (Steidl and Veverka, 2015). Nonetheless, the impact of LDT-VTA stimulation in social behaviors remains completely undisclosed.

Importantly, the LDT was shown to directly enervates the NAc (Dautan et al., 2014, 2016), however, nothing is known about the functional relevance of these projections.

Thus, our aim was to evaluate the importance of the LDT-VTA and LDT-NAc projections in same sex social interaction, by using optogenetics to selectively activate (or inhibit) these specific projections during behavior.

Material and Methods

Animals

Male Wistar Han rats, with 2 months old, were maintained under standard laboratory conditions: an artificial 12 h light/dark cycle (lights on from 08:00 to 20:00 hours), with an ambient temperature of 21±1 °C and a relative humidity of 50–60%; standard diet (4RF21, Mucedola SRL) and water were given *ad libitum*. After cannula implementation animals were individually housed.

Health monitoring was executed according the FELASA guidelines (Nicklas et al., 2002), confirming the Specified Pathogen Free health status of sentinel animals maintained in the animal room. All procedures were approved by the Ethics Committee of the Life and Health Sciences Research Institute and were conducted in accordance with European Union Directive 2010/63/EU. Animal facilities and the people involved in animal experiments were certified by the Portuguese regulatory entity – Direção-Geral de Alimentação e Veterinária (DGAV).

Viral constructs

Constructs were packaged in purified adeno-associated viral vector serotype 5 (pAAV), by the Gene Therapy Center Vector Core (UNC) center. pAAV5–EF1a–WGA–Cre–mCherry, pAAV5–EF1a–DIO–hChR2–eYFP and pAAV5-EF1a-DIO-eNpHR3.0-eYFP were obtained directly from UNC center (Karl Deisseroth, Stanford University). AAV5 vector titers were 2,1-6,6X10^{12} virus molecules/ml as determined by dot blot.

Surgery

Rats were anaesthetised with 75 mg/kg ketamine (Imalgene, Merial) and 0.5 mg/kg medetomidine (Dorbene, Cymedica). All stereotaxic coordinates were describe relative to bregma. All virus were unilaterally injected, with following coordinates - LDT: - 8.5 mm anteroposterior (AP), + 0.9 mm mediolateral (ML), and - 6.5 mm dorsoventral (DV); VTA: - 5.3 mm AP, + 0.6 mm ML, - 7.8 mm DV; NAc: +1.5 mm AP, + 0.9 mm ML, and - 6.0 mm DV from bregma.

The virus solution containing purified adeno-associated viral vector serotype (pAVV5), under the control of an EF1-a premotor, coding the cre-WGA-mCherry, was injected in the NAc or in the VTA (pAAV5-EF1a-mCherry-IRES-WGA-Cre). This virus is a transsynaptic tracing virus, which allow to target neuronal subpopulation based on their synaptic connectivity to a downstream or

upstream region. It was used in conjunction with a DIO (doublefloxed inverse orf) class of targeting virus. This cre-dependent DiO-ChR2-eYFP (pAAV5-EF1a-DIO-hChR2(H134R)-eYFP), for excitation, or cre-dependent DiO-eNpHR3.0-eYFP (pAAV5-EF1a-DIO-eNpHR3.0-eYFP), for inhibition of the pathway, was injected in the LDT. The control (eYFP) group of the experiment was only injected with virus in the LDT. Afterwards, it was implanted an optic fiber (200 μm core fiber optic; Thorlabs) with 2.5 mm stainless steel ferrule (Thorlabs) in the VTA or in the NAc of these animals. Because previous data from our laboratory have shown in different behavioral assays, no differences between eYFP animals with ferrule implanted in the VTA or in the NAc, we considered all in the same control group.

All ferrule were implanted 0.1 mm above the DV coordinate. They were secured to the skull with the use of 2.4 mm screws (Bilaney) and dental cement (C&B kit, Sun Medical). In the end, animals were sutured and let to recover for at least, two weeks, before initiation of the behavioral assays.

Laser delivery

Connect to the ferrule, during the behavior test was a fiber optic, suspended above the behavioral apparatus, to allow animals freely move while receiving the stimulation. The fiber was connected to a 473 nm (excitation) laser or to a 589 nm laser (inhibition). All laser output was manipulated by the use of a Master-8 pulse stimulator (Master-8; AMPI). For excitation the following protocol was used: 30 Hz, 15 pulses of 5 ms, 2 in 2 s, with a delivered of 10 mW at the point of the fiber, generated by the 473 nm laser. Optic inhibition consist in 8 s of constant light, with an interval of 2 s with a delivered of 5 mW at the point of the fiber, generated by the 589 nm laser.

Behavioral test

Resident – Intruder test (Social interaction paradigm)

Social interaction test was performed in the home cage of the injected animals (43x27x19 cm^3, with approximately 3 cm of wood shavings covering the floor). The resident (animal injected with the virus) was allow to explore freely the cage for 1 min. Afterwards, the intruder animal was placed inside the cage for 3 min (test session). During this period, the optogenetic protocols were applied. All behavior were recorded using a camera. Time spent in

social interaction was recorded, this include: body sniffing, anogenital sniffing, direct contact (pushing the snout or head underneath the intruder body and crawling over or under intruder body) and close flowing (<1 cm). Each animal performed the social interaction test paired with laser stimulation (ON) and 24 h later, with no stimulation (OFF). Groups were counterbalanced for the order of the stimulation. Change of social interaction was calculated as following: social interaction time with laser ON minus social interaction time with laser OFF.

Novel object interaction (Object interaction paradigm)

The object interaction protocol, was also performed 2 times, with an interval of 24 h, paired with stimulation (ON) and no stimulation (OFF), in a counterbalanced order.bRats were attached to the optic fibre connected to the lasers (473 nm and 589 nm) and placed inside of the home cage, for 1 min, to freely explore. Next, an object was placed inside of the arena cage for 3 min and stimulation was applied. The object consisted of a ceramic black and white sphere. Object interaction time was recorded using a camera. Change of object interaction was calculated as following: object interaction time with laser ON minus object interaction time with laser OFF.

Locomotor activity protocol

Locomotion activity was assessed using the open field arena (43.2cm×43.2cm; Med Associates Inc.). Animals were placed in the center of the arena, connect to the fiber, for 6 min, and were allowed to freely move and optical stimulation was applied. Total distance and time travelled was used as indicator of locomotor activity.

Immunofluorescence

Animals were anesthetized with pentobarbital (Eutasil) and were transcardially transfused with 0.9% of saline followed by 4% of paraformaldehyde. Brains were removed and fixed with 4% of paraformaldehyde. Coronal vibratome sections (50 μm) were incubated with primary antibody goat GFP (1:500, ab6673, Abcam), overnight at 4°C. Appropriate fluorescent secondary antibody was used (AlexaFluor-488 anti-goat, 1:1000, Life Technologies) for 2 h at room temperature. All sections were mounted on microscope slides and images were taken by confocal microscopy (Olympus FluoViewTMFV1000).

Histology

For histological determination of the fiber implantation, coronal sections (50 µm) were cut in vibratome (Leica), mounted and stained with hematoxylin, to help to delimit regional boundaries, before mounting and coversliping.

Statistical Analysis

Data was presented as mean ± standard error of the mean (SEM). Statistical analysis was performed using Graphpad Prism 6.01 software. Data was verified for Gaussian distribution. Two group comparisons were achieved by using Student t-test. When no Gaussian distribution was assumed, a nonparametric test (Mann-Whitney U-test or Wilcoxon Signed-Rank test) was used. Statistical assessment for multiple comparison were achieved by using two way ANOVA, followed by Bonferroni *post-hoc* tests. Statistical significance was set at $p<0.05$.

Results

Optogenetic stimulation of LDT inputs to the VTA

In the experimental group, a construct containing a cre-dependent channel-rhodopsin (ChR2) fused to eYFP (pAAV5-EF1a-DIO-hChR2(H134R)-eYFP) was injected in the LDT; in combination with injection of pAAV5-EF1a-mCherry-IRES-WGA-Cre in the VTA. This last construct contains a wheat germ agglutinin (WGA)-Cre recombinase fusion, which allows transsynpatic migration of cre protein into local axon terminals and transport to upstream somata (Dong et al., 2011; Yuan et al., 2011), enabling the expression of cre-dependent ChR2. Control animals (eYFP), were injected with the pAAV5-EF1a-DIO-hChR2(H134R)-eYFP vector in the LDT only. To stimulate the ChR2-expressing LDT axons in the VTA, we have implanted the optic fiber above the VTA to allow the delivery of a 473 nm light (Fig.1A). All animals were checked for correct cannula placement (Sup. Fig.1) and viral expression was confirmed (Sup. Fig. 2).

First, animals were tested in a resident-intruder test. Each rat was tested twice, in two separated days, with a different intruder. After 1 min of habituation, intruder was placed inside the home cage, for 3 min, paired with either light-off (OFF) or light-on (ON) with a frequency of 30 Hz (15 pulses of 5 ms), with an interval of 2 s (Fig.1B). The order was counterbalanced between animals.

We observed an increase in social interaction (social interaction time ON – social interaction time OFF) of LDT-VTA stimulated animals (Fig.1C, t_{17}=3.283, p=0.004; Fig.1E, ChR2-VTA: t_{11}=6.125, p=0.001). Within eYFP group results, no differences were observed (Fig.1D, t_{7}=0.533 , p=0.605).

To confirm that the observed increase in interaction was not due to a novelty effect, we also evaluated the impact of LDT-VTA stimulation in modulating the interaction time with an object using the same paradigm. Upon LDT-VTA stimulation, no differences were observed in object interaction between groups (Fig.1F, t_{14}=0.155, p=0.879). Also, no differences were observed in object interaction with laser ON and with laser OFF, in both groups (Fig.1G, eYFP: t_{10}=0.544, p=0.599; Fig.1H, ChR2-VTA: Z=3, p=0.813).

To control for the effects of LDT-VTA stimulation in locomotion, we placed animals in an open field arena, and stimulated these projections throughout the 6 min of test, similarly as in the social interaction test. No significant differences were found between groups on distance and

time travelled, though there is a trend for increased locomotion in ChR2-VTA animals (Fig.1I, Distance: U=5, p=0.171; Fig.1J; Time: U=6, p=0.233). Regarding, the distance travelled throughout the duration of the test, as expected, time as an impact in the distance travelled (Fig.1K, $F_{(35,28)}$=3.117, p<0.001). No differences were observed between groups (Fig.1K, $F_{(1,8)}$=3.709 , p=0.09).

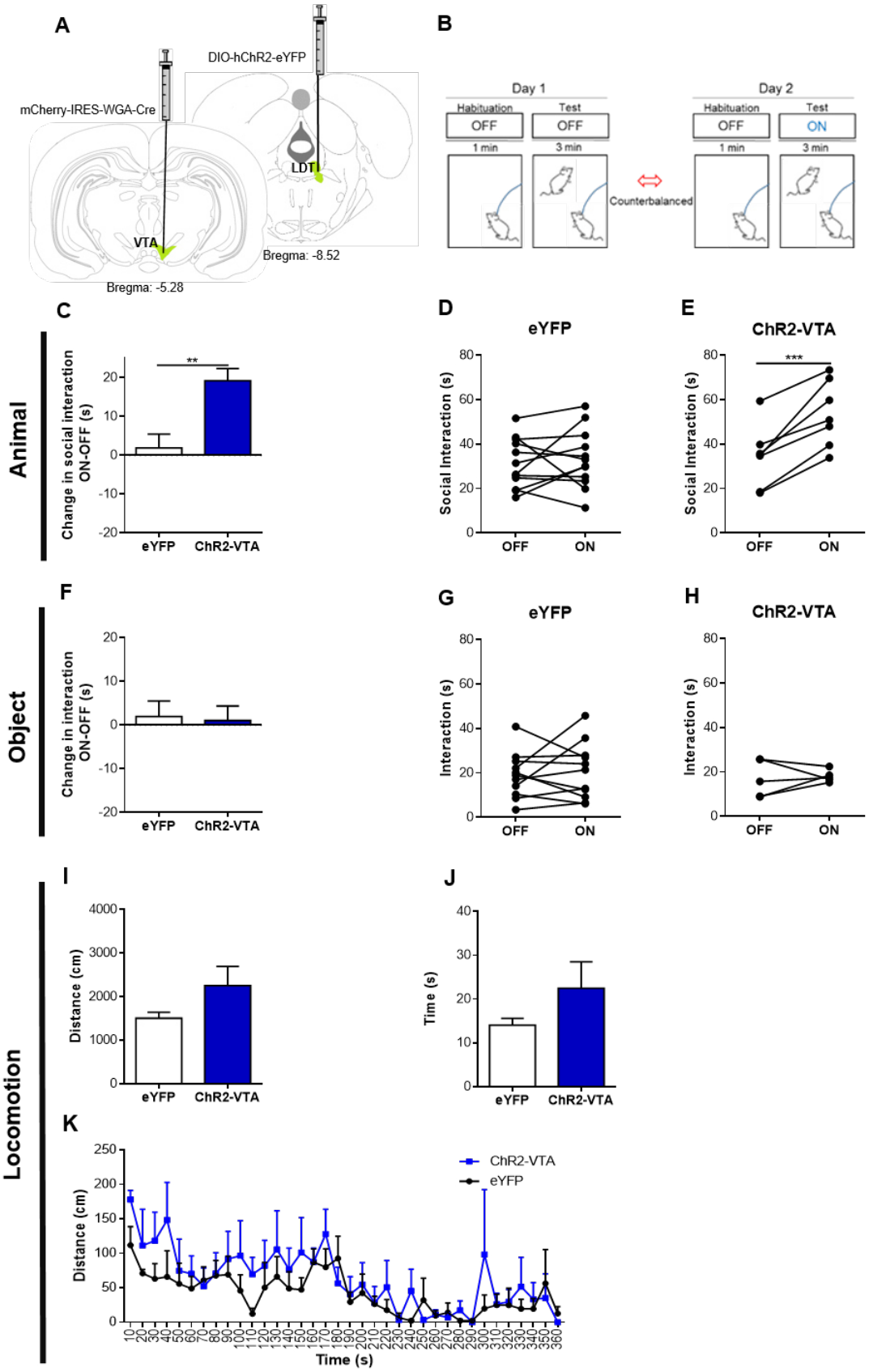

Figure 1. Effects of optogenetic stimulation of LDT-VTA pathway in social interaction. (A) Experimental design. We injected pAAV5-EF1a-mCherry-IRES-WGA-Cre in the VTA and pAAV-EF1a-DIO-hChR2(H134R)-eYFP in the LDT. WGA-cre migrates from the VTA to directly-connected regions, namely the LDT, inducing the expression of cre-

dependent ChR2 (or eYFP). **(B)** Schematic design of the home-cage resident-intruder behavioral procedure. After 1 min of habituation, 3 min social interaction period was counterbalanced, with a 24 h of interval, between ON and OFF laser conditions. Optical stimulation parameters used were: 473nm light delivered in 30 Hz burst (15 pulses, 5 ms each) every 2 s (Excitation). **(C)** Summary of light-evoked changes in social interaction after excitation of the LDT-VTA pathway. Phasic stimulation of LDT projection neurons into the VTA induces an increase in social interaction in comparison with eYFP group (n_{eYFP}= 12, $n_{ChR2\text{-}VTA}$=7). Summary of individual responses of LDT-VTA stimulation in eYFP **(D)** and ChR2-VTA **(E)** animals during the resident-intruder paradigm. **(F)** Summary of light-evoked changes in object interaction after optical stimulation of the LDT-VTA pathway. No differences were found between groups (n_{eYFP}= 11, $n_{ChR2\text{-}VTA}$=5). Summary of individual responses of LDT-VTA stimulation in eYFP **(G)** and ChR2-VTA **(H)** animals during objection interaction. **(I,J)** Open field locomotion results. No differences were found in distance (n_{eYFP}= 6, $n_{ChR2\text{-}VTA}$=4) and time travelled (n_{eYFP}= 6, $n_{ChR2\text{-}VTA}$=4) between eYFP and ChR2-VTA stimulated groups, though there is a trend for increase locomotion in ChR2 animals. **(K)** Also, no differences were observed between groups in distance travelled throughout time (n_{eYFP}= 6, $n_{ChR2\text{-}VTA}$=4).

LDT, laterodorsal tegmental nucleus; VTA, ventral tegmental area. Data presented as mean ± SEM; **p<0.01, ***p<0.001.

Optogenetic stimulation and inhibition of LDT inputs into the NAc

Since previous work have suggested that the LDT also projects to the NAc directly (Dautan et al., 2014, 2016), we also decided to evaluate the role of LDT-NAc projections on social behavior.

Following the rational of the experimental groups from the previous experiment, we injected in the LDT the pAAV5-EF1a-DIO-hChR2(H134R)-eYFP virus, and in the NAc the pAAV5-EF1a-mCherry-IRES-WGA-Cre virus (Fig.2A). We next stimulated LDT-NAc projections during the resident-intruder paradigm (30 Hz, 15 pulses of 5 ms, with an interval of 2 s).

Activation of LDT-NAc projections did not induce any change on social interaction time (Fig.2C, t_{25}=0.521, p=0.607). Together, no differences were observed between social interaction time when laser is ON or OFF (Fig.2D, eYFP: t_7=0.533, p=0.605; Fig.2D, ChR2-NAc: t_{14}=0.234, p=0.818). Besides, we have tested different stimulation protocols with no effects in social interaction (Table 1).

Change on Social Interaction time ON-OFF (s)			
Protocol (Stimulation)	**eYFP**	**ChR2-NAc**	**Statistics**
20Hz, 15 pulses, interval of 5 s	4,766±0,782	-7,676±6,108	t_8= 2,201 p=0,0780
30Hz, 30 pulses, interval of 2 s	-10,15± 8,597	-5,195±5,847	t_{10}=0,496 p=0,631
20 Hz, 80 pulses, interval of 15 s	-13,83± 5,014	-0,927± 6,057	t_8=1,286 p=0,235

Table 1. Effects of different stimulation protocols in social behavior. No differences were observed with 3 different stimulation protocol: 20 Hz, 15 pulses with an interval of 5 s (n_{eYFP}=5; $n_{ChR2\text{-}NAc}$=5); 30 Hz, 30 pulses with an interval of 2 s (n_{eYFP}=5; $n_{ChR2\text{-}NAc}$=7); 20 Hz, 80 pulses, interval of 15 s (n_{eYFP}=3; $n_{ChR2\text{-}NAc}$=7).

Data presented as mean ± SEM.

Identical outcome was observed during the object interaction paradigm (Fig.2F, t_{23}=1.796, p=0,087). No difference was observed when comparing laser ON with the laser OFF in object interaction time (Fig.2G, eYFP: t_{10}=0.544, p=0.599; Fig.2H, ChR2-NAc: t_{13}=2.061, p=0.06). Likewise, this stimulation did not induce any effect on locomotion activity (Fig.2I, Distance: U=16, p=0.439; Fig.2J, Time: U=15, p=0.439). As expected, distance travelled was different throughout time (Fig.2K, $F_{(35,385)}$=1.551, p=0.027). No differences were observed between groups (Fig.2K, $F_{(1,11)}$=2.549x10^{-7} p=0.999).

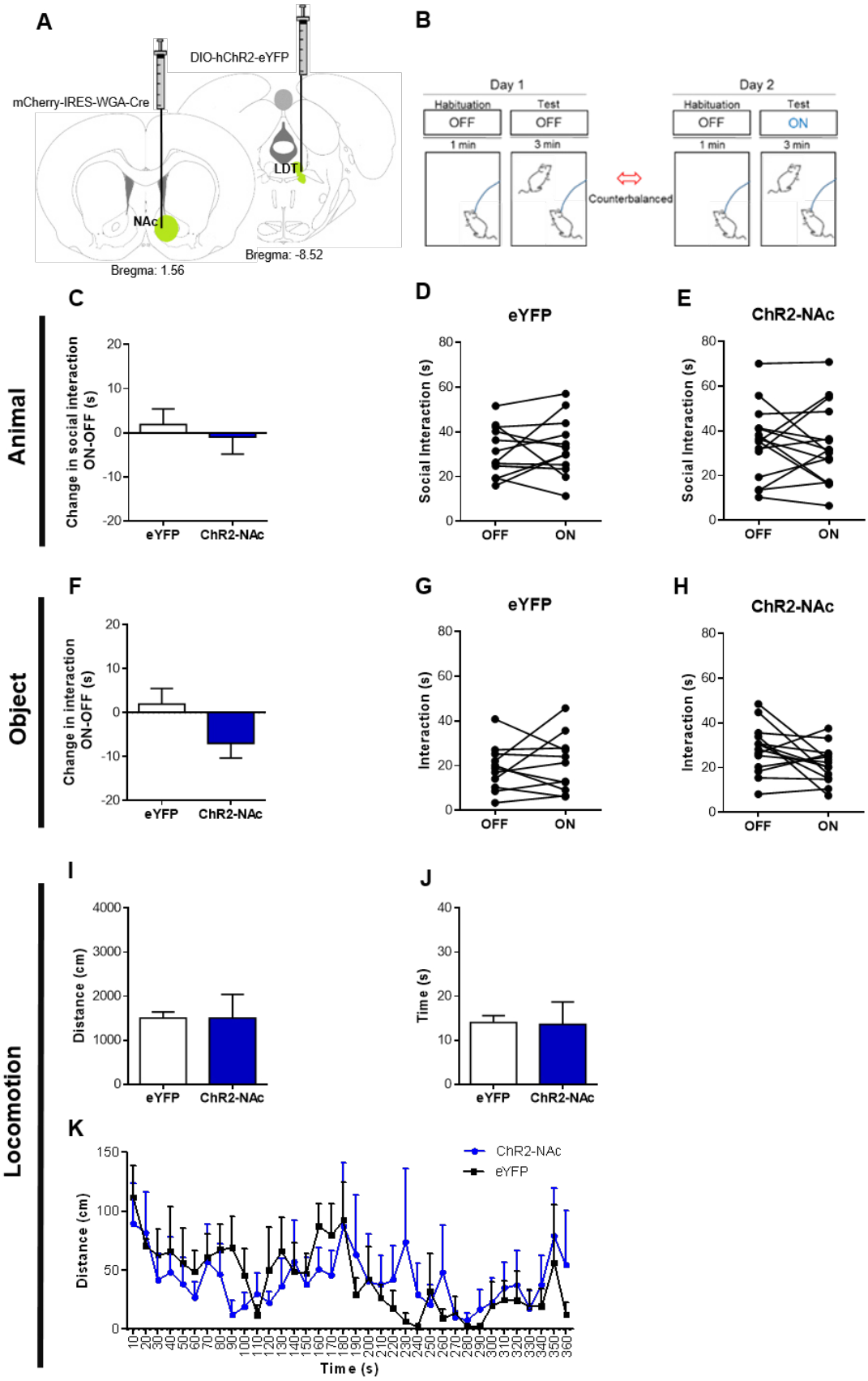

Figure 2. Effects of optogenetic stimulation of LDT-NAc pathway in social interaction. (A) Injection of pAAV5-EF1a-mCherry-IRES-WGA-Cre in the NAc and pAAV5-EF1a-DIO-hChR2-eYFP in the LDT. (B) Schematic design

of the home-cage resident-intruder behavioral procedure. After habituation, 3 min social interaction period was counterbalanced, between ON and OFF light conditions, with a 24 h of interval. Optical stimulation parameters (Excitation) used were: 473nm light delivered in 30 Hz burst (15 pulses, 5 ms each) every 2 s. **(C)** Stimulation of LDT projection neurons into the NAc does not induce any difference in social interaction (n_{eYFP}= 12, $n_{ChR2\text{-}NAc}$=15). **(D,E)** Summary of individual responses of LDT-NAc stimulation during resident-intruder paradigm in eYFP and ChR2 individual animals, respectively. **(F)** Summary of object interaction light-evoked changes. No differences were found between groups (n_{eYFP}= 11, $n_{ChR2\text{-}NAc}$=14). **(G,H)** Summary of LDT-NAc stimulation in eYFP and ChR2 individual animals, respectively, during objection interaction test. **(I,J)** LDT-NAc optical excitation during the locomotor activity paradigm, leads to no differences in distance (n_{eYFP}= 6, $n_{ChR2\text{-}NAc}$=7) and time travelled (n_{eYFP}= 6, $n_{ChR2\text{-}NAc}$=7) between eYFP and ChR2 group. **(K)** No differences were observed between groups in distance travelled throughout time (n_{eYFP}= 6, $n_{ChR2\text{-}NAc}$=7).

LDT, laterodorsal tegmental nucleus; NAc, nucleus accumbens. Data presented as mean ± SEM.

Because nothing is known about LDT-NAc biological role in social behaviors (if any), we have also decided to perform inhibition experiments. The experimental group was injected with the pAAV5-EF1a-mCherry-IRES-WGA-Cre virus in the NAc, and in the LDT with pAAV5-EF1a-DIO-eNpHR3.0-eYFP (Fig.3A). Control group was injected only with pAAV5-EF1a-DIO-eNpHr3.0-eYFP in the LDT. All animals were checked for correct cannula placement (Sup. Fig.1) and viral expression was confirmed (Sup. Fig.2).

The inhibition of eNpHR-expressing LDT neurons in the NAc, was performed by delivery of a 589 nm laser with a pulse of 8 s with an interval of 2 s. Inhibition of LDT-NAc projections does not modulate social and novel-object interaction (Fig.3C, Social: t_7=0.631, p=0.536; Fig.3F, Object: t_{17}=0.717, p=0.483). No differences were observed between the social (Fig.3D, eYFP: t_7=0.533, p=0.605; Fig.3E, eNpHR: t_7=0.377, p=0.717) and object interaction time (Fig.3G, eYFP: t_{10}=0.544, p=0.599; Fig.3H, eNpHR: t_7=0.064, p=0.541). However, we observed an slightly increase in distance travelled (Fig.3I, U=11, p=0.107), and an increase in time travelled in a locomotor activity paradigm (Fig.3J, U=8.5 , p=0.047). Throughout time, distance travelled was statistically different (Fig.3K, $F_{(35,420)}$=3.556, p<0.001). No differences were observed between groups (Fig.3K, $F_{(1,12)}$=2.489, p=0.141).

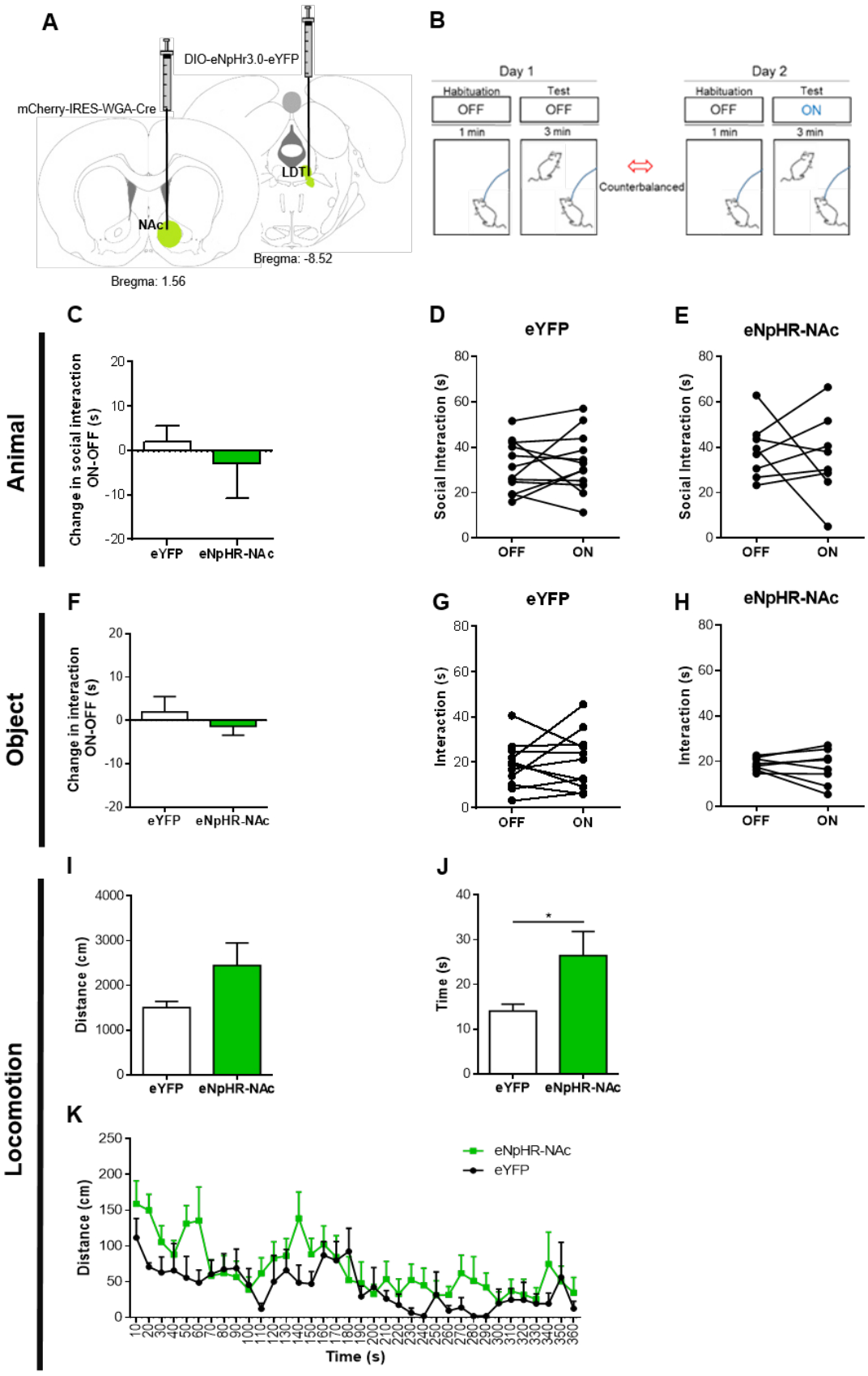

Figure 3. Effects of optic inhibition of LDT-NAc pathway in social behavior. (A) Injection of pAAV-EF1a-mCherry-IRES-WGA-Cre into NAc and pAAV5-EF1a-DIO-eNpHr3.0-eYFP into the LDT. (B) Schematic design of the home-cage resident-intruder behavioral procedure. Three min social interaction period, after habituation, was

counterbalanced, between ON and OFF light conditions, with a 24 h of interval. Optical stimulation parameters (Inhibition) used were: 589nm light was delivered in 1 pulse of 8 s, every 2 s. **(C)** Inhibition of LDT projection neurons into the NAc does not induce any difference in social interaction (n_{eYFP}= 12, $n_{eNpHR\text{-}NAc}$=8). **(D,E)** Summary of LDT-NAc optogenetic inhibition, during resident-intruder paradigm, in eYFP and eNpHR-NAc individual animals, respectively. **(F)** Summary of object interaction light-evoked changes. No differences were found between groups (n_{eYFP}= 11, $n_{eNpHR\text{-}NAc}$=8). **(G,H)** Summary of LDT-NAc inhibition in eYFP and eNpHR-NAc individual animals, respectively, during objection interaction test. **(I,J)** LDT-NAc optical inhibition during the locomotor activity paradigm, leads to no differences in distance (n_{eYFP}= 6, $n_{eNpHR\text{-}NAc}$=8) and a moderate increase in the time travelled (n_{eYFP}= 6, $n_{eNpHR\text{-}NAc}$=8) between eYFP and eNpHR-NAc group. **(K)** Group has no effect on distance travelled through time (n_{eYFP}= 6, $n_{eNpHR\text{-}NAc}$=8).

LDT, laterodorsal tegmental nucleus; NAc, nucleus accumbens. Data presented as mean ± SEM; *$p<0.05$.

Discussion

Ours results demonstrate that optogenetic activation of LDT-VTA projections induces a modest increase in same sex social interaction in freely-behaving animals. Conversely, LDT projections to the NAc neurons do not seem to play a crucial role in this type of interaction.

Strong evidence suggest that the VTA plays an important role in social behaviors in humans (Depue and Morrone-Strupinsky, 2005; Groppe et al., 2013) and in rodents (Curtis and Wang, 2005; Gil et al., 2013; Gunaydin et al., 2014; Northcutt and Nguyen, 2014). Optogenetic studies have observed that VTA TH neurons activity is involved in bidirectional modulation of same sex social interaction (Gunaydin et al., 2014). The NAc is the main recipient of DAergic inputs from the VTA (Beckstead et al., 1979; Fallon and Moore, 1978). Activation of VTA TH projection to the NAc, increases social interaction in freely-behaving mice (Gunaydin et al., 2014). Moreover, pharmacological studies have shown that DA is implicated in affiliative behaviors (Aragona et al., 2006; Borges et al., 2013; Curtis and Wang, 2005; Gingrich et al., 2000). Additionally, it has been observed during social same sex encounter an increase in NAc spiking (Gunaydin et al., 2014).

Still, little is known about the impact of upstream regions in the modulation of social behavior. Studies have shown that LDT contributes to regulation of mesolimbic DA signalling (Forster and Blaha, 2000; Forster et al., 2002; Lodge and Grace, 2006). Indeed, LDT sends cholinergic, glutamatergic, and GABAergic neurons inputs to the midbrain VTA. LDT neurons were found preferentially synapse onto DA neurons in lateral VTA projections to the NAc lateral shell (Lammel et al., 2012).

In fact, LDT mediates a divergent impact on mesoaccumbens neurons that is likely to excite DA cells and inhibit GABA neurons (Omelchenko and Sesack, 2005b). Consistent with this, electrical stimulation of LDT leads to an increase of DA release in the NAc, which is blocked by glutamate or acetylcholine (Ach) receptor antagonists applied to the VTA (Blaha et al., 1996; Forster and Blaha, 2000). Indeed, these ACh projections mediate DA cell firing, DA release, and are involved in reward and psychostimulant-induced locomotor activity (Dobbs and Mark, 2012).

Moreover, the stimulation of LDT-VTA projections induce an increase in the time spent in stimulation-paired compartment on the conditioning place preference and reinforces operant responding, together, with no effect on open field assays for anxiety or locomotor activity

(Lammel et al., 2012; Steidl and Veverka, 2015; Xiao et al., 2016). Indeed, both cholinergic and glutamatergic LDT-VTA projections lead to place preference, however nothing is known about the impact of GABAergic LDT-VTA projections on these behaviors (Lammel et al., 2012; Xiao et al., 2016).

Despite these important evidences showing that LDT-VTA (and less so LDT-NAc) circuit is important for reward, not much is known about the role of this circuit in social behaviors.

We observe an increase in social interaction upon LDT-VTA stimulation, but not in object interaction, suggesting that the LDT can play a role in this type of behaviors. Regarding the bidirectional modulation of LDT-NAc pathway, we did not observe any changes in social or object interaction. We applied 3 additional protocols of excitation, but none induced any effect on social behavior (Table 1; protocols are: 1) 20 Hz, 15 pulses, interval of 5 s; 2) 30 Hz, 30 pulses, interval of 2 s; 3) 20 Hz, 80 pulses, interval of 15 s). Of course that one can argue that we may no be activating these neurons at a correct frequency or temporal window. Therefore, it would be interesting to measure LDT and NAc electrophysiological activity during the test, in order to understand how this circuit responds to social interaction. Calcium imaging of specific neuronal populations of these regions could also help elaborate the temporal dynamics of neuronal firing in this regions: when and at what frequency these neurons fire during social encounters? Immediately before interaction? During? After? In this perspective, these results could guide us to select an appropriate stimulation protocol.

One other possibility is that this circuit may be involved in other types of social behaviors, namely different sex social behaviors or parental behaviors. However, still there is not much information regarding the modulation of this pathways in other social dimensions. Only a recent study have observed that infusion of ghrelin into the LDT increases, whereas infusion of ghrelin receptor 1A (GHSR-1A) antagonist decreases the preference for female mouse as well as sexual behavior in sexually naïve male mice, suggesting that LDT can be important in mediating sexual behavior (Prieto-Garcia et al., 2015). Furthermore, lesions of the cholinergic projections to the VTA decreases sexual interaction and preference for a female in sexually naïve animals (Kippin and van der Kooy, 2003). Supportively, previous studies have found a role for ghrelin signalling within the LDT, where GHS-R1A are expressed on cholinergic neurons projecting onto VTA-DA neurons (Dickson et al., 2010), besides, ghrelin administration into the LDT increases alcohol

intake (Jerlhag et al., 2009) as well as induces a concomitant release of VTA-Ach and NAc-DA (Jerlhag et al., 2012).

In summary, we believe that more studies are needed to dissect the neuronal basis of social behaviors using similar strategies as we used here. Moreover, considering the complexity of social behaviors, additional social tests should be performed to evaluate the role of these specific projections in this type of behaviors.

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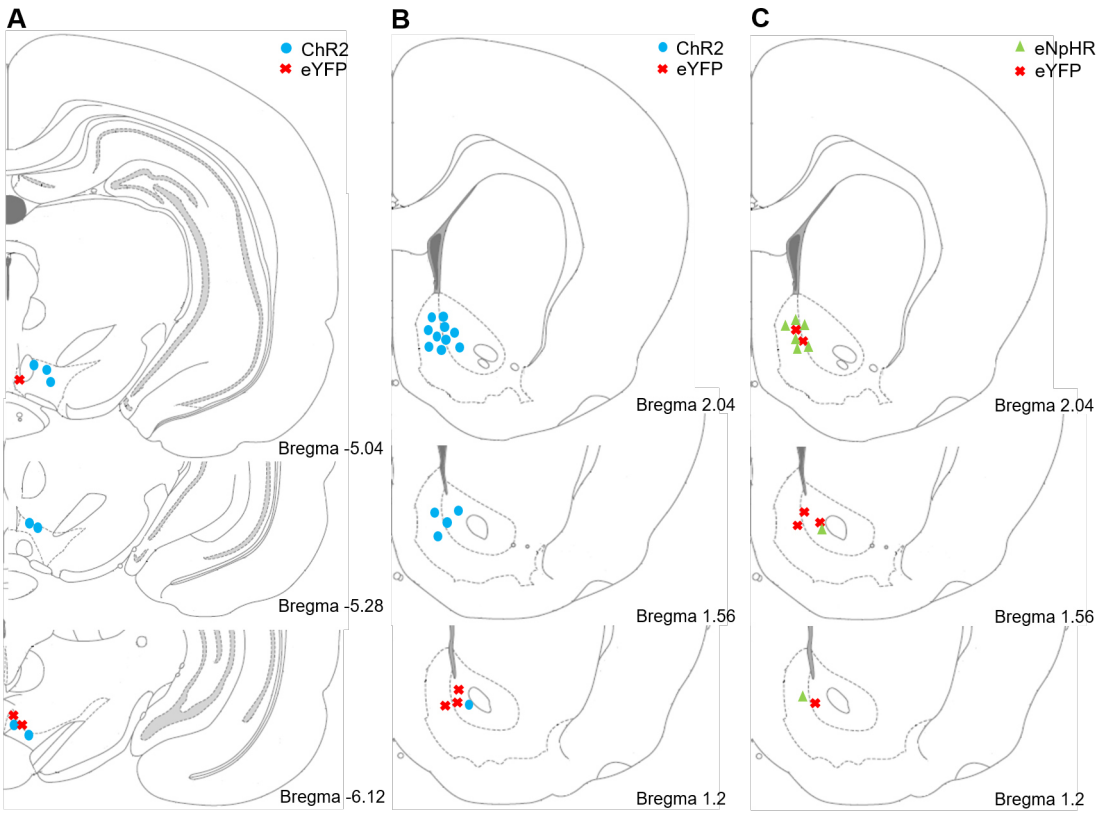
Supplementary Information

Borges S, Coimbra B, Soares-Cunha C, Sousa N, Rodrigues AJ

Impact of optogenetic modulation of LDT-NAc and LDT-VTA pathways in social behaviors

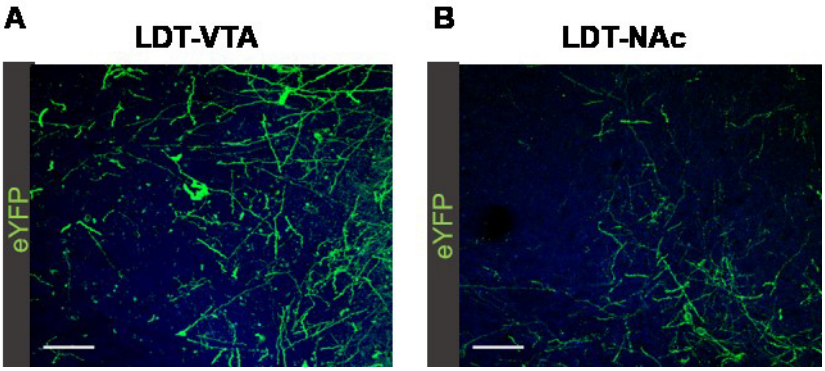
Supplementary Figure 1



Supplementary Figure 1. Histological confirmation of optical fiber sites of all animals. (A) Coronal sections from bregma containing the VTA. Center of the optical fiber placements in the VTA for the animals injected with ChR2 (n=7; blue circles) and eYFP (n=3; red crosses). (B) Coronal sections from bregma containing the NAc. Center of the optical fiber placements in the NAc for all animals injected with ChR2 (n=15; blue circles) and eYFP (n=3; red crosses). (C) Center of the optical fiber placements in the NAc for all animals injected with eNpHR (n=8; blue circles) and eYFP (n=6; red crosses).

VTA, Ventral tegmental area; NAc, nucleus accumbens.

Supplementary Figure 1



Supplementary Figure 2. Confocal images of eYFP expressing cells in the LDT. (A) Sections containing the ChR2-eYFP or eNpHR-eYFP injected virus in the VTA or **(B)** in the NAc.

LDT, laterodorsal tegmental nucleus; VTA, ventral tegmental area; NAc, nucleus accumbens.

CHAPTER 3.2

Borges S, Cunha-Soares C, Coimbra B, Sousa N, Rodrigues AJ

Role of dopamine receptor D2-expressing neurons in social behavior

Role of dopamine receptor D2-expressing neurons in social behavior

Borges S[1,2], Soares-Cunha C[1,2], Coimbra B[1,2], Sousa N[1,2] and Rodrigues AJ[1,2]

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Abstract

Social behavior is a highly rewarding event; therefore, it is modulated by neural systems implicated in other rewards such as food, sex or drugs of abuse. Though the mesolimbic system has been linked to social interaction, little is known about the role of nucleus accumbens (NAc) different neuronal populations in these behaviors. At least one study has shown that optogenetic activation of dopamine receptor D1 (D1R)-expressing neurons in the NAc leads to increase in social interaction time. On the other hand, less explored is the role of dopamine receptor D2 (D2R)-expressing neurons in these types of behaviors.

In the present study, we used optogenetics to study the role of NAc D2R-expressing neurons in social behaviors. Interestingly, activation of NAc D2R-expressing neurons increases social interaction in control animals, in a resident-intruder home cage test. Furthermore, it rescues social impairments previously observed in an *in utero* glucocorticoid (iuGC)-exposed animal model.

In sum, these findings suggest that accumbal D2R-expressing neurons play a relevant role in social behaviors, and that additional studies have to be made in order to evaluate the contribution of other different types of NAc neurons in this type of behaviors.

Introduction

Neurons in the nucleus accumbens (NAc) regulate a variety of behaviors and functions, which includes motivational processes and incentive salience (Berridge, 2007; Floresco, 2015; Salamone and Correa, 2012). The NAc is composed mainly by medium spiny neurons (MSNs) which are separated into those expressing dopamine receptor D1 (D1R, D1-MSNs), constituting the direct pathway, and those expressing dopamine receptor D2 (D2R, D2-MSNs), integrating the indirect pathway (Gerfen and Surmeier, 2011; Surmeier et al., 1996, 2011). Previous studies support the existence of an opposite function between these two types of MSNs (Albin et al., 1989; Graybiel, 2000). Indeed, activation of D1-MSNs is correlated with positive rewarding events, while activation of D2-MSNs is related with aversion (Hikida et al., 2010; Kravitz et al., 2012; Lobo et al., 2010). However, recent data from other laboratories and ours has shown that this functional/behavioral bias is not so evident (Cui et al., 2013; Soares-Cunha et al., 2016). We previously showed that activation of either NAc D1R- or D2R-expressing neurons enhances motivation in a progressive ratio task (Soares-Cunha et al., 2016).

Furthermore, other converging lines of evidence implicate the dopaminergic (DAergic) system in social behaviors. DA has been associated as neuromodulator in same-sex affiliative behaviors (Puglisi-Allegra and Cabib, 1997; Robinson et al., 2002, 2011).

In juvenile rats, stimulating NAc DA neurotransmission enhances social play, whereas blocking NAc DA receptors reduces it (Manduca et al., 2016). In adult same-sex interaction, recent studies have shown that activation or inhibition of tyrosine-hydroxylase (TH) neurons from ventral tegmental area (VTA) to the NAc leads to an increase or decrease of social interaction, respectively (Gunaydin et al., 2014). Moreover, optogenetic activation of D1R in the NAc leads to an increase in social interaction time (Gunaydin et al., 2014).

Considering the previously mentioned evidence, here, we have the purpose of unroll the importance of NAc D2R-expressing neurons in social interaction. For this, we took advantage of optogenetic tools for testing the casual significance of circuits, by selectively activating D2-MSNs in freely moving rodents during the execution of complex social behaviors.

Material and Methods

Animals

Male Wistar Han rats (2 months old at the beginning of the tests) were used. Animals were maintained under standard laboratory conditions: artificial 12-h light/dark cycle (lights on from 08:00 to 20:00 hours), with an ambient temperature of 21±1°C and a relative humidity of 50–60%; rats were individually housed after cannula implantation; standard diet (4RF21, Mucedola SRL) and water were given *ad libitum*.

In the case of the model of *in utero* exposure to glucocorticoids (iuGC), pregnant Wistar han rats were individually housed under the same standard laboratory conditions, but with a different diet (standard diet 4RF21, Mucedola SRL). Subcutaneous injections of dexamethasone at 1 mg/kg (iuGC animals) or vehicle - sesame oil (control – CONT animals) were administered on days 18 and 19 of pregnancy. On postnatal day 21, progeny was weaned according to prenatal treatment and gender. At 2 months old, male rats, after cannula implementation, were individually housed.

All behavioral experiments were performed during the dark period of the light/dark cycle. Health monitoring was performed according to FELASA guidelines (Nicklas et al., 2002), confirming the Specified Pathogen Free health status of sentinel animals maintained in the same animal room. All procedures were conducted in accordance with European Regulations (European Union Directive 2010/63/EU). Animal facilities and the people directly involved in animal experiments were certified by the Portuguese regulatory entity – Direção-Geral de Alimentação e Veterinária (DGAV). All protocols were approved by the Ethics Committee of the Life and Health Sciences Research Institute.

Constructs and virus

eYFP, hChR2(H134R)-eYFP and eNpHR3.0-eYFP were cloned under the control of D2R minimal promoter region (Drd2; ENSRNOG00000008428; Gene ID: 24318), which included 1,540 bp upstream of the first (non-coding) exon (kindly provided by Dr. Karl Deisseroth, Stanford University)(Zalocusky et al., 2016). Constructs were packaged in AAV5 serotype by the Gene Therapy Center Vector Core (UNC) center. AAV5 vector titers were 3.7-6x10^{12} virus molecules/ml as determined by dot blot.

Surgery

Rats were anaesthetised with 75 mg/kg ketamine (Imalgene, Merial) and 0,5 mg/kg medetomidine (Dorbene, Cymedica). A midline incision was made down the scalp. All stereotaxic coordinates were describe relative to bregma. Virus was unilaterally injected, into the NAc, with the following coordinates: + 1.2 mm anteroposterior (AP), + 1.2 mm mediolateral (ML), and - 6.5 mm dorsoventral (DV) from bregma. Craniotomy was made using a drill.

The needle was placed in the NAc with the AAV5 solution containing eYFP (control), hChR2(H134R)-eYFP (excitation), or with eNpHR3.0-eYFP (inhibition). After the injection the needle was kept for more 10 min and slowly removed.

Afterwards, an optic fiber (200 μm core fiber optic; Thorlabs) with 2.5 mm stainless steel ferrule (Thorlabs) was implanted, 0.1mm above the DV coordinate used for the viral injection, in the NAc. They were secured to the skull with the use of 2.4 mm screws (Bilaney) and dental cement (C&B kit, Sun Medical). Finally, animals were sutured and let to recover for at least, three weeks, before initiation of the behavioral assays.

Laser delivery

An optic fiber, placed in suspension above the behavioral apparatus was connected to the animal's ferrule at the beginning of the behavioral test. This allowed animals to freely move while stimulation was applied. The optic fiber was connected to a 473 nm laser or to a 589 nm laser. All laser output was manipulated by a Master-8 pulse stimulator (Master-8, AMPI). For excitation the following protocol was used: 40 Hz, 40 pulses of 12.5 ms, every 10 s, with light power of 10 mW at the tip of the fiber, generated by the 473 nm laser. Optic inhibition consisted of 8 s of constant light, with an interval of 2 s with power of 5 mW at the tip of the fiber, generated by the 589 nm laser.

Behavioral test

Resident – Intruder test (Social interaction paradigm)

Social interaction test was performed in the rat's home cage (43 x 27 x 19 cm^3, with approximately 3 cm of wood shavings covering the floor). The resident (optically stimulated animal) was allow to freely explore the home cage for 1 min - habituation. Afterwards, an intruder

was placed inside the resident's home cage for 3 min - test. During this period, optogenetic stimulation was applied. All behaviors were recorded using a camera. Time in social interaction consisted on: body sniffing, anogenital sniffing, direct contact (pushing the snout or head underneath the intruder body and crawling over or under intruder body) and close following (<1 cm).

The behavioral test was performed 2 times by each animal, with an interval of 24 h. In one session the resident was paired with stimulation (ON), and on the other with no stimulation (OFF). Sessions with stimulation or with no stimulation were counterbalanced in all groups. Change in social interaction was calculated as follows: difference between social interaction time with laser ON and social interaction time with laser OFF.

Novel object interaction (Object interaction paradigm)

The object interaction protocol was also performed twice, with an interval of 24 h, paired with stimulation (ON) and no stimulation (OFF), in a counterbalanced order. The optogenetically-stimulated animal was allowed to freely explore the home cage, for 1 min. Following that, stimulation was applied and a ceramic black and white sphere (object) was placed inside the arena during 3 min. Object interaction time was recorded using a camera. Change of object interaction was calculated as following: difference in time spent exploring the object with laser ON and time spent exploring the object with laser OFF.

Three-chamber sociability test

Three-chamber sociability apparatus consists in a Plexiglas rectangular box (150 cm x 50 cm x 40 cm) without a top. The center compartment was smaller (30 cm x 50 cm) than the other two equal-size compartments (60 cm x 50 cm). The inverted cylindrical enclosures (diameter 18 cm, height 40 cm) were placed in each of the end compartments and inside it was placed an animal during testing sessions. The apparatus was cleaned with 10% ethanol between sessions.

At the beginning of the behavioral session, an optogenetically-stimulated animal was placed in the middle compartment and was allowed to freely explore the apparatus for 5 min. In the second 5 min session, a control rat was placed inside the inverted enclosure (social compartment), and an empty enclosure was placed in the opposite compartment (non-social compartment). The optogenetically-stimulated animal was place in the home cage for 1-2 min

while experimenter placed the stimulus rat inside the inverted enclosure, and was then put back in the behavioral arena to freely explore both chambers for 5 min. The amount of time the test animal spent in each compartment was measured. Each animal performed the sociability test 2 times separated by 24 h, with one session paired with optical stimulation (ON) and one with no stimulation (OFF).

Afterwards, the same test protocol was repeated in the 2 following days; however, this time an object with the same format as a rodent was place inside one of the enclosures (object compartment). The amount of time the test animal spent in object and non-object compartment was measured. Groups were counterbalanced for order of light stimulation as well as side assigned as the social/object zone.

Locomotor activity protocol

Locomotion activity was assessed using the open field arena (43.2 cm × 43.2 cm; Med Associates Inc.). Animals were placed in the center of the arena, connected to the optic fiber, and were allow to freely move for 6 min. Optogenetic protocols used were the same as the ones applied in the social and object paradigms. Total distance and time travelling were used as indicators of locomotor activity.

Ultrasonic vocalizations analyses

An ultrasound microphone (Avisoft Bioacoustics) was placed above the behavior apparatus during social and object interaction paradigm. Ultrasonic vocalizations (USVs) were recorded using an Avisoft Recorder (version 5.1.04). The following settings were used: sampling rate: 250 000; format: 16 bit. For analysis, recordings were transferred to Avisoft SASLab Pro (version 5.1.22; Avisoft Bioacoustic). This program produce spectrograms of USVs, with a frequency resolution of ~1.2 kHz and a temporal resolution of ~0,4 ms, by conducting a fast Fourier transformation (256 FFT length, 100% frame, Hamming window filter, and 50% time window overlap).

Detection of 50 kHz USVs was supplied by an automated threshold-based algorithm (threshold: - 40 dB) and a hold time mechanism (hold time: 5 ms). Lower cut-off frequency of 40 kHz was used to eliminate background noise. Only calls with duration between 0.2 ms and 0.8

ms were counted. USVs were also inspected manually to ensure that, when necessary, calls not detected automatically could be subsequently counted.

In vivo anesthetized electrophysiology recordings

A different subset of animals injected with the virus was anaesthetised with urethane (1,44 g/kg, Sigma) four weeks post surgery. The total dose was administered in three separate intraperitoneal injections, 15 min apart. Adequate anaesthesia was confirmed by the lack of withdrawal responses to hind limb pinching. A recording electrode coupled with fiber optic patch cable (Thorlabs) was placed in the NAc, following anatomical coordinates: +1.2 mm AP, +1.2 mm ML, and -6.0 to -7.0 mm DV from bregma (Paxinos and Watson, 2007), using a stereotaxic frame (David Kopf Instruments) with non-traumatic ear bars (Stoeling).

Extracellular neural activity in the NAc was recorded with a tungsten electrode (tip impedance 5–10 Mat 1 kHz). Recordings were amplified and filtered by the Axoclamp (Molecular Devices) (low-pass filter at 500 Hz and high-pass filter at 0.5 kHz). Spikes of single neuron were discriminated, and digital pulses were detected by laboratory interface and software (CED micro 1401 interface, SPIKE 2 software; Cambridge Electronic Design) and led to a computer. The DPSS 473 nm laser system or DPSS 589 nm laser system (CNI), controlled by a stimulator (Master-8, AMPI) were used for light delivery. For excitation the following protocol was used: 40 Hz, 40 pulses of 12.5 ms, every 10 s, 10 mW at the point of the fiber, generated by the 473 nm laser. Optic inhibition consist in 8 s of constant light, with an interval of 2 s, 5 mW at the point of the fiber, generated by the 589 nm laser.

Electrophysiological data analysis

For electrophysiology data analysis, firing rate histograms were calculated for the baseline (60 s before stimulation), stimulation period and after stimulation period (60 s after the end of stimulation).

The cells were considered as responsive or not responsive to the stimulation on the basis of their firing rate change with respect to the baseline period. Neurons showing a firing rate higher or lower than 20% from the mean frequency of the baseline period were considered as responsive (Benazzouz et al., 2000; Soares-Cunha et al., 2016).

Immunofluorescence

Animals were anesthetized with pentobarbital (Eutasil) and were transcardially perfused with 0.9% of saline followed by 4% of paraformaldehyde. Brains were removed and fixed in 4% of paraformaldehyde for 24 h. Coronal vibratome sections (50 μm) were incubated with primary antibodies mouse anti-D2 receptor (1:400, B-10, Santa Cruz Biotechnology) or with mouse anti-D1 receptor (1:300, NB110-60017, Novus Biological), for 48 h or 24 h at room temperature (RT), respectively. Appropriate fluorescent secondary antibody was used for 2 h at RT (AlexaFluor-594 anti-mouse, 1:1000, Life Technologies). Afterwards, sections were wash and incubated with the primary antibody goat anti-GFP (1:500, ab6673, Abcam) overnight at 4°C, followed by incubation with fluorescent secondary antibody for 2 h at RT (AlexaFluor-488 anti-goat, 1:1000, Life Technologies). All sections were mounted on microscope slides and images were taken using a confocal microscopy (Olympus FluoViewTMFV1000).

Histology

For histological determination of the fiber sites localization, coronal sections (50 μm) were cut in vibratome (Leica), mounted and stained with hematoxylin, to help to delimit regional boundaries, before mounting and coversliping.

Statistical Analysis

Data was presented as mean ± standard error of the mean (SEM). Statistical analysis was performed using Graphpad Prism 6.01 software. First, data was verified for normality distribution. Comparisons between two groups were achieved by using Student t-test. When no Gaussian distribution was assumed, a nonparametric test (Mann-Whitney U-test or Wilcoxon Signed-Rank test) was used. More than two groups were analysed by using the ANOVA. Statistical significance was set at $p<0.05$.

Results

Optogenetic stimulation of nucleus accumbens D2R-expressing neurons

NAc D2R-expressing neurons were transduced with channel-rhodopsin (ChR2) fused with a eYFP fluorescent tag (hChR2(H134R)-eYFP) under the control of D2R minimal promoter. This group was entitled the CONT-D2-ChR2 group, whereas the CONT-D2-eYFP group, the control animals, were injected with a similar viral vector, but only expressing eYFP and not ChR2. To stimulate NAc D2R-expressing neurons, an optic fiber was implemented in the NAc to allow the delivery of light (Fig.1A).

First, we observed that optical activation of D2R-expressing neurons in the NAc lead to an increase in accumbal firing rate (Fig.1B, $\chi^2(3)=13.80$, p=0.001; baseline vs during stimulation: p=0.003; baseline vs after stimulation: p=0.906; during vs after stimulation: p=0.076), with 56.8% of cells presenting an increase in activity and 6.8% decreased activity, while 36.4% of cells did not respond (Fig.1C). After stimulation, 25% of cells presented an increase activity and 9.1% decreased activity, while 65.9% of cells did not respond, when compared with baseline neuronal activity (Fig.1C).

A different group of animals was then tested in a resident-intruder test. Each rat was tested twice, in two separated days, with a different intruder (Fig.1D). After 1 min for the animal to freely explore the arena, an intruder was placed inside the home cage, for 3 min, period in which both CONT-D2-ChR2 and CONT-D2-eYFP rats were paired with either no optical stimulation (OFF) or with optical stimulation (ON) (with the following settings: frequency: 40 Hz, number of pulses: 40, pulse duration: 12.5 ms, interval: 10 s).

We observed an increase in change in social interaction (social interaction time ON – social interaction time OFF) in CONT-D2-ChR2 group, when compared with CONT-D2-eYFP control animals (Fig.1E, $t_{27}=4.119$, p=0.0003). Regarding the effect of the stimulation within groups, in CONT-D2-ChR2-expressing we observed an increase in social interaction time with the laser ON compared with the laser OFF (Fig.1G, $t_{14}=4.055$, p=0.001). Surprisingly, in CONT-D2-eYFP group, we observed a trend for a decrease in social interaction time, with the laser ON (Fig.1F, $t_{13}=2.137$, p=0.052).

Regarding the 50 kHz USVs emitted during the social interaction paradigm, no differences were observed in the number of USVs emitted with the laser ON and laser OFF

(Fig.1H, t_{17}=0.116, p=0.909). However, unexpectedly, CONT-D2-eYFP group presented a tendency to increase the number of calls emitted with laser ON when compared with laser OFF (Fig.1I, t_{20}=1.915, p=0.07). A tendency for an increased number of USVs with optical stimulation was observed in the CONT-D2-ChR2 group (Fig.1J, t_{14}=1.807, p=0.092).

In the two subsequent days, animals were exposed to the same interaction paradigm, but this time using an object as "intruder". This experiment was performed in order to verify if the increase in interaction observed in stimulated CONT-D2-ChR2 rats was due to an increased interest for socializing with other animals, or simply a general increase in exploratory/novelty seeking behavior. Upon NAc D2R-optical stimulation, no differences were observed in object interaction time between groups (Fig.1K, t_{25}=0.211, p=0.835; Fig.1L, CONT-D2-eYFP: U=86, p=0.595; Fig.1M, CONT-D2-ChR2: U=84, p=0.986). In addition, regarding the emitted calls during object interaction, no differences were observed (Fig.1N, U=13, p=0.295; Fig.1O, CONT-D2-eYFP: Z=-6, p=0.688; Fig.1P, CONT-D2-ChR2: Z=9, p= 0,438).

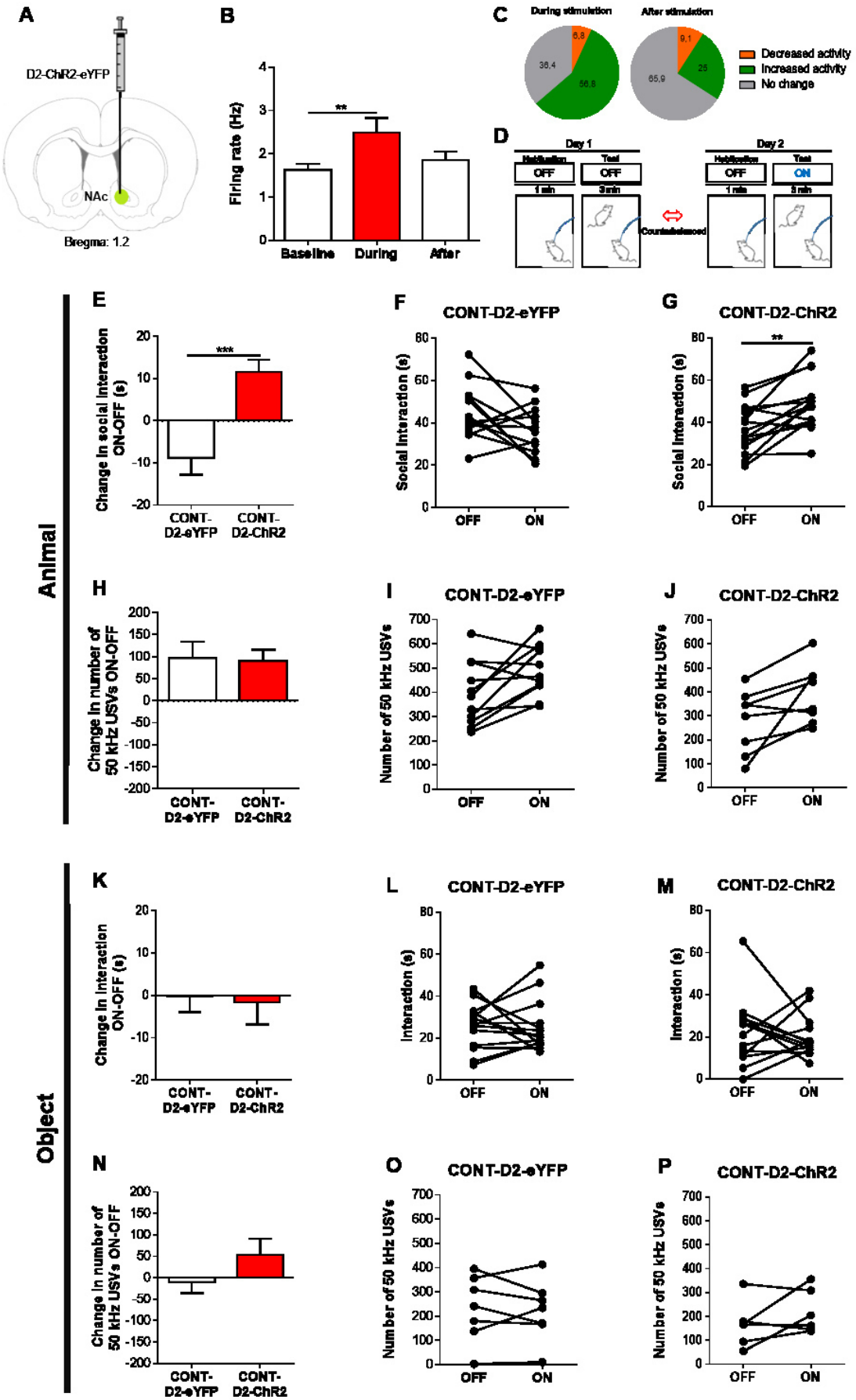
A
D2-ChR2-eYFP
NAc
Bregma: 1.2
B
Firing rate (Hz)
**
Baseline
During
After
C
During stimulation
After stimulation
6,8
36,4
56,8
9,1
25
65,9
Decreased activity
Increased activity
No change
D
Day 1
Habituation
Test
OFF
OFF
1 min
3min
Day 2
OFF
ON
Counterbalanced
Animal
E
Change in social interaction ON-OFF (s)
***
CONT-D2-eYFP
CONT-D2-ChR2
F
CONT-D2-eYFP
Social Interaction (s)
OFF
ON
G
CONT-D2-ChR2
**
H
Change in number of 50 kHz USVs ON-OFF
I
CONT-D2-eYFP
Number of 50 kHz USVs
J
CONT-D2-ChR2
Object
K
Change in Interaction ON-OFF (s)
L
CONT-D2-eYFP
Interaction (s)
M
CONT-D2-ChR2
N
Change in number of 50 kHz USVs ON-OFF
O
CONT-D2-eYFP
P
CONT-D2-ChR2

Figure 1. Effects of optogenetic stimulation of NAc D2R-expressing neurons in social and object interaction. (A) Experimental design. We injected AAV5-D2-hChR2(H134R)-eYFP in the NAc. **(B)** *In vivo* electrophysiological recordings. Increase in accumbal firing rate during optogenetic stimulation. Optical stimulation parameters used were: 473 nm light delivered in 40 Hz burst (40 pulses, 12.5 ms each pulse - Excitation) (n=38 cells, n=4 animals). **(C)** More than half of the cells increase firing rate (56.8%), 6.8% decrease and 36.4% did not respond during stimulation. After the optical stimulation, 65.9% of cells do not change, 25% increase and 9.1% decrease neuronal activity when compared with baseline activity. **(D)** Schematic design of the home-cage resident-intruder behavioral procedure. After 1 min of habituation, 3 min social interaction period was counterbalanced, with a 24 h of interval, between ON and OFF laser conditions. **(E)** Summary of light-evoked changes in social interaction after excitation of the NAc D2R-expressing neurons (40 Hz, every 10 s). Phasic stimulation of accumbal D2R-expressing neurons induces an increase in social interaction in comparison with CONT-D2-eYFP group ($n_{CONT\text{-}D2\text{-}eYFP}$= 14, $n_{CONT\text{-}D2\text{-}ChR2}$=15). Summary of individual responses of NAc stimulation in CONT-D2-eYFP **(F)** and CONT-D2-ChR2 **(G)** animals during the resident-intruder paradigm. **(H)** Summary of light-evoked changes in number of 50 kHz USVs emitted during the resident-intruder paradigm after optical stimulation of the D2R-expressing neurons in the NAc. No differences were found between groups ($n_{CONT\text{-}D2\text{-}eYFP}$=11, $n_{CONT\text{-}D2\text{-}ChR2}$=8). Summary of individual responses of NAc stimulation in CONT-D2-eYFP **(I)** and CONT-D2-ChR2 **(J)** animals during social interaction. **(K)** Summary of stimulation-evoked changes in object interaction after excitation of the accumbal D2R-expressing neurons. No differences were found between groups ($n_{CONT\text{-}D2\text{-}eYFP}$=14, $n_{CONT\text{-}D2\text{-}ChR2}$=13). Summary of individual responses of optical stimulation of NAc D2R-expressing neurons in CONT-D2-eYFP **(L)** and CONT-D2-ChR2 **(M)** animals during objection interaction. **(N)** Summary of stimulation-evoked changes in number of 50 kHz USVs emitted after excitation of the accumbal D2R-expressing neurons, during object interaction paradigm. No differences were found between groups ($n_{CONT\text{-}D2\text{-}eYFP}$=7, $n_{CONT\text{-}D2\text{-}ChR2}$=6). Summary of individual responses (number of calls) of NAc D2R-expressing neurons excitation in CONT-D2-eYFP **(O)** and CONT-D2-ChR2 **(P)** animals during objection interaction.

NAc, nucleus accumbens; D2R, dopamine receptor 2; USVs, ultrasonic vocalizations. Data presented as mean ± SEM; **p<0.01, ***p<0.001.

To evaluate other aspects of social behavior, we performed the three-chamber sociability test (Fig.2A). Each trial had 5 min of habituation to freely explore the apparatus, followed, by another 5 min of either laser OFF or laser ON (test sessions). During the test sessions, the tested rats were presented with an intruder or an object inside of the inverted enclosure. There were no differences in the number of entries in the intruder-paired chamber between groups (Fig.2B, $F_{(1,30)}$=0.994, p=0.327) and in the time spent in the social arena (Fig.2C, $F_{(1,30)}$=0.051, p=0.823). However, it seems that stimulation had an effect on time spent (Fig.2C, $F_{(1,\ 30)}$= 4,761, p=0.037) but not in the number of entries in the social arena (Fig.2C, $F_{(1,\ 30)}$= 0,397, p=0.533). Within groups no differences were observed between laser OFF and ON (Fig.2B, number of entries: CONT-D2-eYFP: t_{30}=0.142, p>0.999; CONT-D2-ChR2: t_{30}=1.069, p>0.999; Fig.2C, time spent:

CONT-D2-eYFP: t_{30}=1.058, p>0.999; CONT-D2-ChR2: t_{30}=2.058, p=0.290). In addition, no differences were observed within laser OFF (Fig.2B, number of entries: t_{30}=1.297, p>0.999, Fig.2C, time spent: t_{30}=0.294, p>0.999) or within laser ON results (Fig.2B, number of entries: t_{30}=0.113, p>0.999, Fig.2C, time spent: t_{30}=0.613, p> 0.999).

Concerning the number of entries and time spent in the object arena, laser OFF-ON was similar between groups (Fig.2D, number of entries: $F_{(1,30)}$=1,205, p=0,281; Fig.2E, time spent: $F_{(1,30)}$=0.474, p=0.496). However, it was still observed an effect of group in time spent (Fig.2E, $F_{(1,30)}$=4.631, p=0.039), but not in number of entries in the arena (Fig.2D, $F_{(1,30)}$=0.994, p=0.327).

Within groups no differences were observed between laser OFF and ON (Fig.2D, number of entries: CONT-D2-eYFP: t_{30}=1.234, p> 0,999; CONT-D2-ChR2: t_{30}=0.291, p> 0.999; Fig.2E, time spent: CONT-D2-eYFP: t_{30}=0,083, p> 0.999; CONT-D2-ChR2: t_{30}=1.092, p> 0.999). Within laser OFF and laser ON sessions (Fig.2D, number of entries: OFF: t_{30}=0.247, p>0.999; ON: t_{30}=1.235, p>0.999; Fig.2E, time spent: OFF: t_{30}=2.094, p=0.269; ON: t_{30}=0.949, p>0.999) no differences were observed between groups.

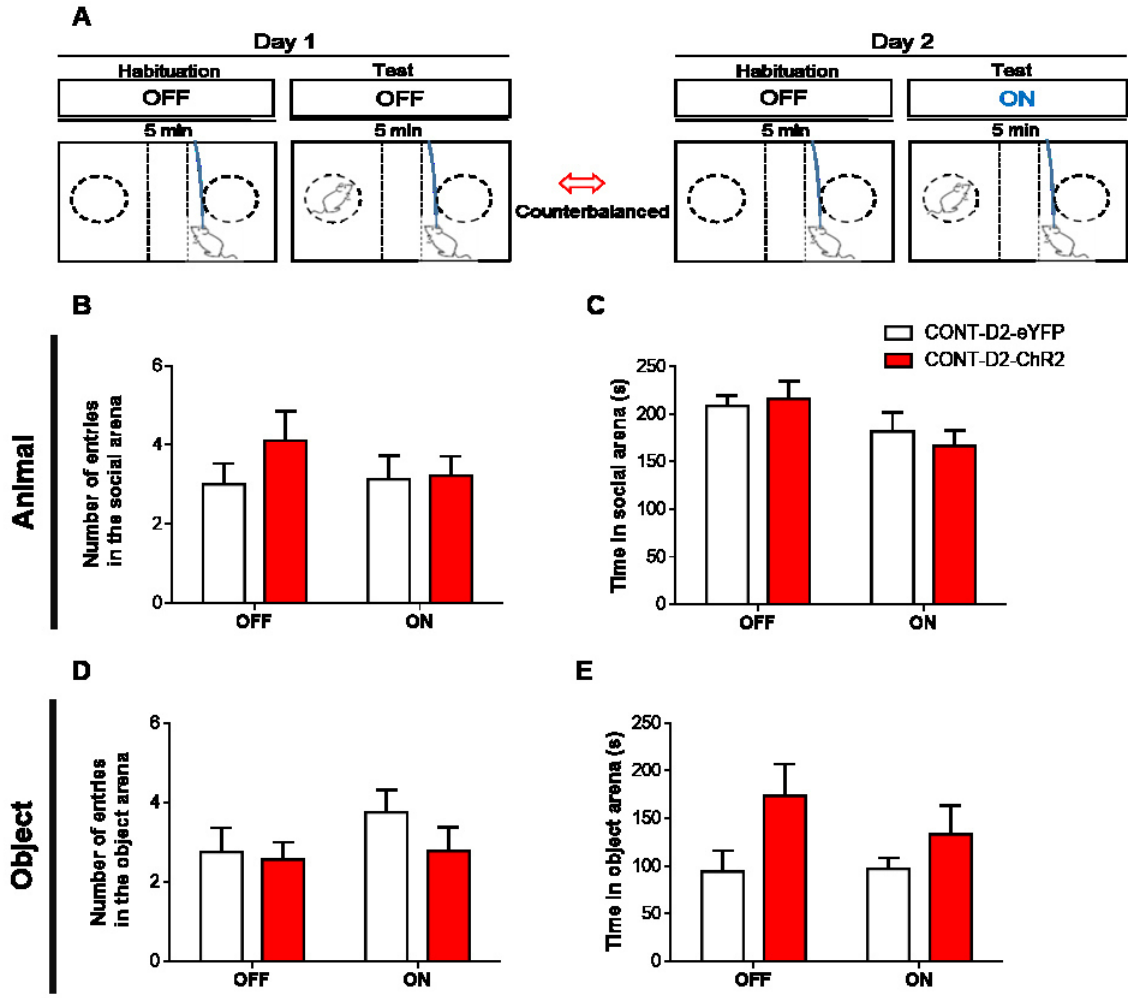

Figure 2. Effects of optogenetic stimulation of NAc D2R-expressing neurons in the three-chamber sociability test. (A) Schematic design of the three-chamber sociability behavioral procedure. After 5 min of habituation to the apparatus, animals performed a 5 min social interaction session (with laser ON or laser OFF, with an interval of 24 h), counterbalanced with the intruder animal/object placed inside an inverted enclosure. The order was counterbalanced for laser ON and laser OFF session, as well as for placement of the enclosed animal/object in the testing arena. Optical stimulation parameters used were: 473 nm light delivered in 40 Hz burst (40 pulses, 12.5 ms each) every 10 s (Excitation). **(B)** Number of entries and time spent **(C)** in the social zone of the arena during the test session. Stimulation of accumbal D2R-expressing neurons had no effect in the number of entries and time spent in the social arena, when comparing laser ON/OFF and experimental group ($n_{CONT\text{-}D2\text{-}eYFP}$= 8, $n_{CONT\text{-}D2\text{-}ChR2}$=9). **(D)** Number of entries and time spent **(E)** in the object zone of the arena during the test session. No differences were observed between groups ($n_{CONT\text{-}D2\text{-}eYFP}$= 8, $n_{CONT\text{-}D2\text{-}ChR2}$=9).

NAc, nucleus accumbens; D2R, dopamine receptor 2. Data presented as mean ± SEM.

Moreover, optic stimulation of NAc D2R-expressing neurons did not change locomotor activity; no differences were found between groups, neither on distance or time travelled during

the 6 min of the test (Fig.3A, Distance: U=90, p=0.969; Fig.3B, Time: U=89, p=0.931; Fig.2K, Time: $F_{(35, 875)} = 2.640$, $p<0.001$; Group: $F_{(1, 25)} = 0.006$, $p= 0.939$).

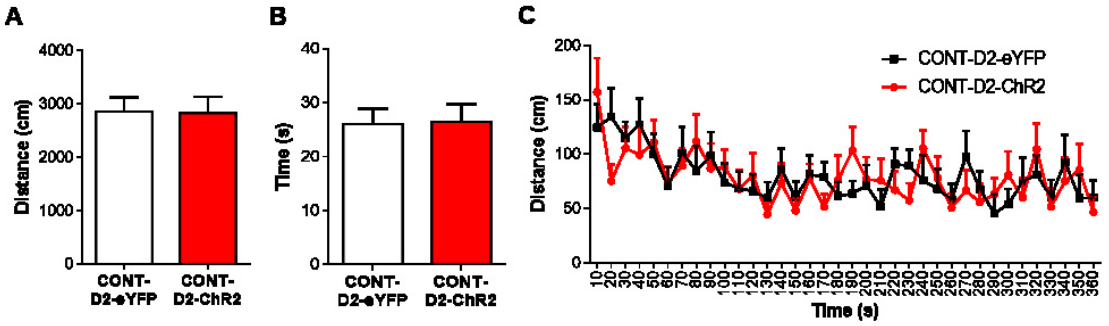

Figure 3. Effects of optogenetic stimulation of accumbal D2R-expressing neurons in locomotor activity. No differences were observed in distance **(A)** and time travelled **(B)** in the open field, between CONT-D2-eYFP and CONT-D2-ChR2, upon excitation of NAc D2R-expressing neurons. Optical stimulation parameters used were: 473 nm light delivered in 40 Hz burst (40 pulses, 12.5 ms each) every 10 s (Excitation). **(C)** Time had an impact in the distance travelled throughout the duration of the test. No differences were observed between groups ($n_{CONT\text{-}D2\text{-}eYFP}$= 14, $n_{CONT\text{-}D2\text{-}ChR2}$=13).

NAc, nucleus accumbens; D2R, dopamine receptor 2. Data presented as mean ± SEM.

Regarding inhibition of accumbal D2R-expressing neurons, the experimental groups, were injected with either the AAV5-D2-eNpHR3.0-eYFP (inhibition, CONT-D2-eNpHR) or the AAV5-D2-eYFP (control, CONT-D2-eYFP) virus in the NAc (Fig.4A). This group was entitled the CONT-D2-eNpHR group. Optical inhibition lead to a decrease in NAc firing rate (Fig.4B, $\chi^2(3)=14.91$, p=0.001; baseline vs during stimulation: p=0.003; baseline vs after stimulation: p>0.999; during vs after stimulation: p=0.003). 54.2% of cells presented a decrease activity and 14.6% increased activity, while 31.2% of cells did not respond (Fig.4C). After stimulation, half of cells (50%) did not respond, 29.2% increased activity and 20.8% decreased activity when compared with baseline neuronal activity (Fig.1C).

The inhibition of accumbal D2R-expressing neurons (589 nm laser with a pulse of 8 s with an interval of 2 s) did not affect change in social interaction time and number of calls emitted (Fig.4D, Social: t_{23}=0.86, p=0.398; Fig.4G, USVs: t_{15}=0.617, p=0.546). Laser ON led to a decrease in social interaction time (Fig.4F, t_{20}=3.011, p=0.007), whereas no differences were observed in the number of USVs emitted (Fig.4I, Z=9, p=0,438), in CONT-D2-eNpHR group.

During the object interaction paradigm, no differences were observed in object interaction time and number of 50 kHz USVs (Fig.4J, Object: t_{26}=0.449, p=0.657; Fig.4M, USVs:

U=20.5, p=0.975). Within the CONT-D2-eNpHR group, no differences were observed in object interaction time and number of 50 kHz calls, when comparing Laser OFF with Laser ON sessions (Fig.4L, Object: t_{26}=0.868, p=0.394; Fig.4O; USVs: Z=-9, p= 0,438).

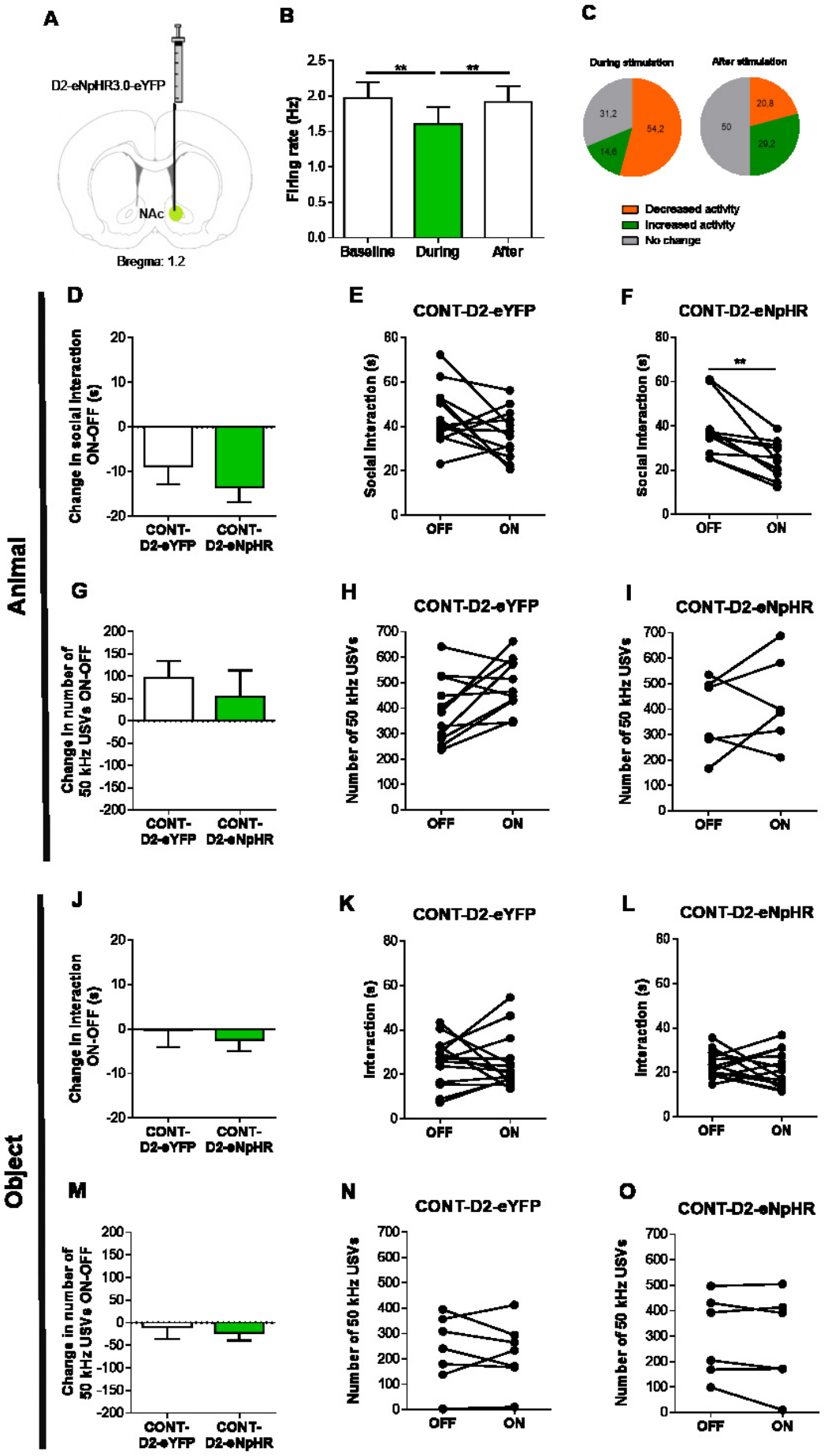
A
D2-eNpHR3.0-eYFP
NAc
Bregma: 1.2
B
Firing rate (Hz)
Baseline
During
After
C
During stimulation
After stimulation
31.2
54.2
14.6
50
20.8
29.2
Decreased activity
Increased activity
No change
Animal
D
Change in social interaction ON-OFF (s)
CONT-D2-eYFP
CONT-D2-eNpHR
E
CONT-D2-eYFP
Social Interaction (s)
OFF
ON
F
CONT-D2-eNpHR
G
Change in number of 50 kHz USVs ON-OFF
H
CONT-D2-eYFP
Number of 50 kHz USVs
I
CONT-D2-eNpHR
Object
J
Change in interaction ON-OFF (s)
K
CONT-D2-eYFP
Interaction (s)
L
CONT-D2-eNpHR
M
Change in number of 50 kHz USVs ON-OFF
N
CONT-D2-eYFP
Number of 50 kHz USVs
O
CONT-D2-eNpHR

Figure 4. Effects of optogenetic inhibition of NAc D2R-expressing neurons in social and object interaction. (A) Experimental design. AAV5-D2-eNpHR3.0-eYFP (or eYFP) containing virus was injected in the NAc. **(B)** *In vivo* electrophysiological recordings. Decrease in the NAc firing activity during optogenetic inhibition. Optical parameters used were: 589 nm light delivered during 8 s (n=48 cells, n=4 animals). **(C)** More than half of the cells decrease firing rate (54.2%), 14.6% increase and 31.2% did not respond during stimulation. After optical stimulation, 50% of cells do not change, 29.2% increase and 20.8% decrease neuronal activity when compared with baseline activity. **(D)** Summary of light-evoked changes in social interaction (8 s of constant light every 2 s). Inhibition of accumbal D2R-expressing neurons had no effect in social interaction compared with CONT-D2-eYFP group ($n_{CONT\text{-}D2\text{-}eYFP}$= 14, $n_{CONT\text{-}D2\text{-}eNpHR}$=11). Summary of individual responses of NAc inhibition in CONT-D2-eYFP **(D)** and CONT-D2-eNpHR **(E)** animals during the resident-intruder paradigm. CONT-D2-eNpHR presented a decrease in total time in social interaction in the laser ON session. **(F)** Summary of light-evoked changes in the number of 50 kHz USVs emitted during the social interaction assay after optical inhibition of the D2R-expressing neurons in the NAc. No differences were found between groups ($n_{CONT\text{-}D2\text{-}eYFP}$=11, $n_{CONT\text{-}D2\text{-}eNpHR}$=6). Summary of individual responses of NAc stimulation in CONT-D2-eYFP **(G)** and CONT-D2-eNpHR **(H)** animals during resident-intruder interaction. **(I)** Summary of stimulation-evoked changes in object interaction after inhibition of the accumbal D2R-expressing neurons. No differences were found between groups ($n_{CONT\text{-}D2\text{-}eYFP}$=14, $n_{CONT\text{-}D2\text{-}eNpHR}$=14). Summary of individual responses of optical stimulation of NAc D2R-expressing neurons in CONT-D2-eYFP **(J)** and CONT-D2-eNpHR **(K)** animals during objection interaction. **(L)** Summary of stimulation-evoked changes in the number of 50 kHz USVs emitted after inhibition of the accumbal D2R-expressing neurons, during object interaction paradigm. No differences were found between groups ($n_{CONT\text{-}D2\text{-}eYFP}$-7, $n_{CONT\text{-}D2\text{-}eNpHR}$=6). Summary of individual responses (number of calls) of NAc D2R-expressing neurons inhibition in CONT-D2-eYFP **(M)** and CONT-D2-eNpHR **(N)** animals during objection interaction.

NAc, nucleus accumbens; D2R, dopamine receptor 2; USVs, ultrasonic vocalizations. Data presented as mean ± SEM; **p<0.01.

Surprisingly, NAc D2R-expressing neurons inhibition lead to a decrease in distance and time spent travelled in a locomotor activity assay (Fig.5A, Distance: U=39, p=0.006; Fig.5B, Time: U=31, p= 0.001). Regarding the distance travelled throughout the duration of the test, both time and group had a significant effect (Fig.5C, Time: $F_{(35,\ 910)}$=2.372, p<0.0001; Group: $F_{(1,\ 26)}$= 9.130, p= 0.006).

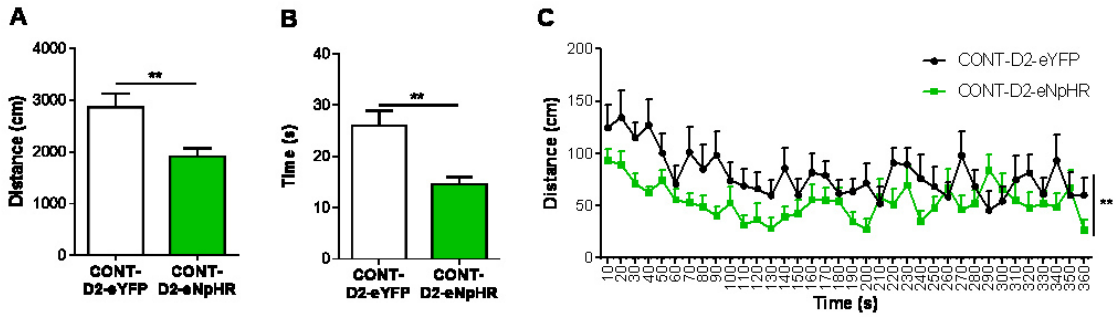

Figure 5. Effects of optogenetic inhibition of accumbal D2R-expressing neurons in locomotor activity. Inhibition of NAc D2R-expressing neurons lead to a decrease in distance **(A)** and time travelled **(B)** in the open field paradigm. Optogenetic parameters used were: 589 nm light delivered in during 8 s every 2 s. **(C)** Time and group has an impact in the distance travelled throughout the duration of the test ($n_{CONT\text{-}D2\text{-}eYFP}$= 14, $n_{CONT\text{-}D2\text{-}eNpHR}$=14).

NAc, nucleus accumbens; D2R, dopamine receptor 2. Data presented as mean ± SEM; **p<0.01.

Rescue of social deficits in an *in utero* glucocorticoid exposure (iuGC) model

We then assessed if NAc D2R-expressing neurons stimulation was sufficient to normalize the impaired social interaction present in a rat model of iuGC exposure. These animals present hypodopaminergia in the mesolimbic circuit, upregulation of NAc D2R expression levels and a significant impairment in social behaviors (Borges et al., 2013; Rodrigues et al., 2012).

Control and iuGC animals were unilaterally injected with the D2-ChR2-eYFP construct and submitted to *in vivo* electrophysiological recordings, social/object interaction and locomotor activity. As expected, after the results from the CONT-D2-ChR2 group, optical stimulation lead to an increase in NAc neuronal activity (Sup. Fig.1A, $\chi^2(3)=10.5$, p=0.005; baseline vs during stimulation: p=0.056; baseline vs after stimulation: p>0.999; during vs after stimulation: p=0.009). We observed that more than half of the cells (56.8%) presented an increase in neuronal activity and 6.8% showed a decrease in activity, while 36.4% of cells did not respond (Sup. Fig.1B). After stimulation, 65.9% of cells returned to basal activity, 25% presented increased activity and 9.1% decreased activity (Sup. Fig.1B).

As previously shown by our laboratory, iuGC rats presented a decrease in social interaction time (Fig.6A, t_{51}=3.006, p=0.004) however, no differences in USVs emitted were observed (Fig.6B, t_{28}=0.899, p=0.376), when comparing with the CONT group.

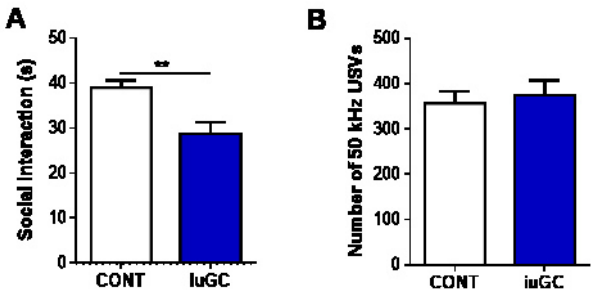

Figure 6. *In utero* glucocorticoid exposed (iuGC) animals present social deficits. (A) iuGC group presented a decrease in total time spent in social interaction, when compared with the CONT group (n_{CONT}= 40, n_{iuGC}=13). **(B)** No differences were observed between groups in the number of 50 kHz USVs emitted during the social encounter (n_{CONT}= 15, n_{iuGC}=15).

iuGC, *in utero* glucocorticoid exposure; USVs, Ultrasonic vocalizations. Data presented as mean ± SEM; **p<0.01.

During the social interaction paradigm, differences were observed in changes in social interaction time between groups (Kruskal-Wallis=10.09, p=0.006). No differences were observed in changes in social interaction time between CONT-D2-eYFP and iuGC-D2-eYFP (Fig.8A, p> 0,9999). iuGC-D2-ChR2 group showed an increase in change in social interaction time when compared with CONT-D2-eYFP (p=0.006), while a small trend was observed when comparing with the iuGC-D2-eYFP group (p=0.085). Within groups, we observed no difference in iuGC-D2-eYFP group, a trend in CONT-D2-eYFP group, and an increase in the iuGC-D2-ChR2 group, between OFF and ON sessions (Fig.8B, CONT-D2-eYFP: t_{13}=2.137, p= 0.0522; Fig.8C, iuGC-D2-eYFP: Z=-5, p=0.687; Fig.8D, iuGC-D2-ChR2: Z=36, p=0.008).

Regarding the 50 kHz USVs, no differences were observed between groups (Fig.8E, Kruskal-Wallis=0.03, p=0.985; CONT-D2-eYFP vs iuGC-D2-eYFP: p>0.999; CONT-D2-eYFP vs iuGC-D2-ChR2: p>0.999; iuGC-D2-eYFP vs iuGC-D2-ChR2: p>0.999). Within groups, no differences were observed in the number of calls emitted when comparing laser OFF and Laser ON, in iuGC-D2-eYFP and iuGC-D2-ChR2 (Fig.8G. iuGC-D2-eYFP: Z=15, p=0.156; Fig.8H, iuGC-D2-ChR2: t_7=1.569, p=0.161). However, unexpectedly, CONT-D2-eYFP group presented a slight increase in number of calls emitted with laser ON when compared with laser OFF (Fig.8F, t_{20}=1.915, p=0.07).

No differences were observed in object interaction time (Fig.8I, Kruskal-Wallis=1.736, p=0.42; CONT-D2-eYFP vs iuGC-D2-eYFP: p=0.684; CONT-D2-eYFP vs iuGC-D2-ChR2: p>0.999; iuGC-D2-eYFP vs iuGC-D2-ChR2: p=0.685) and number of calls (Fig.8M, Kruskal-Wallis=0.538, p=0.779; CONT-D2-eYFP vs iuGC-D2-eYFP: p>0.999; CONT-D2-eYFP vs iuGC-D2-ChR2: p>0.999;

iuGC-D2-eYFP vs iuGC-D2-ChR2: p>0.999). Moreover, no differences were observed in objection interaction time within groups (Fig.8J, CONT-D2-eYFP: Z=1, p>0.999; Fig.8K, iuGC-D2-eYFP: Z=-9, p=0.313; Fig.8L, iuGC-D2-ChR2: Z=2, p=0.945) and the number of calls emitted (Fig.8N, CONT-D2-eYFP: Z=-6, p=0.687; Fig.8O, iuGC-D2-eYFP: Z=-1, p>0.999; Fig.8P, iuGC-D2-ChR2: Z=8, p=0.641), when comparing Laser OFF and Laser ON sessions.

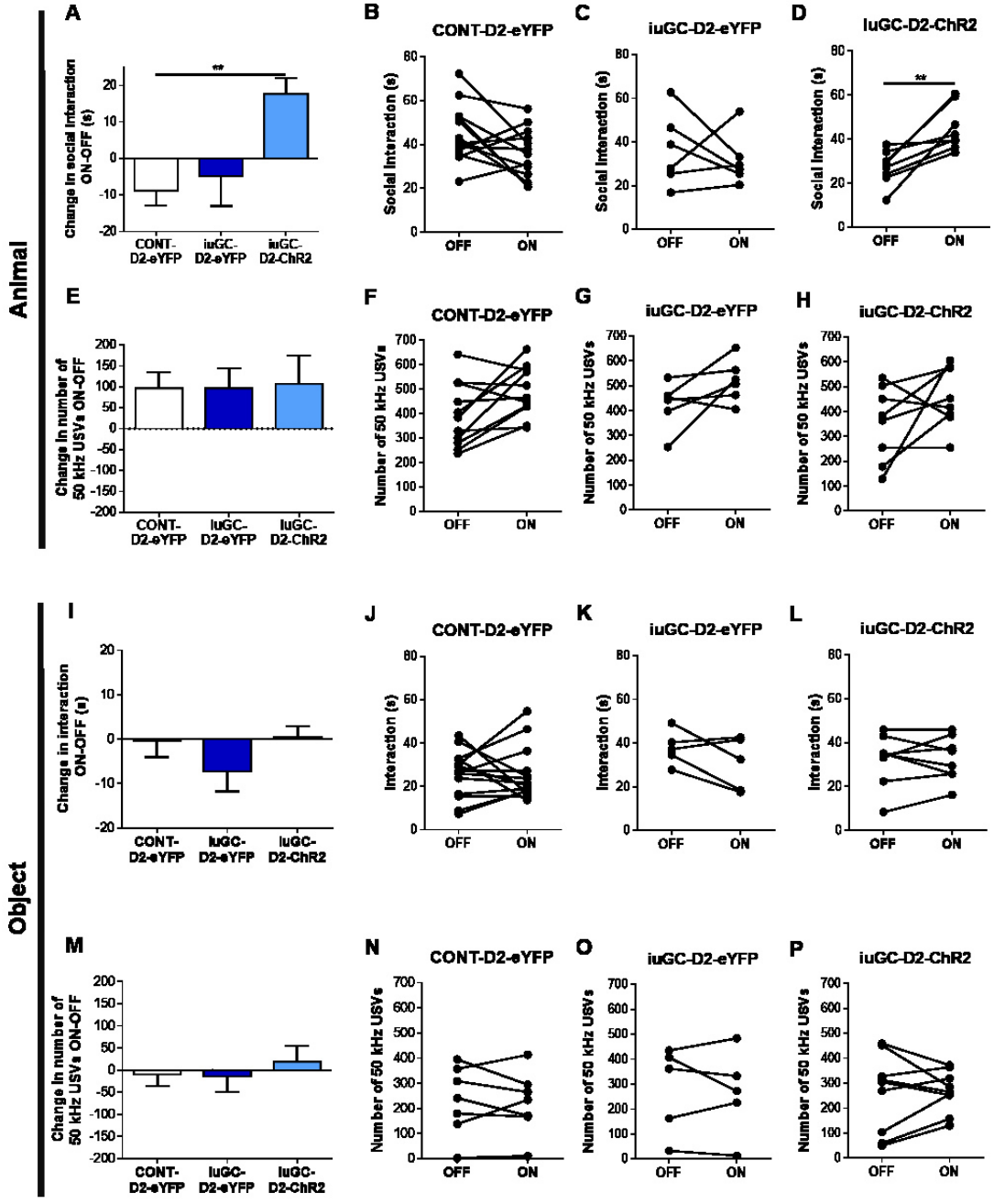

Figure 7. Effects of optogenetic stimulation of NAc D2R-expressing neurons in the iuGC animal model, during social and object interaction. (A) Optical stimulation parameters used were: 473 nm light delivered in 40 Hz burst (45 pulses, 12.5 ms each) every 10 s (Excitation). Excitation of accumbal D2R-expressing neurons rescues the social deficits observed in the iuGC animal model ($n_{CONT\text{-}D2\text{-}eYFP}$= 14, $n_{iuGC\text{-}D2\text{-}eYFP}$= 6, $n_{iuGC\text{-}D2\text{-}ChR2}$=9).

Summary of individual responses in social interaction after excitation of the NAc D2R-expressing neurons of CONT-D2-eYFP **(B)**, iuGC-D2-eYFP **(C)** and iuGC-D2-ChR2 group **(D)**. In the iuGC-D2-ChR2 group, was observed an increase in social interaction time, when comparing laser ON with laser OFF sessions. **(E)** Summary of light-evoked changes in number of 50 kHz USVs emitted during the resident-intruder paradigm after optical stimulation of the D2R-expressing neurons in the NAc. No differences were found between groups ($n_{CONT\text{-}D2\text{-}eYFP}$= 11, $n_{iuGC\text{-}D2\text{-}eYFP}$= 6, $n_{iuGC\text{-}D2\text{-}ChR2}$=8). Summary of individual responses (number of 50 kHz USVs) of stimulation of NAc D2R-expressing neurons in CONT-D2-eYFP **(F)**, iuGC-D2-eYFP **(G)** and iuGC-D2-ChR2 **(H)** animals during social interaction. No differences were observed within groups. **(I)** Excitation of accumbal D2R-expressing neurons has no effect in changes in object interaction ($n_{CONT\text{-}D2\text{-}eYFP}$=14, $n_{iuGC\text{-}D2\text{-}eYFP}$=5, $n_{iuGC\text{-}D2\text{-}ChR2}$=8). Summary of individual responses in object interaction after excitation of the NAc D2 neurons of CONT-D2-eYFP **(J)**, iuGC-D2-eYFP **(K)** and iuGC-D2-ChR2 group **(L)**. No differences were observed within groups when comparing laser ON with laser OFF sessions. **(M)** Summary of light-evoked changes in number of 50 kHz USVs emitted during the novel object interaction after optical stimulation of the D2R-expressing neurons in the NAc. No differences were found between groups ($n_{CONT\text{-}D2\text{-}eYFP}$=7, $n_{iuGC\text{-}D2\text{-}eYFP}$=5, $n_{iuGC\text{-}D2\text{-}ChR2}$=8). Summary of individual responses (number of 50 kHz calls) of stimulation of NAc D2 neurons in CONT-D2-eYFP **(N)**, iuGC-D2-eYFP **(O)** and iuGC-D2-ChR2 **(P)** animals during object interaction. No differences were observed within groups.

NAc, nucleus accumbens; D2R, dopamine receptor 2; iuGC, *in utero* glucocorticoid exposure; USVs, Ultrasonic vocalizations. Data presented as mean ± SEM; **p<0.01.

In the locomotor activity test, activation of the NAc D2R-expressing neurons did not produce any effect in distance travelled (Fig.9A, Kruskal-Wallis=2.885, p=0.236; CONT-D2-eYFP vs iuGC-D2-eYFP: p>0.999; CONT-D2-eYFP vs iuGC-D2-ChR2: p=0.269; iuGC-D2-eYFP vs iuGC-D2-ChR2: p>0.999) and time (Fig.9B, Kruskal-Wallis=2.274, p=0.321; CONT-D2-eYFP vs iuGC-D2-eYFP: p>0.999; CONT-D2-eYFP vs iuGC-D2-ChR2: p=0.436; iuGC-D2-eYFP vs iuGC-D2-ChR2: p>0.999). Regarding the distance travelled throughout the test, time but not group was significantly different (Fig.9C, Time- $F_{(35, 875)}$ = 1.528, p=0.027; Group- $F_{(2, 25)}$= 1.232, p= 0.309).

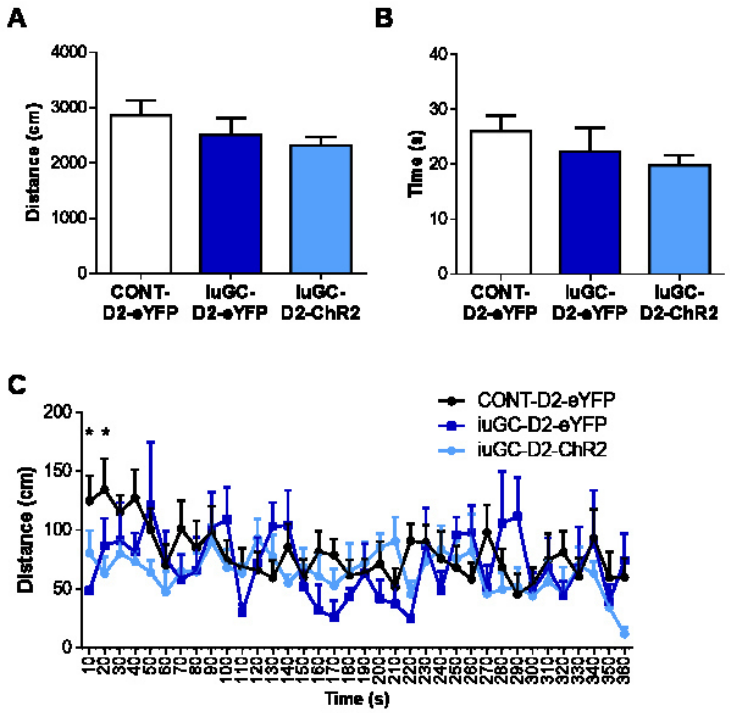

Figure 8. Effects of optogenetic excitation of accumbal D2R-expressing neurons in locomotor activity. No differences were observed in distance **(A)** and time travelled **(B)** in the open field, between CONT-D2-eYFP, iuGC-D2-eYFP and iuGC-D2-ChR2, upon excitation of NAc D2 neurons ($n_{CONT\text{-}D2\text{-}eYFP}$=14, $n_{iuGC\text{-}D2\text{-}eYFP}$=5, $n_{iuGC\text{-}D2\text{-}ChR2}$=9). **(C)** Time has an impact in the distance travelled throughout the duration of the test. No major differences were observed between groups (though at time 10 and 20 s we observe a decrease in distance travelled, when comparing CONT-D2-eYFP, of iuGC-D2-eYFP and iuGC-D2-ChR2, respectively).

NAc, nucleus accumbens; D2, dopamine receptor 2. Data presented as mean ± SEM; *p<0.05.

At the end of the experiment, animals were sacrificed, brains were extracted and prepared for microscopy. All animals were checked for correct cannula placement (Sup. Fig.2; Sup. Fig.3) and viral expression was confirmed (Sup. Fig. 4).

Discussion

Ours results demonstrate that optogenetic activation of NAc D2R-expressing neurons may influence adult same–sex social interaction in freely-behaving animals. These findings provide some hints on which pathways can be involved in behaviors relevant for autism spectrum disorder, obsessive-compulsive disorders, schizophrenia and anxiety social behaviors.

Previous studies suggest that the striatum plays an important role in many characteristics of social behaviors, both in humans (Aharon et al., 2001; Bhanji and Delgado, 2014; Davey et al., 2009; Fisher et al., 2006; Gunther Moor et al., 2010; Hughes and Beer, 2012; Spreckelmeyer et al., 2009; Zink et al., 2008)(Puglisi-Allegra and Cabib, 1997; Robinson et al., 2002, 2011) and in rodents (Manduca et al., 2016; Pellis et al., 1993). The striatum is divided in the dorsal and ventral part, often named as caudate-putamen and NAc, respectively. The dorsal division is subdivided into a medial and a lateral portion, and the nucleus accumbens into core and shell areas. Ninety-five percent of neurons in the NAc are MSNs containing mainly D1R or D2R (though some populations express both).

The NAc mediates the hedonic/pleasurable properties of rewards and plays a ubiquitous role in behaviors related to obtaining them, such as incentive salience and motivational processes (Berridge, 2007; Floresco, 2015; Salamone and Correa, 2012). Indeed, previous studies have shown that enhancing dopamine (DA) transmission in this region increases the "wanting" of animals to work for food, without changing the "liking" for the taste of the sweet food (Robinson et al., 2002; Wise, 2004). On the other hand, blunting DA transmission attenuates the "wanting" of animals to work for rewards (Salamone et al., 2003; Wise, 2004). Moreover, the NAc is also important in the modulation of same-sex interaction (Gunaydin et al., 2014; Manduca et al., 2016). Nonetheless, it is not known the specific role of the NAc D2R-expressing neurons on adult social behavior.

Injections of selective dopamine D1R and D2R receptor agonists suppressed juvenile social play behavior (Siviy et al., 1996). However, injections of non-selective dopamine receptor agonist induce an increase (Beatty et al., 1984; Vanderschuren et al., 2008) as well a decrease in social play behavior (Niesink and Van Ree, 1989). Furthermore, studies reported the role of D2R in the NAc for the formation of a partner preference in female voles. Microinjection of a D2R antagonist into the NAc (but not the prelimbic cortex) blocked the formation of a partner preference in mating voles, whereas a D2R agonist facilitated formation of a partner preference

in the absence of mating. Mating facilitated partner preference formation lead to an increase of approximately 50% in extracellular DA in the NAc (Gingrich et al., 2000).

However, with more precise techniques, such as optogenetic, studies of the ventral and dorsal striatum suggest that activation of D1-MSN or D2-MSN mediates distinct types of behaviors (e.g., approach vs avoidance) (Kravitz et al., 2012; Lobo et al., 2010). Indeed, optogenetic stimulating of D1R-expressing neurons induced persistent reinforcement, whereas stimulating D2R-expressing neurons induced transient punishment (Kravitz et al., 2012), in both operant and a place-preference tasks. However, our laboratory has shown that optogenetic activation (or inhibition) of NAc D2R-neurons during reward-predictive cues strongly enhances (or diminishes) motivation (Soares-Cunha et al., 2016).

Here in, we observed that optogenetic activation/inhibition of NAc D2R-expressing neurons leads to increase/decrease in social interaction. On the other hand, no effect in the object interaction time and in the number of 50 kHz USVs emitted during both paradigms. Regarding activation of NAc D2R-expressing neurons during the chamber sociability test, no differences were observed. A likely explanation for this lack of effect is that the animals are isolated for some weeks (since surgery), which may lead to near maximal levels of social behavior. Indeed, optogenetically stimulated animals (Laser OFF, social isolated animals) presented an increase in time spent in the social arena when compared with animals pair housed (no isolation) (Sup. Fig.5; t_{23}=2.506, p=0.02).

These findings were further extended by showing that activating NAc D2R-expressing neurons in a model of *in utero* glucocorticoid exposure that presents D2R dysfunction (Borges et al., 2013; Rodrigues et al., 2012) rescues social deficits of these animals.

In summary, these studies highlight the important role of D2R in the modulation of the motivation for social interaction, by pinpointing the NAc as a critical site of action.

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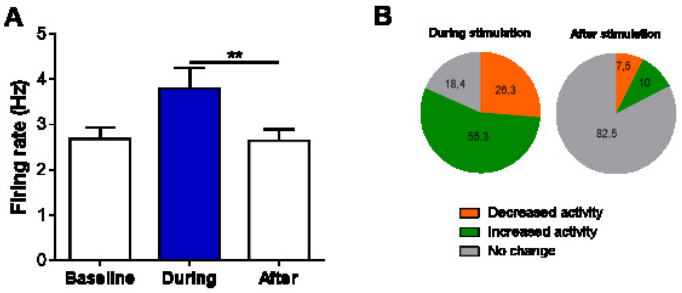
Supplementary Information

Borges S, Cunha-Soares C, Coimbra B, Sousa N, Rodrigues AJ

Role of dopamine receptor D2-expressing neurons in social behavior

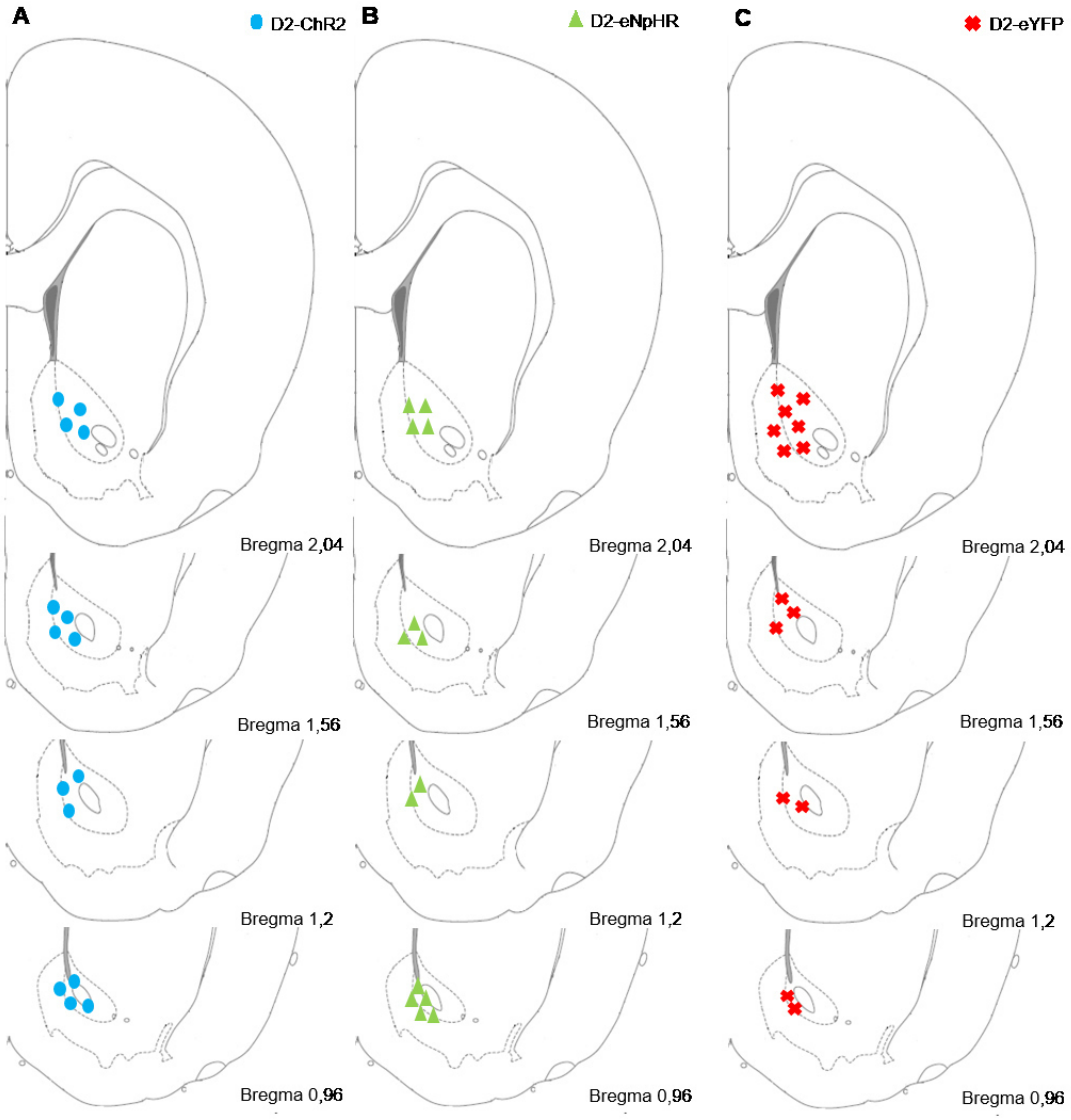
Supplementary Figure 1



Supplementary Figure 1. ***In vivo*** **electrophysiological recordings in iuGC rats. (A)** Optical excitation increase the NAc firing activity in iuGC-D2-ChR2 animals. Stimulation parameters used were: 473 nm light delivered in 40 Hz burst (40 pulses, 12.5 ms each pulse - Excitation) (n=38 cells, n=4 animals). **(B)** More than half of the cells increase firing rate (55.3%), 26.3% decrease and 18.4% did not respond. After stimulation, 10% increase, 7.5% decrease and the remaining 82.5% of cells return to baseline activity.

iuGC, *in utero* glucocorticoid exposure. Data presented as mean ± SEM; **p<0.01.

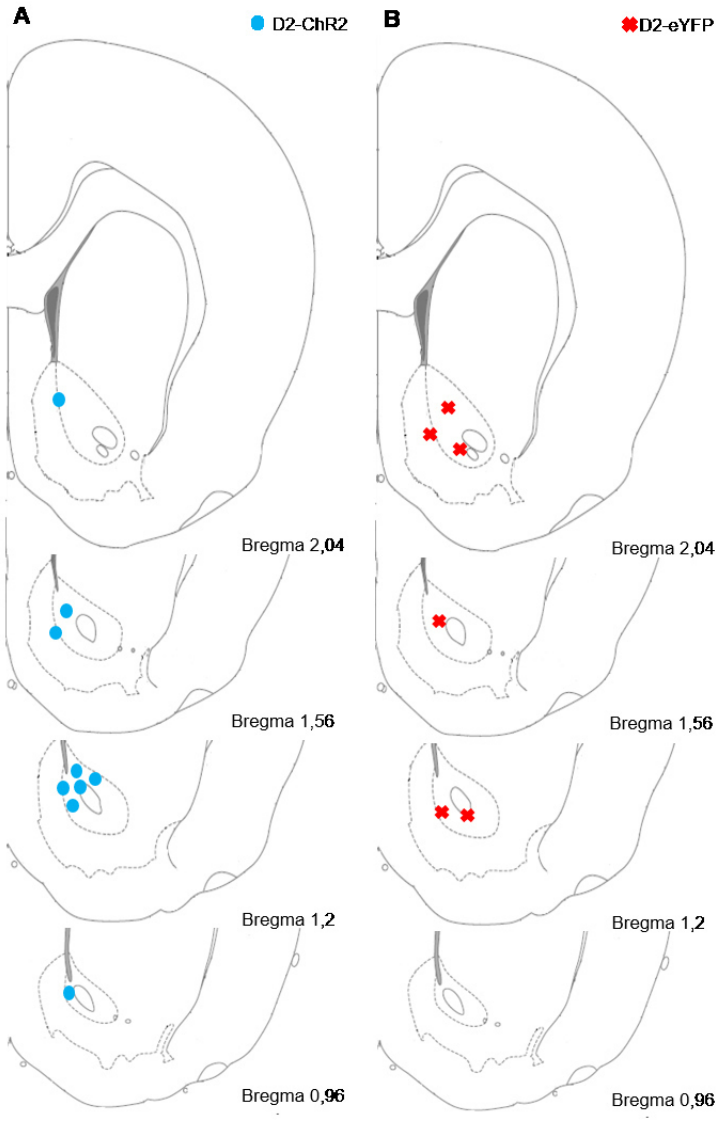
Supplementary Figure 2



Supplementary Figure 2. Histological verification of optical fiber sites in CONT groups. Coronal sections from bregma containing the NAc. Center of the optical fiber placements in the NAc for all the animals injected with **(A)** D2-ChR2 (n=15; blue circles), **(B)** D2-eNpHR (n=14; green triangles) and **(C)** D2-eYFP (n=14; red crosses).

NAc, nucleus accumbens.

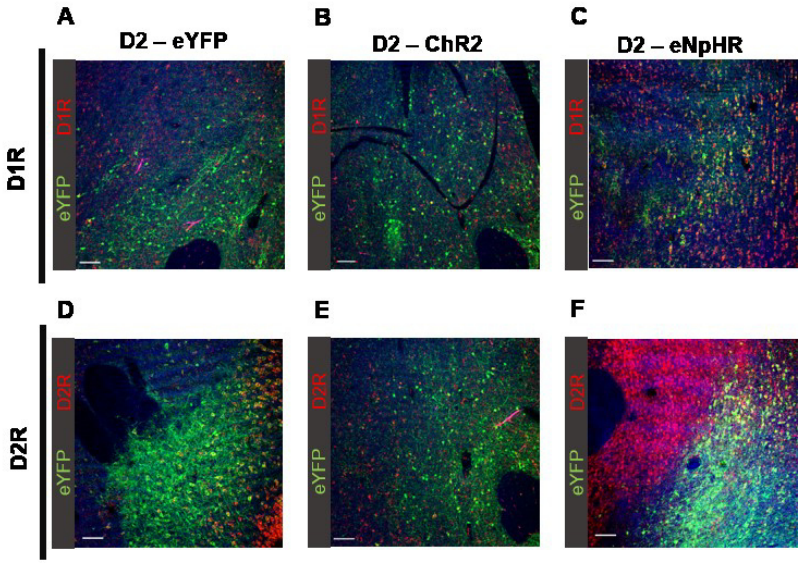
Supplementary Figure 3



Supplementary Figure 3. Histological verification of optical fiber sites in iuGC groups. Coronal sections from bregma containing the NAc. Center of the optical fiber placements in the NAc for all the animals injected with **(A)** D2-ChR2 (n=9; blue circles) and **(B)** D2-eYFP (n=6; red crosses).

NAc, nucleus accumbens; iuGC, *in utero* glucocorticoid exposure.

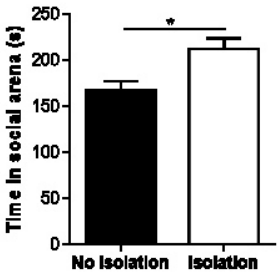
Supplementary Figure 4



Supplementary Figure 4. Immunofluorescence against eYFP and D1R or D2R in the NAc. Brain sections containing the NAc. Representative double immunofluorescence using antibodies against eYFP and D1R of an animal injected with AAV5-D2-eYFP **(A)**, AAV5-D2-ChR2-eYFP **(B)**, or with AAV5-D2-eNpHR3.0-eYFP **(C)**. Representative double immunofluorescence image against eYFP and D2R of an animal injected with AAV5-D2-eYFP **(D)**, AAV5-D2-ChR2-eYFP **(E)**, or with AAV5-D2-eNpHR3.0-eYFP **(F)**. Scale bar: 100 μ m.

NAc, nucleus accumbens; D2R, dopamine receptor 2; D1R, dopamine receptor 1.

Supplementary Figure 5



Supplementary Figure 5. Effects of isolation on time in social arena in the three-chamber sociability test. After 5 min of habituation, animals were exposed to 5 min of social interaction period, with the intruder animal placed inside of an inverted enclosure. Isolation leads to an increase in time spent in the social arena when compared to not isolated animals ($n_{no\ isolation}$=8, $n_{isolation}$=17).

Data presented as mean ± SEM; *p<0.05.

CHAPTER 4

Discussion

Discussion

Throughout this dissertation we have used a series of behavioral, molecular and optogenetic techniques in order to identify new molecular correlates and neuronal pathways involved in anxiety and social behavior. For this, we took advantage of an animal model of in utero glucocorticoid (iuGC) exposure that presents an anxious behavior and social deficits (Borges et al., 2013; Oliveira et al., 2006). The behavioral alterations induced by the prenatal exposure to GCs were correlated with a series of alterations at structural and molecular level in brain regions that have been shown to be relevant for anxiety and social behaviors, which we will discuss in the following sections.

Specifically, exposure to iuGC in rodents induced an increase in the number of choline-acetyltransferase (ChAT)-positive cells in laterodorsal (LDT) and pedunculopontine tegmental nucleus (PPT), however, no differences were observed in the volume and total number of cells. When exposed to an aversive stimulus, iuGC animals presented an increase in the number of c-fos +/ChAT+ cells in both LDT and PPT regions, suggesting that these animals react to aversive situations in a different manner, which is in agreement with the anxious behaviour observed.

After, taking advantage of the optogenetics technique, we stimulated specific pathways and observed their impact in social behavior. Social behavior is highly rewarding (Trezza et al., 2011; Vanderschuren et al., 2016), therefore, its expression is modulated through neural system implicated in other rewards such as food, sex and drugs of abuse (Siviy and Panksepp, 2011; Trezza et al., 2010). Previous studies have shown that the LDT-ventral tegmental area (VTA) projection generate reward (Lammel et al., 2012; Steidl and Veverka, 2015). Indeed, upon optogenetic stimulation of LDT-VTA projections, we observed an increase in social interaction time. On the contrary, the recently discovered LDT-nucleus accumbens (NAc) projection does not seem to modulate same sex social interaction time.

Because previous evidence showed that optical stimulation of dopamine receptor D1 (D1R)-expressing neurons, in the NAc, leads to an increase in social behavior (Gunaydin et al., 2014), we also decided to evaluate the role of dopamine receptor D2 (DR2)-expressing neurons. Optogenetic activation of NAc DR2-expressing neurons also induce an increase in same sex social interaction time. This data goes in accordance with results from our laboratory that have shown that activation/inhibition of NAc D2R–expressing neurons enhances/decreases motivation

in a progressive ratio task (Soares-Cunha et al., 2016). Together, this data suggests that the functional/behavioural dychotomy between D1 and D2 neurons is not so strict.

Animal model of *in utero* glucocorticoid exposure

Different models of stress or GC exposure have been used to study anxiety, which vary in the day, duration and doses of exposure to stress hormones. In this work, we injected a synthetic glucocorticoid - dexamethasone (1mg/kg) in days of 18 and 19 of gestation. Previous studies from our lab have demonstrated that this exposure in utero can have long-term effects on the offspring.

Studies have shown a relationship between prenatal glucocorticoid exposure and depression, increased anxiety and impaired social behavior and memory, both in rodents (Borges et al., 2013; DeKosky et al., 1982; Oliveira et al., 2006; Roque et al., 2011) and in humans/non-humans primates (Hauser et al., 2008; Hirvikoski et al., 2012; Trautman et al., 1995).

Moreover, prenatal exposure to GCs caused impaired hypothalamic-pituitary-adrenal (HPA) axis (Oliveira et al., 2006; de Vries et al., 2007), alter the mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) expression (Levitt et al., 1996) and can influence entire neurotransmitter systems (Oliveira et al., 2006, 2012, Rodrigues et al., 2011, 2012; Virdee et al., 2014).

The use of this iuGC model is also of clinical relevance. This model was created with the intention to mimic the clinical situation of administration of synthetic GCs to pregnant woman in risk of preterm labor. This administration reduces the incidence of respiratory distress syndrome (RDS), intraventricular hemorrhage, necrotizing enterocolitis (NEC) and respiratory support, intensive care admissions and systemic infections in the first 48 hours of life and leads to neonatal mortality improvement (Miracle et al., 2008).

Although experimental studies with animals in comparison to studies with humans are much easier to control for variables, there are many differences in species response to GC, so some caution has to be made in interpreting the rodent data.

Effects of iuGC treatment on anxiety behavior

Firstly, is important to describe what is considered to be normal levels of anxiety and a pathological state. Anxiety is defined as “a psychological, physiological, and behavioral state

induced in animals and humans by a threat to well-being or survival, either actual or potential" (Steimer, 2002). When present to a threat is follow by an increase in arousal, expectancy, autonomic and neuroendocrine activation, also occurs specific behavior transition from ongoing behaviors, such as exploration, feeding, to an escape or other defensive behaviors. This changes has the intent to facilitate coping with an adverse or unexpected situation.

However, when anxiety is prolonged in time and/or disproportional in magnitude can become a pathological state. Pathological anxiety can also be a consequence of predisposing factors, which can result from numerous gene-environment interactions during development (particularly during the prenatal period), and experience (life events). Moreover, is important to distinguish anxiety from fear behaviors. Fear is characterized by a response to an immediate, real danger, while anxiety is characterized by response to potential threat, this means a potential but not a real danger (Steimer, 2002). Here, we looked for the impact of prenatal GC exposure in the developing of anxiety behavior in the offspring, in the mesopontine cholinergic system and in the bed nucleus of the stria terminalis (BNST) network.

We took particular attention to the mesopontine system because it has been shown to be responsible for the initiation of 22 kHz ultrasonic vocalizations (USVs) (Brudzynski, 2001), and iuGC animals present a significant increase in the number of aversive calls in an anxiogenic environment (Chapter 2.1). Stimulation of the LDT induced species-typical 22 kHz USVs comparable to those emitted in natural situations. These 22 kHz calls induced could be antagonized by atropine or scopolamine, antagonist of muscarinic receptors, applied into the cholinoceptive strip. The 22 kHz USVs, also named low-frequency vocalizations, are emitted when exposed to predators (Blanchard et al., 1991), during intermale aggression (Vivian and Miczek, 1993), when presented to an aversive stimuli, like startling noises (Kaltwasser, 1990), or unescapable foot-shocks (Antoniadis and McDonald, 1999; Borta et al., 2006; Vivian and Miczek, 1993; Wöhr et al., 2005).

Previous studies have shown that the low-frequency calls are not only emitted during the aversive event, but also when present to a stimulus associated with such experiences (Antoniadis and McDonald, 1999; Borta et al., 2006; Cuomo et al., 1988; De Vry et al., 1993; Molewijk et al., 1995; Wöhr et al., 2005). Additionally, the 22 kHz USVs are associated with a negative affective state analogous to anxiety and depression. Indeed, anxious animals, assessed by their anxiety behavior in the elevated plus maze, emitted more negative calls during the fear-

conditioning training than animals characterized as less anxious (Borta et al., 2006). Regarding the effect of stress on USVs emitted there is a tremendous variation. Neonatal isolation or maternal separation leads to a decreased in the number of fear-conditioned 22-kHz USVs in adult males (Kosten et al., 2005, 2006). Moreover, chronic restraint stress increase 22 kHz USVs in response to acute footshock, while chronic footshock stress had the opposite effect (Swiergiel et al., 2007). Interestingly, animals with inherently low positive affect (as measured by tickling induced appetitive 50 kHz calls) emitted more negative USVs following chronic variable stress exposure (Mällo et al., 2009). Consistently, aversive experiences during early development lead to increased emission of 22 kHz USV in adulthood (Yee et al., 2012).

We observed that iuGC exposure leads to anxious behavior and increase in fear behavior. Furthermore, we found increased ChAT expression in the LDT and PPT of iuGC animals in a basal situation. Moreover, the increase in the number of 22 kHz USVs emitted and freezing during the fear conditioning paradigm was associated with an increase of ChAT/c-fos expression neurons in the LDT and PPT, when compared with control animals. This suggests an emotional overreactivity of iuGC animals to adverse stimulus, which could be partially explained by the differences in the mesopontine cholinergic pathway. One way to prove this would be to stimulate/inhibit the mesopontine cholinergic neurons in iuGC animals using optogenetics or drugs to evaluate if it is able to rescue the behavior of these animals.

The bed nucleus of the stria terminalis (BNST) is a critical node in the stress response neurocircuitry and plays a significant role in anxiety behavior. Lesions in the large anterior BNST, reduce corticotropin releasing factor (CRF) mRNA expression in the paraventricular nucleus of the hypothalamus (PVN), attenuate plasma corticosterone response and decrease PVN c-fos mRNA (Choi et al., 2007, 2008). Additionally, lesions in the anterior BNST, preceded by a conditioned stress paradigm, lead to an attenuation of prolactin, adrenocorticotropic hormone (ACTH) and corticosteroids levels (Gray et al., 1993). Lesions in the posterior BNST show a contrasting effect increasing the levels of CRF mRNA and increasing the number of AVP mRNA-producing PVN parvocellular neurons (Herman et al., 1994). In response to acute stress, lesions in the posterior BNST lead to an increase in plasma ACTH, corticosterone, stress-induced PVN c-fos mRNA, CRF and AVP mRNA expression when compared with sham animals (Choi et al., 2007). Collectively

these results confirm that the posterior BNST region is involved in the inhibition of the HPA axis, whereas the anterior BNST nuclei are involved in HPA axis excitation.

Stress can result in structural and morphological changes in the BNST, which are associated with synaptic changes. Chronic unpredictable stress induce anxiety-like behavior and lead to increases in BNST volume correlated with an increase in dendritic length (but not in the Amy) (Pego et al., 2008). In fact, previous from our laboratory has found that iuGC exposure leads to increase in anteromedial BNST division volume, but did not affect total number of cells in the BNST. In the anteromedial BNST division, the iuGC treatment leads to an increase in the total dendritic length, however, no effect was observed in spine densities, even when a separate analysis of mature and immature forms (Oliveira et al., 2012). This data may eventually explain the HPA axis impairment and hyperanxious phenotype observed in these animals.

In chapter 2.2, we aimed to understand the impact of iuGC exposure in the BNST network. With the help of single-cell electrophysiology we were able to record the activity of neurons located in the anterior BNST (aBNST) while stimulating the infralimbic cortex (ILCx) or the central nucleus of the amygdala (CeA). This technique, allowed us to have a more integrative perspective on the effects of iuGC exposure in the BNST activity.

Previous work showed that ILCx stimulation leads to an hyperactivation of BNST neurons (Massi et al., 2008). As expected, we observed excitation of aBNST neurons upon ILCx electric stimulation. No differences were observed between iuGC and CTR animals. In addition, we determined if the application of a tetanic activation on the ILCx at a physiologically relevant frequency (Jackson et al., 2001), triggers neuroplastic changes in the aBNST neurons. This stimulation in control mice leads to a long-term depression (LTD) in the aBNST neurons. However, we did not observe any effect of this stimulation, in both control and iuGC animals, a finding that needs to be further confirmed. Still, it has been previously observed that acute stress reverses the LTD to long-term potentiation (LTP) (Glangetas et al., 2013).

In iuGC animals, it has been previously observed a decrease volume of CeA, though no changes were observed in the estimated number of cells. CeA neurons displayed a significant dendritic atrophy when compared to controls, however, no changes was found on the densities of total, mature and immature spines (Oliveira et al., 2012). Studies have shown that stimulation of CeA leads to inhibition of BNST neurons, the same was observed in CONT and iuGC animals, though no differences were observed between groups.

Next, we assessed the impact of GC treatment in the VTA region, since previous work has reported that BNST projects to and exerts a strong excitatory influence on the firing of dopaminergic (DAergic) neurons within the VTA (Georges and Aston-Jones, 2001, 2002). In response to a foot-shock, the glutamatergic BNST-VTA neurons present an increase in their activity, contrary to GABAergic BNST-VTA neurons (Jennings et al., 2013). iuGC exposure induced a reduction in proliferation in the VTA, reduced number of tyrosine-hydroxylase positive cell in the VTA, leading to decrease in the DAergic innervation to the NAc (Leao et al., 2007; Rodrigues et al., 2012). Despite the morphological and neurochemical changes previously observed, we observed no differences in the firing rate and burst firing of DAergic neurons from the VTA between groups. Yet, since these neurons encode "reward prediction error", we may have to observe their activity during reward-related behaviors and not in anesthetized animals in order to observe any changes.

Yet, because the data was particularly surprising, especially in the BNST activity, we have evaluated if the anxious phenotype was also present in the Sprague Dawley strain exposed to iuGC. Importantly, iuGC treatment does not induce a hyperanxious state in Sprague Dawley strain, contrary to Wistar Han. This may due to genetic differences or different susceptibilities between strains, which could potentially explain the lack of differences in the BNST activity.

In summary, in this part of dissertation we indicate that prenatal GC treatment affects the mesopontine cholinergic pathway, which in turn can underlie the anxious behavior and enhanced stress reactivity observed in these animals.

Modulation of social behavior

As mention before, social behavior, present in all mammalian species, is highly rewarding (Trezza et al., 2011; Vanderschuren et al., 2016), and as such, is regulated through neural system also implicated in other types of reward such as food, sex and drugs of abuse (Siviy and Panksepp, 2011; Trezza et al., 2010). It has long been acknowledged that reward processing depends on mesocorticolimbic dopamine (DA) systems, comprising DA neurons in the VTA and their projections to the NAc, amygdala, prefrontal cortex (PFC), and other forebrain regions (Kelley and Berridge, 2002). The VTA is important to regulate reproductive behaviour and parental behaviuor (Dulac et al., 2014; Hansen et al., 1991; Sirinathsinghji et al., 1986).

More recently, studies show that activation or inhibition of VTA tyrosine hydroxylase (TH) neurons leads to an increase or decrease of same sex social interaction, respectively (Gunaydin et al., 2014).

One of inputs to the VTA is the LDT, indeed, optogenetic stimulation of the LDT neurons that project to the VTA trigger reward associated behaviors, enhances conditioned place preference (Lammel et al., 2012) and operant responding in rodents (Steidl and Veverka, 2015). Nevertheless, the impact of LDT-VTA stimulation in social behaviors remains completely undisclosed. Furthermore, LDT also directly enervates the NAc (Dautan et al., 2014), however, nothing is known about the functional relevance of these projections. In chapter 3.1, we assessed the impact of optogenetic stimulation of LDT- VTA and LDT-NAc projections in the same-sex social behaviors. In a home-cage resident-intruder social interaction, excitation of LDT-VTA projection leads to a moderate increase in social interaction time, on the other hand, had no effect on object interaction time.

Regarding the LDT-NAc projections, bidirectional modulation of these projections has no effect on social (and object) interaction. However, inhibition of this projection leads to an increase in locomotor activity, which was surprising because the NAc is usually not associated with locomotor activity.

Despite these promising results, more studies need to be performed. Firstly, it would be interesting to inhibit the LDT-VTA projections during social behaviors. Secondly, we could evaluate animals' behaviour in different social paradigms to observe in which dimension this projections are more relevant (social motivation, social recognition or aggressiveness).

Additionally, we have evaluated the role of NAc D2R-expressing neurons in a social interaction paradigm. It is frequently presumed that D1 and D2 medium spiny neurons (D1-MSN, D2-MSN) play opposite roles in behavior. Activation of D1-MSNs is correlated with positive rewarding events, whereas the D2-MSNs activation is related with aversion (Hikida et al., 2010; Kravitz et al., 2012; Lobo et al., 2010). Nonetheless, recently data from our laboratory have shown that this functional/behavioural bias is not so strict. We show that optogenetic activation/inhibition of NAc D2-MSNs enhances/decreases motivation in a progressive ratio task (Soares-Cunha et al. 2016).

It has been shown that NAc modulation of D1 or D2 neurons can modulate social behaviors (Beatty et al., 1984; Gunaydin et al., 2014; Holloway and Thor, 1985; Humphreys and Einon, 1981; Manduca et al., 2016; Niesink and Van Ree, 1989; Siviy et al., 1996; Trezza and Vanderschuren, 2009; Vanderschuren et al., 2008). Dopamine receptor antagonists reduced social play (Beatty et al., 1984; Holloway and Thor, 1985; Humphreys and Einon, 1981; Niesink and Van Ree, 1989; Siviy et al., 1996; Trezza and Vanderschuren, 2009), whereas both increases and decreases in social play have been reported after treatment with dopamine receptor agonists, (Beatty et al., 1984; Niesink and Van Ree, 1989; Siviy et al., 1996; Vanderschuren et al., 2008).

Directly block of either D1 or D2 NAc receptors reduced social play in animals highly motivated to play as a result of longer social isolation before testing (Manduca et al., 2016). Optogenetic activation of D1 in the NAc leads to increase in adult same-sex social interaction time (Gunaydin et al., 2014). Increased striatal D2 receptors resulted in social deficits in behavior and vocalizations that emerge between adolescence and adulthood (Kabitzke et al., 2015). Mice exhibiting higher D2 receptor expression are more aggressive than mice with lower D2 receptors (Couppis et al., 2008).

Moreover, humans with social anxiety disorder presented a reduced dopamine D2 receptor binding in the striatum (Schneier, 2000; Schneier et al., 2008).

Regarding other forms of social interaction, D2 receptor antagonists disrupt maternal behavior and, in isolated rat pups, reduce USVs that promote maternal interaction (Curry et al., 2013). The interaction between oxytocin and D2 type receptors in NAc shell facilitates intraspecific attachment, but D1 type appears to facilitate parental attachment (Coria-Avila et al., 2014).

Moreover, activation of D1 type receptors prevents the formation of attachment toward a familiar partner, whereas activation of D2 type receptors facilitates it, in the rostral shell of NAc from male voles (Aragona et al., 2006). Indeed, males that have established a selective and long-lasting preference present a significant upregulation in D1 like receptors in the rostral shell.

We observed that optical activation of D2R-expressing neurons in the NAc leads to an increase in adult same sex social interaction time. This stimulation was behaviorally specific since no differences were observed in object interaction time and in locomotor activity. Regarding D2-MSN inhibition, we observe a decrease in social interaction time in the D2-eNpHR group when

comparing laser ON with laser OFF. No differences were observed in object interaction time and number of 50 kHz USVs emitted in both paradigms, however, we do observe a decrease in locomotion activity.

In summary the data suggest that NAc D2R-expressing neurons have an important role in same sex, non-aggressive, social behaviors. However, would be important to assess the impact of this modulation on other types of social paradigms.

Effects of iuGC treatment on social behavior

It has been shown that stress during specific neurodevelopmental periods have the capability to alter adult social behaviors. Social interaction was decrease in animals exposed to prenatal stress (Borges et al., 2013; de Souza et al., 2013), neonatal stress (Jia et al., 2009; Wei et al., 2013) and peripubertal exposure to physical stressors (Márquez et al., 2013). However, juvenile social stressors did not affect, while others have found to increase this aspect of social behavior (Shimozuru et al., 2008; Wommack et al., 2004; Workman et al., 2011).

Moreover, in adulthood, prenatal (de Souza et al., 2013), neonatal (Franklin et al., 2011; Yu et al., 2013) and juvenile exposure to stressors (Márquez et al., 2013; Naert et al., 2011; Vidal et al., 2011) leads to disruption of social motivation. These studies demonstrate that early life stress is capable to decrease measures of social motivation and the expression of social behaviors.

Administration of either corticosterone or dexamethasone on the first days of life reduce social play (Meaney et al., 1982), whereas others have found an enhance juvenile play (Kamphuis et al., 2004). We have previously observed that iuGC exposure leads to a decrease in social play behavior (Borges et al., 2013). Additionally, these animals present a reduction in volume of NAc, with significant changes in spine density and neuronal morphology. As well a decrease in DA levels in the NAc, accompanied by an increase in the expression of D2 receptors (Oliveira et al., 2012; Rodrigues et al., 2012). Moreover, oral gavage of DA precursor levodopa (L-DOPA) leads to a rescue of the depressive-like behavior and social deficits present in the iuGC exposed animals (Borges et al., 2013).

Since we observe an increase in social interaction while stimulating the D2R-expressing neurons in the NAc, we decided to try to rescue the social deficits observed in the iuGC animals. In chapter 3.2, we observed that social deficits induced by iuGC exposure were rescued by

optical activation of D2 neurons in the NAc. Despite these promising results, much has still to be done. First, one could assess the impact of iuGC exposure in other social dimensions (social recognition, aggressiveness, sexual and parental behaviors). Second, we could evaluate D2 (and D1) neuronal activity during social behaviors using in vivo electrophysiology or calcium imaging.

Conclusions

In this present dissertation we observed:

1. iuGC exposure leads to present hyperanxiety, increased fear behavior, and hyper-reactivity to negative stimuli.;

2. iuGC programmes the mesopontine cholinergic system;

3. iuGC exposure does not induce an anxious behavior in Sprague-Dawley strain, contrary to Wistar Han strain;

4. Activation of LDT-VTA projections leads to an increase in social interaction time, but has no effect on object interaction in naïve animals;

5. Optogenetic activation/inhibition of the LDT-NAc does not produce any alteration in social and object interaction;

6. Optical activation of NAc D2R-expressing neurons leads to an increase in adult same-sex, non-aggressive, social interaction;

7. iuGC exposure leads to social deficits in adult animals, however, stimulation of NAc D2R-expressing neurons rescued these impairments.

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APPENDICE

APPENDIX 1

Borges S, Ventura-Silva AP, Sousa N, Georges F, Pêgo JM and Rodrigues AJ

BNST – Bridging aNxiety, Stress and addiction

BNST – Bridging aNxiety, Stress and addicTion

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Abstract

Stress is linked to the pathophysiology of psychiatric illness, including anxiety and addiction. The bed nucleus of the stria terminalis (BNST) has been associated with these disorders due to its core position in the neural network that underlies stress-related and motivation functions. The BNST is strongly innervated by multiple between and within sub-regions of the BNST as well as by extrinsic inputs (glutamatergic, GABAergic, noradrenergic, dopaminergic, serotoninergic) giving rise to a part of its complexity. Synaptic plasticity in this region could in part underlie the persistent behavioral alterations in generalized anxiety and addiction. In this review, we will examine how stress modulates the BNST circuitry and its role on the comorbidity between anxiety and addiction. Together these studies will provide a foundation for the importance of the BNST in stress-related disorders.

1. Introduction

The bed nucleus of the stria terminalis (BNST) was described in the 1920's by J.B. Johnston and evidence gathered along the years strongly shows that the complexity of this brain region is inversely correlated with its small size. The BNST, one of the components of the extended amygdala, is composed of different subnuclei with distinct developmental origins and neurochemical profile. Additionally, it has a substantial number of afferents and efferents, which provides a challenge to study the behavioral and biological functions related to this brain region, and contribute to the claim that the BNST is one of the most complex structures in the entire central nervous system. Nevertheless, the BNST has been recognized as a key brain region in regulating the stress response, playing an important role in the effects of maladaptive stress, namely in the development of anxiety and addiction.

The BNST subdivisions have many different projections to and from distinct brain regions. The connectome of the BNST in respect to its specific subdivision are summarized in table I-III. The BNST receives innervation from several cortical regions (the infralimbic cortex (ILCx), prelimbic cortex, insula cortex, entorhinal cortex and caudal orbital prefrontal cortex (PFC)), hippocampus (Hipp), parabrachial nucleus (PB), accessory olfactory bulb, main olfactory bulb, ventral tegmental area (VTA), periaqueductal gray (PAG), nucleus tractus solitaries (NTS), nucleus ventral lateral medulla (VLM), ventral noradrenergic bundle (VNAB), locus coeruleus (LC), dorsal raphe (DRN), the amygdala (Amy) (Brennan and Keverne, 2004; Commons et al., 2003; Dong et al., 2001a; Dong et al., 2001b; Forray and Gysling, 2004; Kim et al., 2013; Li et al., 2012; Li and Cho, 2006; Massi et al., 2008; Meloni et al., 2006; Myers et al., 2014; Myers et al., 2005) (Table I). The BNST is strongly connected to stress-responsive regions such as the paraventricular nucleus of the hypothalamus (PVN), an element of the hypothalamic-pituitary-adrenal axis (HPA), which controls stress-induced glucocorticoids release (Champagne et al., 1998; Dong et al., 2001a; Dong and Swanson, 2006b; Roland and Sawchenko, 1993). On the other hand, the BNST is interconnected with limbic structures, namely the central amygdala (CeA), nucleus accumbens (NAc) and VTA, being an important mediator in the expression of emotional aspects of behavior (both aversive and rewarding) (Dong et al., 2001b; Dong and Swanson, 2004; Georges and Aston-Jones, 2001, 2002; Jalabert et al., 2009; Jennings et al., 2013; Kim et al., 2013; Li and Cho, 2006; Walker et al., 2009) (Table II).

This privileged anatomical position between these two circuitries (stress and aversion/reward) imposes the BNST as a relevant region in the development of stress-related disorders such as anxiety and addiction (Dong et al., 2001b; Dong and Swanson, 2006b; Stamatakis et al., 2014; Ventura-Silva et al., 2012b). Importantly, anxiety and addiction are both triggered by stress and the risk for both disorders is higher in individuals with altered HPA axis function (Avery et al., 2016; Koob, 2009). These disorders are often comorbid; anxious individuals are prone to drug-seeking behavior and addicts commonly present anxiety disorders. The role of the BNST in anxiety-related behavior has been firmly established in the last years (Davis et al., 2010; Walker et al., 2003), and emerging evidence strongly suggests that this brain region is crucial in some stages of addiction, including withdrawal related-anxiety and relapse (Silberman et al., 2013; Stamatakis et al., 2014; Wenzel et al., 2014). Consequently, understanding the circuitry of the BNST and how this is affected by stress will be key to understanding both anxiety and addiction. The heterogeneous nature of the BNST presents a challenge, in the sense that different sub-regions and cell types of the BNST create two divergent circuitries with opposing behavioral outcomes.

In the present review, we will focus on the role the BNST in the adaptive and maladaptive stress response and discuss evidence suggesting that this brain region is the link between the comorbidity of anxiety and addiction.

2. The Bed Nucleus of the stria terminalis (BNST): small but complex

In the last decades, a lot has been learnt about the structural and functional organization of the BNST, showing that although small, it is a very complex area. Currently, two main divisions are recognized in the BNST, the anterior and posterior divisions, separated by the entrance of the fibers of the stria terminalis in the BNST, and can be further divided into medial and lateral subdivisions (Table III). To date, 18 different subnuclei of the BNST have been reported, based on their cytoarchitecture, chemoarchitecture and connectivity (Lebow and Chen, 2016) (Table III). The medial subdivision is composed by the anterodorsal (AD) and anteroventral (AV) areas. The last area includes the dorsomedial (dm), dorsolateral (dl), magnocellular (mg) and ventral (v) nuclei. The lateral division is composed by the anterolateral (AL), juxtacapsular (ju), oval (o), rhomboid (rh) and fusiform (fu) nuclei and by the subcomissural zone (sc). The posterior division is divided by the dorsal (d), principal (pr), interfascicular (if) and transverse (tr) nuclei (Table III).

The BNST receives adrenergic, noradrenergic, serotonergic, dopaminergic, glutamatergic and GABAergic inputs and also presents GABAergic and cholinergic interneurons, with GABAergic neurons being the major neuronal population in the BNST (reviewed in (Stamatakis et al., 2014)). In the BNST there are cells (co-)expressing a wide variety of neuropeptides including neuropeptide Y (NPY), corticotropin releasing factor (CRF), enkephalin, dynorphin, substance P, galanin, vasopressin and oxytocin (Lebow and Chen, 2016; Stamatakis et al., 2014; Veinante and Freund-Mercier, 1997). This is another evidence that the BNST is an intricate and complex structure with multiple anatomical parts that have different neurochemical and interconnectivity patterns.

Regarding the electrophysiology properties of the BNST, a full characterization of the neuronal populations of each nucleus is still lacking, probably due to the complexity of the system. Indeed, most electrophysiological studies done in this region are focused on the anterior division of the BNST. It has been reported in experiments using both *in vivo* (Georges and Aston-Jones, 2002; Massi et al., 2008) and *ex vivo* preparations (Daniel and Rainnie, 2016; Dumont et al., 2005; Dumont et al., 2008); that there are electrophysiologically distinct populations of neurons in the BNST. It is known that anterior BNST neurons have an heterogeneous firing rate and heterogeneous responses to numerous neurotransmitters, including opiates, norepinephrine, acetylcholine, oxytocin, and serotonin (Daniel and Rainnie, 2016; Hazra et al., 2011). In the particular case of the anterolateral BNST neuronal population, it appears to be very heterogeneous in terms of electrophysiological proprieties between the dorsal and ventral and within the subdivisions (Egli and Winder, 2003). In *ex vivo* experiences, the dorsal BNST neurons, as opposed to the ventral BNST, appear to be under tonic inhibition (Egli and Winder, 2003). Interestingly, previous studies have shown that the dorsal BNST actually projects to subnuclei of the ventral BNST. The anterolateral BNST has morphologically distinct neuronal subpopulations, however, 70–90% of neurons can be categorized as medium-sized spiny GABAergic neurons (Hazra et al., 2011), which may explain the electrophysiological proprieties of these neuronal groups.

Studies using patch clamp recordings showed that at least three distinct cell types (Type I–III) exist in the anterior BNST, based primarily on their membrane currents, namely hyperpolarization-activated nonspecific cation current (I_h), low-threshold calcium current (I_T), transient voltage-dependent potassium current (I_A), and the inward rectifying current ($I_{K(IR)}$) (Daniel and Rainnie, 2016; Egli and Winder, 2003). Although the neurons of the anterolateral BNST

presented a similar resting membrane potential and input resistance, the I_T and I_h currents were differentially distributed (Egli and Winder, 2003), suggesting that synaptic input to these cells may be differentially integrated. An interesting study combining whole cell patch clamp recording with single cell transcriptomic analysis has shown that these neurons also present distinct gene expression profiles of ion channel subunits, which may account for their different electrophysiological properties (Daniel and Rainnie, 2016; Hazra et al., 2011).

Consistent with this *in vitro* experiment, using *in vivo* electrophysiology of the anterior BNST, we identified three different groups of neurons according to their basal firing rate: low, medium and high (Figure 1B, Kruskal-Wallis ANOVA H'(2)= 65.49, $p<0.001$; Low-Medium: $p<0.001$; Low-High: $p<0.001$; Medium-High: $p=0.124$; Table IV). Interestingly, despite this basal heterogeneity, upon an electric stimulation on the ILCx (0.5 Hz, Methods – Supplements), these different neuronal populations present a homogenous excitatory response (Figure 1C, Response Magnitude- Kruskal-Wallis ANOVA H'(2)= 1.861, p=0.394; Low-Medium: $p>0.999$; Low-High: $p=0.546$; Medium-High: $p=0.661$; Table IV). Regarding the depth from the brain surface and action potential width no differences were found between groups (Figure 1D, Depth- one way ANOVA F(2,69)=1.68, $p=0.193$; Low-Medium: p=0.999; Low-High: $p=0.255$; Medium-High: $p=0.771$; Figure 1E, Width- one way ANOVA F(2,75)=0.126, $p=0.882$; Low-Medium: $p=0.999$; Low-High: $p>0.999$; Medium-High: $p=0.999$). Although the magnitude of response was similar upon ILCx stimulation, we cannot exclude that these neurons fire differently in response to other types of stimulation, adding further complexity to the analysis of BNST neurons.

2.1 Role of the BNST in the stress response

Stress is commonly defined as a challenge to the organism's homeostasis. The stress response is the body's reaction to this challenge inducing physiological (e.g. metabolic, hormonal, etc.) and behavioral (e.g. fight or flight reaction) changes that are required for the return of the organism to its homeostatic balance. A cascade of events leads to the activation of a series of systems, namely the HPA axis and the central limbic stress-loop (Avishai-Eliner et al., 2002) in order to achieve such response.

The response to a "systemic or interoceptive" stressor leads to a visceral change, which does not require the cognitive processing associated with limbic regions. This response starts with the activation of the PVN by visceral afferents, which releases CRF to the anterior pituitary

where it stimulates the synthesis and release of adrenocorticotropic hormone (ACTH) into the bloodstream. In turn, ACTH induces the synthesis and release of corticosteroids (i.e. cortisol in humans or corticosterone in rodents) from the adrenal cortex into the blood stream. Corticosteroids are able to bind to two types of receptors, the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR) leading to changes in genetic transcription in several tissues. MRs show high affinity for corticosterone/cortisol and are fully occupied at basal levels whereas low affinity GRs are more likely to be occupied when plasma corticosterone is elevated, as in a stress period. This receptor also has high affinity for synthetic glucocorticoids (GCs), namely dexamethasone.

The response to a "processive or exteroceptive" stressor involves the activation of the limbic stress-loop pathway. The limbic afferents projecting to the PVN include the PFC, Hipp, Amy and the BNST. Importantly, the BNST also receives processed stressor input from upstream regions of the brain involved in cognitive processing, such as the PFC and the Hipp, and in 'emotional' information processing, such as the Amy. Thus, 'cognitive' or 'emotional' stress signals converge on the BNST, which acts as a relay station between upstream regions and the HPA axis (Avishai-Eliner et al., 2002).

It has been shown that the BNST can either excite or inhibit HPA activity depending on the activated region of the BNST (Casada and Dafny, 1991; Dunn, 1987; Herman et al., 1994; Ventura-Silva et al., 2012b). Stimulation of the lateral BNST decreases the HPA axis activity, measured by a decrease in basal corticosterone secretion. While other studies have shown that stimulation of the medial BNST increased HPA activity in anesthetized rats (Dunn, 1987).

Lesions studies have also shown the heterogeneity of the BNST in stress response. Lesions in the large anterior BNST, reduce CRF mRNA expression in the parvocellular PVN, attenuate plasma corticosterone response and decrease PVN c-fos mRNA (Choi et al., 2008; Choi et al., 2007). Additionally, lesions in the anterior BNST, preceded by a conditioned stress paradigm, lead to an attenuation of prolactin, ACTH and corticosteroids levels (Gray et al., 1993). Lesions in the posterior BNST show a contrasting effect increasing the levels of CRF mRNA and increasing the number of AVP mRNA-producing PVN parvocellular neurons (Herman et al., 1994). In response to acute stress, lesions in the posterior BNST lead to an increase in plasma ACTH, corticosterone, stress-induced PVN c-fos mRNA, CRF and AVP mRNA expression when compared with sham animals (Choi et al., 2007). This suggests that the posterior BNST inhibits the HPA axis response to stress through modulation of PVN excitation (Table V). Collectively these

results confirm that the posterior BNST region is involved in the inhibition of the HPA axis, whereas the anterior BNST nuclei are involved in HPA axis excitation. Moreover, lateral BNST lesions decrease the response to stress, while medial BNST lesions are ineffective (Gray et al., 1993) (Table V).

To date, several studies have suggested that the BNST mediates behavioral responses to acute and chronic aversive stimuli/stress (Casada and Dafny, 1991; Daniel and Rainnie, 2016; Walker et al., 2003). First, as mentioned, the BNST is activated in response to acute stress. In addition, BNST modulates anxiety-related behaviors in several animal models (Hammack et al., 2004; Walker et al., 2003). Lesions, optogenetic inhibition, as well as pre-test infusions of glutamatergic antagonists or cobalt chloride (CoCl(2)), a nonselective synapse blocker, into the BNST to disrupt its function, reduce anxiety behavior and autonomic responses to stress (Crestani et al., 2009; Kim et al., 2013; Resstel et al., 2008). Moreover, the BNST is engaged in behavioral responses elicited by punishing stimulus and aversively conditioned contexts, reinforcing its proposed role in anxiety (Greenwood et al., 2005). Furthermore, the BNST can also mediate behavioral adaptation to chronic stress exposure (Pego et al., 2008; Vyas et al., 2003).

Stress can result in structural and morphological changes in the BNST, which are associated with synaptic changes. In fact, chronic exposure to negatively valued processive stressors (chronic unpredictable stress) induce anxiety-like behavior and lead to increases in BNST volume correlated with an increase in dendritic length (but not in the Amy) (Pego et al., 2008). Additionally, exposure to elevated levels of corticosteroids during a specific period of gestation induces a long-lasting hypertrophy in the BNST (Oliveira et al., 2012), eventually explaining the HPA axis impairment and hyperanxious phenotype observed in these animals. Coherently, chronic immobilization stress leads to an increase in dendritic branching in the BNST but not the CeA. These results suggest a role for dendritic remodeling of BNST neurons in stress-induced facilitation of anxiety (Vyas et al., 2003).

Together, these data show the functional diversity of the BNST regions and their different roles in integrating and processing limbic information in response to stress, and suggesting that excitatory or inhibitory information from upstream regions are guided through functional different cell groups of the BNST.

2.2. Role of the BNST in anxiety behavior

Anxiety is characterized by a sensation of discomfort and apprehension in response to unconditioned diffuse cues. It is a natural response to stressful stimuli, whereas pathologic anxiety is prolonged in time and/or disproportional in magnitude. Anxiety disorders develop when symptoms occur without any recognizable stimulus or when the stimulus does not justify such a reaction. They cause poor quality of life, increase mortality and are associated with significant costs to society. Although genetics can play a role in the development of anxiety disorders, adverse life experiences have been the most consistently reported contributor for their development found to date (Fernandes and Osorio, 2015).

Some of the neurological circuits, which are involved in the regulation of the stress response, coincide with the neurocircuitry of anxiety, namely the extended Amy, including the BNST. Whereas the BNST is associated with anxiety, Amy is more related to fear (Davis et al., 2010), although this is an oversimplification of the behavioral findings. For example, in a recent optogenetic study, it was shown that activation of the BLA augments anxiety, while activation of the projection from the BLA to the CeA decreased anxiety (Tye et al., 2011). More information about the role of amygdala in anxiety is described elsewhere (reviewed in (Davis, 1992)) and will not be discussed in the light of this review.

The BNST and the CeA are intricately connected (Dong et al., 2001a; Dong and Swanson, 2006a) suggesting that their interactions modulate behavioral output, but also they present a similar connectivity. Both structures receive excitatory inputs (Dong et al., 2001a) from the BLA, they are reciprocally connected, and their brainstem afferents overlap extensively (Dong and Swanson, 2004, 2006a). Additionally, the CeA and the BNST also present a similar neuropeptide expression profile and morphology (McDonald, 1983; Woodhams et al., 1983). Additionally, the anterior BNST receives GABAergic projections from the CeA, while the posterior region receives projections from the medial amygdaloidal nuclei (MeA).

However, although both regions share similar connectivity, there are clear differences regarding their biological function. For example, humans with generalized anxiety disorder (GAD) present a decrease in AMY activity and increased activity in the BNST (Avery et al., 2016). In accordance, in individuals with anxiety, the BNST showed greater overall recruitment and exaggerated tracking of threat proximity (Avery et al., 2016). On the other hand, in animal studies, stressors and anxiogenic pharmacological agents such as yohimbine, m-chlorophenylpiperazine, caffeine, isoproterenol (a β-adrenoreceptor agonist) and CRF, increase

the level of expression of immediate early gene c-fos in the BNST (Naka et al., 2013; Sahuque et al., 2006; Singewald et al., 2003), further suggesting that increased activity of the BNST is associated with an enhanced state of anxiety. Conversely, injections of glutamate antagonists in the BNST are anxiolytic (Kim et al., 2013).

At the molecular level, CRF seems to be a strong candidate in "signaling" anxiety in the BNST. The dense expression of CRF in cell bodies and fibers in the BNST suggest that CRF plays an important role in the modulation of neural activity within the BNST, CeA and their projection regions. CRF infused into the lateral cerebral ventricle increases startle, which is blocked with either lesions of the lateral BNST or micro-infusion of a CRF antagonist into the anterolateral BNST (Lee and Davis, 1997). Additionally, intra-BNST infusion of CRF, in rodents, enhances anxiety-like behavior in a variety of anxiety paradigms (Liang et al., 1992; Sahuque et al., 2006). Furthermore, the anterolateral BNST also contains CRF-producing neurons that are responsive to stress (Dabrowska et al., 2013) and may play a role in the development of stress-induced anxious behavior. In accordance it has been shown that CRF mRNA in the anterolateral BNST increases after exposure to corticosterone (Makino et al., 1994) or acute foot-shock (Funk et al., 2006b). Moreover, CRF receptor 1 (CRF1) knockout mice exhibit an anxiolytic behavioral profile (Timpl et al., 1998). Indeed, a study by Heinrichs and coworkers (1997) using a CRF1 and CRF receptor 2 (CRF2) antisense oligonucleotides provides evidence that anxiogenic actions of CRF are mediated by CRF1 rather CRF2 receptors (Heinrichs et al., 1997). In fact, subjects submitted to chronic stress present a decrease in CRF1 in the anterior but not in the posterior region of the BNST, with an increase in the CRF2 in the posterior division (Ventura-Silva et al., 2012a). Together, these data suggest that release of CRF and/or activation of CRF receptors in the BNST mediate anxiety-like behavior.

Despite clear evidence that overactivation of the BNST is associated with increased anxiety, the picture is far more complex than initially anticipated, because within the BNST, different nuclei may have opposing roles in anxiety modulation. For example, electrical stimulation of the anterolateral region produces many of the endocrine, cardiovascular and respiratory responses that are normally elicited by anxiogenic stimuli (Casada and Dafny, 1991). Additionally, we have shown that BNST nuclei have distinct activation patterns in response to anxiogenic stimuli: whereas we observe an overactivation of the anterior and dorsomedial nuclei, the posterior division presents a reduction in activation (Ventura-Silva et al., 2012a). Supporting

this view, Kim et al. showed that decreases in activity in the anterodorsal BNST are anxiogenic, while inactivation of the oval nucleus of the BNST is anxiolytic (Kim et al., 2013).

BNST projections to different nuclei may also play distinct roles in anxiety, adding extra complexity to the circuitry. For example, activation of the anterodorsal BNST-lateral hypothalamus projection is anxiolytic, whereas anterodorsal BNST-parabrachial nucleus (PB) projection decreases respiratory rate (a marker of anxiety), but has no effect in the behavior observed in the open field and elevated plus maze (Kim et al., 2013).

The behavioral outcome modulated by the BNST is also dependent on the balance between different neurotransmitters being recruited in the presence of a stimulus. In response to a foot-shock, the glutamatergic BNST-VTA neurons present an increase in their activity, contrary to GABAergic BNST-VTA neurons (Jennings et al., 2013). Altogether, these data show that different neuronal populations within the BNST can modulate distinct features of anxiety.

2.3. Role of the BNST in addiction

Drug addiction is characterized as a relapsing disorder described in three stages: compulsion to seek and take the drug (preoccupation/anticipation), loss of control in intake (binge/intoxication), and development of a negative emotional state (for example, anxiety) reflecting a motivational withdrawal syndrome (withdrawal/negative affect); reviewed in (Koob and Le Moal, 2008). Early phases are controlled by impulsivity and the final phases by compulsion. Impulse control disorders are characterized by an increasing sense of tension or arousal before committing an impulsive act; pleasure, gratification or relief is felt at the time of committing the act, and after the act, there may or may not be regret, self-reproach or guilt . In contrast, compulsive disorders are characterized by anxiety and stress before committing a compulsive repetitive behavior and after the act, relief from the stress. When this shift happens, alteration from an impulsive disorder to a compulsive disorder, it is followed by a change from positive reinforcement to negative reinforcement, driving the motivated behavior (Koob, 2009).

Classical views highlight the BNST as being involved in the later stages of the disorder, mainly in the withdrawal/negative reinforcement, but this perspective is oversimplified since BNST neurons also respond to drugs and present synaptic adaptations upon acute or chronic exposure. For example, acute exposure to several substances of abuse (e.g. alcohol, morphine and cocaine) increases extracellular dopamine in the BNST (Carboni et al., 2000) and activates BNST neurons in a dopamine-dependent manner (Valjent et al., 2004). Moreover, disruption of

dopamine signaling in this region can alter both cocaine and alcohol-seeking behaviors (Eiler et al., 2003; Epping-Jordan et al., 1998). Similarly, injections of GABAA receptor antagonist into the BNST reduces alcohol self-administration (Hyytia and Koob, 1995), and injections of opioid receptor antagonist reduces both alcohol and cocaine self-administration (Eiler et al., 2003; Epping-Jordan et al., 1998).

Synaptic plasticity is associated with changes in the strength of synaptic connections, which underlie some of the maladaptive changes in reward processing induced by drugs of abuse (Dumont et al., 2005; Malenka and Bear, 2004). Drugs of abuse, like alcohol, can induce robust synaptic plasticity in the BNST (Dumont et al., 2005; Dumont et al., 2008; Weitlauf et al., 2004). An increase in excitatory synaptic strength in the BNST was observed after palatable food or cocaine self-administration, but not in subjects that received cocaine or food passively (Dumont et al., 2005). Additionally, chronic morphine treatment increased AMPA-dependent excitatory postsynaptic currents evoked in ventral BNST neurons projecting to the VTA (Dumont et al., 2008). Moreover, chronic intermittent ethanol exposure potentiates the induction of long term potentiation (LTP) in the ventral and dorsolateral part of this brain region (Stamatakis et al., 2014).

Part of the effects of the BNST in the initial stages of addiction may also be explained by bidirectional connections with the VTA, a core region in addiction/reward circuitry. The BNST has glutamatergic and, more important and predominantly, GABAergic projections to dopaminergic VTA neurons (Georges and Aston-Jones, 2001, 2002; Jalabert et al., 2009; Pina and Cunningham, 2016). In rats, stimulation of the BNST potently and consistently activates DAergic VTA neurons (Georges and Aston-Jones, 2001) leading to an increase of dopamine release in the nucleus accumbens. In accordance, microinfusion of glutamate in the ventral BNST increases the activity of DA VTA neurons, while GABA microinfusion inhibits their activation (Georges and Aston-Jones, 2002). In mice, a recent optogenetic study has proposed that the BNST projections inhibit (glutamatergic projection) or excite (GABAergic projection) VTA DAergic neurons (Jennings et al., 2013), through direct and indirect mechanism, respectively. Additionally, GABAergic CRF co-expressing neurons in the oval nucleus of the BNST also project to the VTA (Daniel and Rainnie, 2016), adding to the complexity of the system.

At a behavioral level, this excitatory projection has been associated with physiological and pathological reward-directed behaviors, such as food and cocaine self-administration (Dumont et al., 2005; Grueter et al., 2006) and drug seeking (Aston-Jones and Harris, 2004). Indeed, initial

pharmacological studies have shown that inhibition of BNST and VTA leads to a reduction in cocaine conditioned place preference (Sartor and Aston-Jones, 2012). In addition, optogenetic activation of the anterodorsal BNST-VTA pathway selectively induces conditioned place preference, indicating a positive reinforcement effect of this circuit (Kim et al., 2013). In another study, optogenetic activation of ventral BNST GABAergic terminals in the VTA is rewarding and buffer stress-induced anxiety, while activation of ventral BNST glutamatergic terminals in the VTA is aversive and anxiogenic (Jennings et al., 2013).

These effects may be partially mediated by disturbances in the BNST-VTA neuronal circuit due to chronic drug abuse (Briand et al., 2010; Pina and Cunningham, 2016). For example, during stress-induced reinstatement of cocaine seeking, an increase in the neuronal activation of VTA-projecting BNST neurons was observed (Briand et al., 2010). Inactivation of BNST prevented stress-induced conditioning place preference (CPP) reinstatement and attenuated neuronal activation of VTA neurons and neurons downstream from the VTA (Briand et al., 2010). Optogenetic activation of ventral BNST GABAergic terminals in the VTA buffer stress-induced anxiety (Jennings et al., 2013).

The maladaptive effects of drug abuse in the BNST go beyond predominant GABAergic and glutamatergic populations. Chronic cocaine (Nobis et al., 2011), morphine (Wang et al., 2006) and alcohol exposure recruit the BNST-CRF circuitry (Silberman and Winder, 2013). Conversely, stress-induced reinstatement of drug-related responding seems to depend on the activation of both CRF and norepinephrine (NE) in the CeA (Funk et al., 2006a; Zorrilla et al., 2001) and BNST (Olive et al., 2002); for detailed reviews, see (Shaham et al., 2003; Shalev et al., 2002). Actually, it has been observed that injection of CRF in the BNST induces reinstatement (Erb and Stewart, 1999), while intra-BNST injection of CRF receptor antagonists impairs stress-induced reinstatement of cocaine and morphine seeking behavior (Erb and Stewart, 1999; Wang et al., 2006). Moreover, CRF enhances BNST-VTA glutamatergic projections (Silberman et al., 2013) and engagement of this BNST–CRF circuitry following exposure to drug-associated cues or stressors results in increased signaling from the BNST to the VTA, driving drug seeking behavior (Silberman et al., 2013; Silberman and Winder, 2013).

2.4. BNST: the link between anxiety and addiction?

Anxiety and addiction are often comorbid. As mentioned before, anxious individuals are prone to drug-seeking behavior and addicts commonly present anxiety disorders (Avery et al.,

2016). When considering this comorbidity, it is interesting to notice that in individuals with anxiety disorders, negative reinforcement and not reward, may drive initial drug use (Avery et al., 2016). Although the comorbidity between these two disorders is clear, not much is known about how they are interconnected and identifying the common links between them can provide a better understanding of how they work and provide potential therapeutic targets.

The BNST is a brain region with a central position between anxiety and addiction circuits. The BNST is not only involved in stress and anxiety circuits, due to its privileged connections with limbic structures and the PVN, but it is also involved in addiction circuit, projecting to areas such as the VTA. Although, it has been found that the Amy and BNST present a similar role in addiction , there is some evidence that reinstatement of drug seeking behavior is mediated mainly by the BNST (Erb et al., 2001b; Shaham et al., 2003). Furthermore, the BNST is involved in the anxiogenic effects of cocaine intoxication (Wenzel et al., 2014). In fact, patients with alcohol use disorder, when presented with fearful expressions, have revealed an increased functional connectivity between BNST and Amy (Avery et al., 2016). Hence, it was hypothesized that this facilitates the neural integration of these structures, allowing threatening stimuli to be more efficiently processed within the extended amygdala.

Several reports have shown that manipulation of the BNST and its neurotransmitters can alter the anxious state of rodents (Jennings et al., 2013; Lee et al., 2008; Sajdyk et al., 2008). Interestingly, many of the neurotransmitters present in the BNST are essential for both anxiety and addictive behaviors. Specifically, previous studies have shown the importance of CRF in the BNST in these two behaviors; for example, intra-BNST injections of CRF antagonists can block both stress-induced reinstatement of cocaine and morphine seeking behavior (Erb and Stewart, 1999; Wang et al., 2006), while activation of CRF receptors directly in the BNST induces social anxiety (Lee et al., 2008).

Additionally, studies in rodents have shown that withdrawal from repeated administration of cocaine, ethanol or nicotine produces an anxiogenic-like response, which could be reversed by CRF antagonists (Funk et al., 2006a; George et al., 2007; Koob, 2009). Considering the importance of CRF in both circuits separately, it is not surprising that an imbalance in CRF at the BNST level can contribute to alterations in both anxiety and addiction.

Exposure to stressful events is the major common eliciting factor for the development of addiction and anxiety and in individuals with altered stress reactivity, the risk to develop both disorders is heightened (Avery et al., 2016; Koob, 2009). It has been widely reported the effects

of stress in the development of anxiety and in the last years, many authors have provided evidence that exposure to stress can also have a significant impact in different stages of addiction. Indeed, it has been shown that a psychosocial stressor task produced changes in the activity of the limbic region, including the BNST, in nicotine-addicted participants. These changes were predictive of a subsequent increase of BNST activity when presented to smoking cues (Avery et al., 2016).

Although different drugs of abuse may act through distinct neurotransmitter systems and activate different neuronal populations, they all produce common negative effects during the withdrawal phase, including heightened anxiety and high vulnerability to relapse when subjected to an acute stressor. A state of stress and exposure to stressor have long been associated with relapse and vulnerability to relapse (Koob, 2009). In this context, the BNST plays a pivotal role, considering its relationship with stress-responsive circuits. Indeed, inactivation of the BNST leads to reduction in both cue- and stress-induced reinstatement of cocaine seeking behavior (Buffalari and See, 2011; Erb et al., 2001a) and decreased interest for natural rewards abstinence (Harris and Aston-Jones, 2007). Inactivation of the ventral BNST, as well as disruptions of either CRF or norepinephrine signaling within this sub-region of the BNST, blocks the ability of stress to reinstate drug seeking (Erb et al., 2001a; Erb and Stewart, 1999; Leri et al., 2002; Wang et al., 2006). Furthermore, stress exposure in animals with a history of heroin self-administration leads to an increase in CRF mRNA within the dorsal BNST (Shalev et al., 2001).

Recent studies have focused on how upstream regions modulate the BNST response upon a stress period. As mention before, the BNST acts as a relay station between the medial prefrontal cortex (mPFC) and the VTA, and this pathway is modulated by cannabinoid receptor 1 (CB1). Acute stress leads the switch from long-term depression (LTD) to long-term potentiation (LTP) after exposure to an electric 10 Hz stimulation on the mPFC input to the BNST (Glangetas et al., 2013). However, the injection of a CB1 antagonist in the BNST blocks this LTP observed in stressed animals. Remarkably, CB1 knock-out mice, in baseline conditions, did not exhibit a stable LTD or, upon an acute stress, a stable LTP (Glangetas et al., 2013). Nevertheless, not all studies show an increase in LTP after stress. Acute social isolation stress animals presented a blunting of LTP, although these animals did not present anxiety-like behavior (Conrad et al., 2011). On the other hand, chronic stress leads to LTD in the BNST (McElligott Zé et al., 2010). Still, others have found that chronic stress leads to an increase in the LTP magnitude while others have found a decrease in LTP magnitude in the dorsal BNST (Conrad et al., 2011;

Dabrowska et al., 2013). These discrepancies between studies could be due to animal model, protocol and methodology used to induce stress however they illustrate how stress modulates neurotransmitter systems traditionally associated to addiction behavior.

3. Conclusion

Many studies have shown that addiction and anxiety are often comorbid. But it is not known if one condition is a cause or consequence of the other. There are at least three options that should be considered: (1) addiction is a cause for anxiety disorder; (2) anxiety disorders can lead to addiction and (3) the addiction and anxiety disorders are both caused by common risk factors, namely, stress.

Stress, anxiety and addiction, all share common pathways. A three-level pathway that involves upstream sensorial and regulatory regions, namely PFC and Amy, and downstream effector regions, VTA and PVN, using the BNST as a relay station between these two levels of processing and expression of a behavioral/physiological reaction. Indeed, we have presented the importance of the BNST in the regulation of stress, anxiety and addiction. We hypothesized that the (dis)stress is a common factor in the genesis of anxiety and addiction due to its functional impact on specific BNST nuclei. Nevertheless, since the BNST is a heterogeneous nucleus composed of different types of neurons and complex interconnectivity, the full understanding of its role in the modulation of these two disorders is itself a great challenge for the future.

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Figure Captions

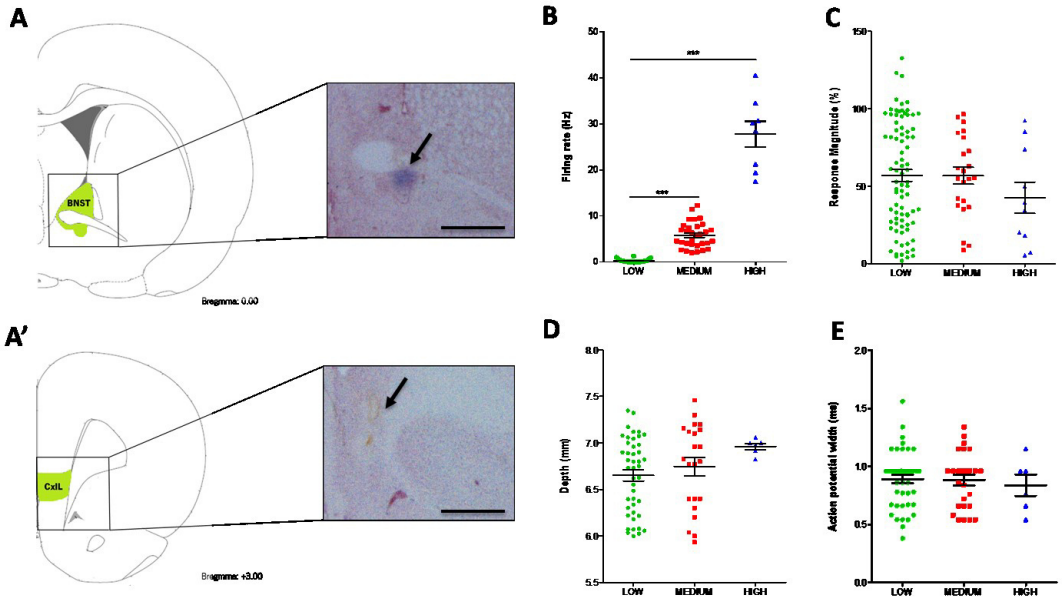

Figure 1 – Bed nucleus of the stria terminalis (BSNT) neuronal heterogeneity. Representative images of coronal sections showing a representative local of recording (blue spot/lesion site) in the anterior BNST **(A)** and infralimbic cortex (CxIL) **(A')**. Numbers represent distance in mm from bregma. **(B)** The anterior BNST neurons were grouped in three different classes according to their firing rate: low, medium and high. **(C)** Upon an electric stimulation of the CxIL (0.5 Hz), the different cell groups present a homogenous excitatory response. No differences were found in depth from the brain surface **(D)** and action potential **(E)**. 26 animals were used (Low: total number of cells= 46; Medium: total number of cells=31; High: total number of cells=7); ***p <0.005.

Afferents	**Type of connections** (main – not exclusive)	**References**
Cortex: caudal ILCx prelimbic Cx insula Cx entrorhinal Cx orbital PFC	Glutamatergic	Massi et al., 2008
Hipp	Glutamatergic	Myers et al., 2014
BLA	Glutamatergic	Kim et al., 2013
PB	Glutamatergic	Dong et al, 2001b Li and Cho, 2006
Acessory and Main Olfactory Bulb	Glutamatergic	Brennan and Keverne,2004
CeA	GABAergic	Dong et al., 2001a Li et al., 2012
VTA	Dopaminergic	Meloni et al., 2006
PAG	Dopaminergic	Meloni et al., 2006
NTS	Noradenergic	Forray and Gysling, 2004
VLM	Noradenergic	Forray and Gysling, 2004
VNAB	Noradenergic	Forray and Gysling, 2004
LC	Noradenergic	Myers et al., 2005
DRN	Serotonergic	Commons et al., 2003

Table I – Overview of the bed nucleus of the stria terminalis (BNST) afferents. Includes the caudal infralimbic cortex (ILCx), prelimbic cortex, insula cortex, entorhinal cortex, caudal orbital prefrontal cortex (PFC), hippocampus (Hipp), the basolateral amygdala (BLA), parabrachial nucleus (PB), accessory olfactory bulb, main olfactory bulb, central nucleus of amygdala (CeA), ventral tegmental area (VTA), periaqueductal gray (PAG), nucleus tractus solitaries (NTS), nucleus ventral lateral medulla (VLM), ventral noradrenergic bundle (VNAB), locus coeruleus (LC), dorsal raphe (DRN), amongst others.

Efferents	Type of connections (main – not exclusive)	References
VTA	Glutamatergic and GABAergic	Georges and Aston-Jones, 2001,2002 Jalabert et al., 2009, Jennings et al., 2013 Kim et al., 2013
PVN	GABAergic and CRFergic	Roland and Sawchenko, 1993 Champagne et al., 1998 Dong et al., 2001a Dong and Swanson, 2006
Hyp	Unknown	Dong and Swanson, 2004
NAc	Unknown	Dong et al., 2001b
PAG	Unknown	Dong et al., 2001b
PB	Unknown	Li and Cho, 2006
CeA	Unknown	Walker et al., 2009

Table II – Overview of the bed nucleus of the stria terminalis (BNST) efferents. Includes the ventral tegmental area (VTA), paraventricular nucleus of the hypothalamus (PVN), hypothalamus (Hyp), nucleus accumbens (Nac), periaqueductal gray (PAG), parabrachial nucleus (PB), central nucleus of amygdala (CeA).

BNST divisions			
Anterior division	Lateral subdivion	Anterolateral (AL)	AL
			Juxtacapsular (ju)
			Oval (o)
			Rhomboid (rh)
			Fusiform nuclei (fu)
			Subcomissural zone (sc)
	Medial subdivision	Anterodorsal (AD)	AD
		Anteroventral (AV)	AV
			Dorsomedial (dm)
			Dorso Lateral (dl)
			Magnocellular (mg)
			Ventral nuclei (v)
Posterior division	Dorsal (d)		
	Principal (pr)		
	Interfascicular (if)		
	Transverse nuclei (tr)		

Table III – Summary of the divisions of the bed nucleus of the stria terminalis (BNST). The BNST is divided in two main divisions, the anterior and posterior divisions. The anterior division can be further divided into medial and lateral subdivisions. The medial subdivision is composed by the anterodorsal (AD) and anteroventral (AV) areas. The last area includes the dorsomedial (dm), dorsolateral (dl), magnocellular (mg) and ventral (v) nuclei. The lateral division is composed by the anterolateral (AL), juxtacapsular (ju), oval (o), rhomboid (rh) and fusiform (fu) nuclei and by the subcomissural zone (sc). The posterior division is divided by the dorsal (d), principal (pr), interfascicular (if) and transverse (tr) nuclei.

BNST neurons recorded	ALL	Low	Medium	High
Number of neurons (n)	85	46	31	7
Spontaneous Firing Rate (Hz)	4,8±0,9	0,25±0,005	5,8±0,5	27,8±2,8
Neurons responsive to stimulation (n)	69	42	23	4
Response magnitude of excitation (% increase)	55,59±3,07	54,3±3,68	56,6±5,4	42,5±10,06

Table IV – Summary of spontaneous firing rate and the effect of electric stimulation of infralimbic cortex (CxIL) on the anterior bed nucleus of the stria terminalis (BNST) neuronal activity. data presented as mean ± SEM; 26 animals were used (Low: total number of cells= 46 ; Medium: total number of cells=31 ; High: total number of cells=7); ***p <0.005.

BNST regions	Approach used	HPA activity	References
anterior BNST	Lesions	Reduce	Choi et al., 2008 Choi et al., 2007
posterior BNST	Lesions	Increase	Herman et al., 1994 Choi et al., 2007
lateral BNST	Lesions	Reduce	Gray et al., 1993
medial BNST	Lesions	No effect	Gray et al., 1993

Table V - Summary of the bed nucleus of the stria terminals (BNST) effects on the hypothalamic–pituitary–adrenal (HPA) axis activity.

Supplementary Information

Borges S, Ventura-Silva AP, Sousa N, Georges F, Pêgo JM and Rodrigues AJ

BNST – Bridging aNxiety, Stress and addiction

Supplementary Methods

Animals

Male Sprague Dawley rats (8 to 12 weeks old) were housed (six per cage) under standard laboratory conditions (22° - 23°C, 50 –55% relative humidity, 12 h light/dark cycle with lights on at 07:00); food and water ad libitum. Total number of animals used was 26. All procedures were conducted in accordance with the European directive 2010-63-EU and with approval from the Bordeaux University Animal Care and Use Committee (N°50120205-A) on animal care and experimentation.

Surgery

Animals were submitted to stereotaxic surgery for the placement of the stimulating and recording electrodes, following anatomical coordinates (Paxinos and Watson, 2007). Surgeries were performed under isoflurane anesthesia as previously described (Georges and Aston-Jones, 2002); body temperature was maintained at approximately 37°C with a homeothermic heating blanket (DC temperature controller, FHC). Lidocaine (100ul) was subcutaneous injected over the skull area before an incision was made through the skin and connective tissues. Two holes were drilled in the skull and stimulating and recording electrodes were placed in the following coordinates: Infralimbic Cortex (CxIL): -3.0 from bregma, 0.5 lateral from midline, -4.5 ventral to brain surface; anterior bed nucleus of the stria terminalis (aBNST): -0.5 from bregma, 1.2 lateral from midline, -6 to -7.5 ventral to brain surface. A reference electrode was fixed in the muscle.

Electrical Stimulation

Bipolar concentric electrode (Phymep) was inserted in the CxIL brain region. The stimulation was administered using a square pulse stimulator (CED micro 1401 interface, SPIKE 2 software, Cambridge Electronic Desing) and a stimulus isolator (DS3, Digitimer). The stimulation consisted of 100 pulses of 0.5Hz with 0.5 ms duration with intensity from 0.2-1 mA.

In vivo anesthetized electrophysiology recordings

Extracellular neural activity in the aBNST was recorded using a glass micropipette (tip diameter, 1–2 µm; 10 –15-MΩ) filled with a 2% pontamine sky blue solution in 0.5 M sodium acetate. Recordings were amplified and filtered by the Axoclamp-2B (low-pass filter at 300 Hz and high-pass filter at 0.5 kHz). Spikes of single neuron were discriminated, and digital pulses

were led to a computer for online data collection using a laboratory interface and software (CED micro 1401 interface, SPIKE 2 software; Cambridge Electronic Design). After isolation a single neuron, before stimulation, spontaneous activity was recorded to establish baseline activity for at least 100 sec. After, single pulses were delivered to the specific brain region every 2 sec, at least, 100 trials were administered per cell.

Histology and microscopy

At the end of each electrophysiological experiment, placements of stimulating and recording electrode tips within the CxIL or aBNST were verified with histological examination of brain tissue. The glass micropipette placement was marked with an iontophoretic deposit of pontamine sky blue dye (20 μA, 30 min). To mark electrical stimulation site, 50μA was passed through the stimulation electrode for 90 secs.

Animals were sacrificed under isoflurane anesthesia and brains were collected and snap frozen in isopentane at -80°C. Coronal sections (30μm) were cut in a cryostat, mounted and stained with neutral red for histological determination of the localization of recording and stimulation sites.

Data Analysis

For electrophysiology data analysis, peristimulus time histograms (PSTHs; 5 ms bin width) of neuronal activity were generated during electrical stimulation of the CxIL, for each neuron recorded. PSTHs were analyzed to determine excitatory and inhibitory epochs. Briefly, the mean and standard deviation (SD) of counts per bin were determined for a baseline period, definite as the 500 ms epoch previous stimulation. The onset of excitation was defined as the first of five bins whose mean value exceeded mean baseline activity by 2 SD, and response offset was determined as the time at which activity had returned to be consistently within 2 SD of baseline. Response magnitudes for excitation were calculated with the following equation: (counts in excitatory epoch) - (mean counts per baseline bin 3 number of bins in excitatory epoch).

Statistical Analysis

Data is presented as mean± standard error of the mean (SEM). Statistical analysis was performed using Graphpad Prism 5.0 software. Data was verified for Gaussian distribution. A-

nova test was used in all the statistical analysis. When no Gaussian distribution was assumed, a nonparametric test (Kruskal-Wallis test) was used. Statistical significance was set at $p<0.05$.

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