Universidade do Minho
Escola de Medicina

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Role of laterodorsal tegmentum in motivation and reward

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Tese de Doutoramento em Ciências da Saúde

Trabalho efetuado sob a orientação da
Doutora Ana João Gomes Rodrigues
e do
Professor Doutor Nuno Jorge Carvalho Sousa

março de 2018

STATEMENT OF INTEGRITY

I hereby declare having conducted my thesis with integrity. I confirm that I have not used plagiarism or any form of falsification of results in the process of the thesis elaboration.

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University of Minho, 29/03/2018

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“Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less.”

Marie Curie

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ABSTRACT

The reward circuit is mainly comprised by dopaminergic projections arising from the ventral tegmental area (VTA) to the nucleus accumbens (NAc), and has been shown to mediate different aspects of reward and reinforcement. Mesopontine neurons of the laterodorsal tegmentum (LDT) tightly modulate the activity of dopaminergic neurons of the VTA, controlling the release of dopamine in the NAc. Interestingly, a recent study has shown that the LDT also sends direct projections to the nucleus accumbens, suggesting both a direct and indirect role in modulating accumbal activity.

In this thesis, we first show that prenatal glucocorticoid exposure (iuCG model) impacts the electrophysiological activity of the LDT-VTA circuit, culminating with a decrease in motivational drive. LDT basal activity was decreased in iuGC-exposed animals, and we observe a bidirectional effect in evoked activity: iuGC animals present a decrease in the magnitude of excitation and an increase in the magnitude of inhibition in the VTA after electrical stimulation of LDT. Importantly, optogenetic activation of LDT-VTA projections rescues this phenotype, suggesting that motivational levels are dependent on this circuit. In line with this, we further manipulated LDT-VTA circuit during multiple phases of reward using optogenetics. We showed that optical LDT-VTA stimulation is able to causally enhance choice preference for a laser-paired reward and induce intracranial self-stimulation.

Next, we investigated the recently described LDT projections to the NAc. This region is comprised by two major populations of GABAergic medium spiny neurons (MSNs), canonically segregated into those expressing dopamine receptor D1 (D1-MSNs), and those expressing D2 (D2-MSNs). Using anatomical, electrophysiological and behavioural experiments, we confirmed the presence of direct cholinergic, glutamatergic and GABAergic projections from the LDT to the NAc, evoking a predominantly excitatory response in accumbal cells. Lastly, we show for the first time that selective activation of LDT-NAc projections enhances motivational drive and induces preference for a laser-associated lever, but only when paired with a food reward. We further demonstrate that specific activation of LDT-NAc cholinergic projections is sufficient for this shift in preference, suggesting that these projections enhance and narrow incentive motivation in rodents.

In conclusion, with this work we showed that i) prenatal glucocorticoid exposure negatively impacts motivation due to impairments in LDT-VTA inputs, and that activation of this circuit rescues

motivational deficits; ii) that LDT also controls NAc activity through direct projections of different natures; and iii) LDT-NAc (cholinergic) projections convey positive reinforcement signals.

RESUMO

O circuito de recompensa é composto principalmente por projeções dopaminérgicas originárias da área tegmental ventral (VTA) para o *nucleus accumbens* (NAc), e está envolvido na percepção de recompensa e reforço positivo. Os neurónios do núcleo tegmental dorso-lateral (LDT) modulam a atividade dos neurónios dopaminérgicos do VTA, controlando a libertação de dopamina no NAc. Um estudo recente mostrou que o LDT também envia projeções diretas para o NAc, sugerindo um papel direto e indireto na modulação da atividade do accumbens.

Nesta tese, mostramos pela primeira vez que a exposição pré-natal a glucocorticóides (modelo iuGC) afeta a atividade electrofisiológica do circuito LDT-VTA, originando uma diminuição da motivação. A atividade basal do LDT estava diminuída nos animais expostos a iuGC, e observamos um efeito bidirecional na atividade evocada do VTA: após a estimulação elétrica do LDT, os animais iuGC apresentavam uma diminuição na magnitude da excitação e um aumento na magnitude da inibição. De salientar que a ativação optogenética das projeções LDT-VTA normaliza esse fenótipo, sugerindo que os níveis motivacionais dependem deste circuito. Em consonância, recorrendo à técnica de optogenética, manipulamos o circuito LDT-VTA durante múltiplas fases de recompensa. A estimulação optogenética do circuito LDT-VTA aumenta a preferência por uma recompensa associada à estimulação e induz *autoestimulação* intracraniana.

De seguida investigamos as projeções do LDT para o NAc descritas recentemente. O NAc é composto por duas populações principais de neurónios espinhosos médios GABAérgicos (MSNs), segregados canonicamente nos que expressam o receptor de dopamina D1 (D1-MSNs) e os que expressam D2 (D2-MSNs). Dados anatómicos, eletrofisiológicos e comportamentais confirmam a presença de projeções diretas do LDT para o NAc colinérgicas, glutamatérgicas e GABAérgicas, que induzem uma resposta predominantemente excitatória no NAc. Por último, demonstramos pela primeira vez que a ativação seletiva das projeções LDT-NAc aumenta o nível motivacional e induz a preferência por uma alavanca associada à estimulação, mas somente quando associada com uma recompensa. Mostramos ainda que a ativação específica das projeções colinérgicas LDT-NAc é suficiente para a mudança de preferência.

Em conclusão, com este trabalho demonstramos que i) a exposição pré-natal a glucocorticóides reduz a motivação devido a défices nas projeções do LDT para o VTA, e que a ativação deste circuito normaliza estes défices; ii) que o LDT também controla diretamente a

atividade do NAc através de projeções diretas de tipos diferentes; e iii) as projeções LDT-NAc (colinérgicas) codificam sinais de reforço positivos.

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ABBREVIATIONS

11β-HSD2: 11β-hydroxysteroid dehydrogenase type 2

A_2a: adenosine receptor 2a

ac: anterior commissure

ACC: anterior cingulate cortex

ACh: acetylcholine

ACTH: adrenocorticotropic hormone

AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

ANOVA: analysis of variance

AP: anteroposterior

AVP: arginine vasopressin

BLA: basolateral amygdala

bp: base pair

cAMP: cyclic adenosine monophosphate

CeA: central amygdala

ChAT: choline acetyltransferase

ChR2: channelrhodopsin 2

CIN: cholinergic interneuron

CNQX: 6-cyano-7-nitroquinoxaline-2,3-dione

CPP: conditioned place preference

CR: calretinin

CRF: continuous reinforcement

CRH: corticotropin-releasing hormone

CS: conditioned stimulus

D1R (Drd1 or D1): dopamine receptor D1

D2R (Drd2 or D2): dopamine receptor D2

DA: dopamine

DAPI: 4′,6-diamidino-2-phenylindole

DAT: dopamine transporter

DREADD: designer receptor activated by designer drugs

DEX: dexamethasone

DLS: dorsolateral striatum

dlVP: dorsolateral ventral pallidum

DMS: dorsomedial striatum

DNA: deoxyribonucleic acid

dOFC: dorsal orbitofrontal cortex

DPSS: diode-pumped solid-state

DV: dorsoventral

eNpHR: enhanced halorhodopsin

eYFP: enhanced yellow fluorescent protein

FELASA: federation for laboratory animal science associations

FR: fixed ratio

FS: fast spiking GABAergic interneuron

FSCV: fast scan cyclic voltammetry

GABA: gamma-aminobutyric acid

GC: glucocorticoid

G-CaMP: (green fluorescent protein)-calcium molecular probe

GAD1: glutamate decarboxylase 1

GAD2: glutamate decarboxylase 2

GFP: green fluorescent protein

GPCR: G protein-coupled receptor

GPe: glubus pallidus external

GPi: globus pallidus internal

Gpr6: G protein-coupled receptor 6

GR: glucocorticoid receptor

h: hour

HPA: hypothalamus-pituitary-adrenal

Hz: hertz

ICVS: Instituto de Investigação em Ciências da Vida e Saúde/Life and Health Sciences Research Institute

IF: Immunofluorescence

ILC: infralimbic cortex

ITI: inter-trial interval

iuGC: in utero exposure to glucocorticoids

kg: kilogram

L-DOPA: levodopa

LCN: lipocalin

LDT: laterodorsal tegmentum

LH: lateral hypothalamus

LHb: lateral habenula

lOFC: lateral orbitofrontal cortex

LTS: low threshold spiking GABAergic interneuron

mAChR: muscarinic acetylcholine receptor

MDT: mediodorsal thalamus

MFB: medial forebrain bundle

mg: milligram

mGluR: metabotropic glutamate receptor

min: minute

ML: mediolateral

mm: millimetre

mPFC: medial prefrontal cortex

MR: mineralocorticoid receptor

ms: millisecond

MSN: medium-sized spiny neuron

mW: milliwatt

NAc: nucleus accumbens

NAcc: nucleus accumbens core

nAChR: nicotinic acetylcholine receptor

NAcs: nucleus accumbens shell

nm: nanometre

NMDA: *N-methyl-D-aspartate receptor*

NPY: neuropeptide Y

OFC: orbitofrontal cortex

PCR: polymerase chain reaction

PIT: Pavlovian-to-instrumental transfer

PLC: prelimbic cortex

PPN: pedunculopontine nuclei

PR: progressive ratio

PTSD: post-traumatic stress disorder

PVN: paraventricular nucleus of the hypothalamus

RTPP: real-time place preference

RMT: rostromedial tegmental nucleus

RR: random ratio

s: second

s.e.m (or SEM): standard error of the mean

SN: substantia nigra

SNc: substantia nigra pars compacta

SNr: substantia nigra pars reticulata

STN: sub thalamic nuclei

t-test: Student's t-test

TAN: tonically active cholinergic interneuron

TH: tyrosine hydroxylase

UCS: unconditioned stimulus

UNC: University of North Carolina

vmVP: ventromedial ventral pallidum

vOFC: ventral orbitofrontal cortex

VGAT: vesicular GABA transporter

VGLUT2: vesicular glutamate transporter 2

VMAT2: vesicular monoamine transporter 2

VP: ventral pallidum

VTA: ventral tegmental area

β2-nAChR: beta2-subunit-containing nicotinic acetylcholine receptor

μg: microgram

μL: microliter

μm: micrometre

THESIS OUTLINE

This thesis is composed of 4 Chapters. Chapter 1 is the General Introduction, and the chapters concerning the experimental work are presented in the form of research articles in Chapters 2 and 3; Chapter 4 is the general discussion of the work. The manuscript of Chapter 2.1 has been published in eLife. The manuscripts in Chapters 2.2 and 3 are in preparation for submission.

Chapter 1 starts with a general introduction to the theme of this dissertation. We describe the brain reward circuit and the major key brain regions of interest in reward processing. Next, we provide a description of the Ventral Tegmental Area (VTA), the Nucleus accumbens (NAc) and the Laterodorsal Tegmentum (LDT), focusing on their different neuronal populations and their role in reward and motivation. Finally, we review evidence about the impact of prenatal exposure to stress or high levels of glucocorticoids in the reward circuit and in behaviour.

In Chapter 2, we focus on LDT projections to the VTA. In Chapter 2.1, we show that *in utero* exposure to high levels of glucocorticoids (iuGC model) decreases motivation by altering the connectivity of the LDT-VTA pathway, and that optogenetic activation of these inputs normalizes this behavioural deficit. In Chapter 2.2, we show that activation of LDT inputs in the VTA is sufficient to induce intracranial self-stimulation and change preference for a laser stimulation-paired reward.

In Chapter 3, we investigate the function of LDT direct inputs to the NAc in reward-related behaviours using optogenetics. We show that LDT-NAc projections are functional and able to enhance motivation for a stimulus-paired reward; and this effect appears to be mainly dependent on cholinergic inputs.

In Chapter 4 we discuss the main findings of this work, present critical inputs on the experiments performed and offer future perspectives.

Chapter 1

General Introduction

1. General introduction

Every organism is innately bound to seek and respond to reward, and their survival depends on it. The ability to recognize, interpret, and respond to reward is critical to motivate animals to seek water and food, to mate and nurture progeny (Koob and Le Moal, 2008; Koob and Volkow, 2010; Russo and Nestler, 2013). Reciprocally, aversive stimuli cause avoidance and generally involve negative emotions, such as dislike and fear, inhibiting the expression of related behaviours.

Many behavioural disorders including addiction, depression and anxiety present deficits on the interpretation and response to positive and negative stimuli (Nestler and Carlezon, 2006). In this context, a large effort has been made in order to identify the neurobiological basis of reward and aversion. For several years, scientists focused primarily on the best-characterized reward circuit in the brain, consisting in projections from midbrain neurons from the ventral tegmental area (VTA) to the nucleus accumbens (NAc). Different players were added to this circuit and recognized as 'brain reward regions', including the amygdala, the medial prefrontal cortex and the hippocampus, amongst others. One system has received particular attention due to the modulatory effects it exerts during reward or aversive processes: the mesopontine tegmentum, comprised by two brainstem areas, the pedunculopontine tegmentum (PPT) and the laterodorsal tegmentum (LDT) (Mena-Segovia, 2016).

Interestingly, several studies have shown that the reward system is particularly affected by both prenatal and postnatal exposure to stress or stress hormones (glucocorticoids – GCs). This exposure can impact brain regions of the reward circuit at a molecular, morphological and functional level, promoting the appearance of neuropsychiatric disorders related to reward behaviours, such as addiction and depression (Lupien et al., 2009).

In this introduction, we will first describe the reward circuit, with a special focus on the VTA and NAc nuclei inputs and outputs and their crucial role in reward-related behaviours. Additionally, we will review important findings related to the neuromodulatory effects of the LDT and its role in the coding and processing of reward. Next, we will provide an overview about the impact of stress/GCs on the reward circuit, which ultimately leads to altered behaviour.

1.1. The reward circuit

In the last years a great advance has been made in identifying brain regions associated with not only natural rewards, such as food, sex or social interaction but also in the context of drugs of abuse. In fact, the majority of the knowledge underlying the reward circuit and reward mechanisms originated from drug use studies and the maladaptive reward process in addiction, including relapse and craving studies, through the use of sophisticated genetic-based opto- and chemogenetic tools to observe and manipulate neuronal activity within specific cell types and circuits.

As mentioned above, the role of dopamine outputs from the VTA to the NAc is well established in reward recognition, approach and consumption, with reports showing that these regions also respond to aversive stimuli (Koob and Le Moal, 2008; Mogenson et al., 1980; Russo and Nestler, 2013). Dopaminergic projections arising in the VTA also innervate the dorsal striatum, the prefrontal cortex (PFC), the hippocampus, the central amygdala (CeA) and basolateral amydala (BLA) (Oades and Halliday, 1987; Swanson, 1982). Apart from these connections, the VTA also sends γ-amino butyric acid (GABA) and glutamate outputs to the NAc and lateral habenula (LHb) (Brown et al., 2012; Root et al., 2014a). These regions form a highly intricate circuit considering that the NAc, receives dense glutamatergic innervation from the PFC, amygdala and hippocampus and, in turn, the PFC, amygdala and hippocampus form reciprocal glutamatergic connections. Moreover, this system also receives cholinergic and glutamatergic inputs from the LDT (Cornwall et al., 1990; Forster and Blaha, 2000; Lodge and Grace, 2006). (Fig.1-2). In this way, the high complexity of this circuit involves, apart from the different regions that play a role in reward processing, distinctive molecular and functional changes in response to rewards. In the following sections, we will provide a general overview of the anatomy of key players in reward processing, the VTA, NAc and LDT, and the role of these regions in rewarding events and reward-related disorders.

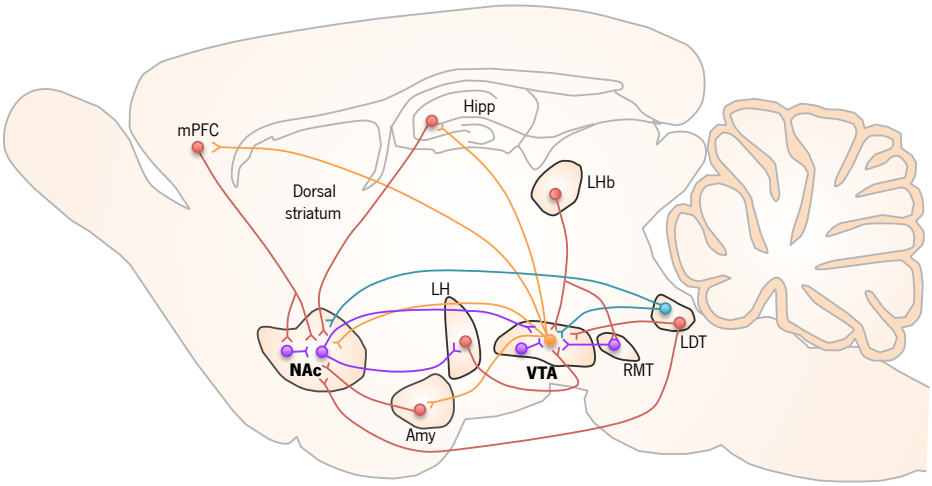

Figure 1 - The brain reward circuit. Summary scheme showing the classical projections between cortical, limbic, midbrain and brainstem nuclei, with a particular focus on connections to and from the VTA and NAc. VTA dopaminergic (orange) projections to the NAc comprise the primary reward system. Additionally, VTA dopamine neurons innervate the mPFC, hippocampus and amygdala, as well as other regions of the thalamus. On the other hand, GABAergic (purple) projections arising in the NAc are able to modulate the VTA directly or indirectly through basal ganglia nuclei such as ventral pallidum (not shown). The NAc receives excitatory glutamatergic (red) inputs from the mPFC, hippocampus, amygdala and thalamus (not represented) and cholinergic and glutamatergic inputs from the brainstem. Accumbal activity is also modulated by different types of interneurons. In turn, the VTA receives direct glutamatergic inputs from the mPFC, LDT, LHb and LH, as well as glutamatergic and GABAergic inputs from the BNST and cholinergic (grey) innervation arising in the LDT. These various inputs control aspects of reward-related perception and memory.

(This scheme was adapted from Russo and Nestler, 2013).

VTA: ventral tegmental area; NAc: nucleus accumbens; mPFC: medial prefrontal cortex; Hipp: hippocampus; Amy: amygdala; LDT: laterodorsal tegmentum; LHb: lateral habenula; LH: lateral hypothalamus; RMT: rostromedial tegmental nucleus.

2. Players of the reward circuit

2.1. Ventral tegmental area (VTA)

The general understanding of the reward circuitry underlying reward-related disorders, such as mood disorders or addiction, begins with the VTA. To accelerate the identification of neurons that make synapses onto VTA cellular subpopulations, several investigators have taken advantage of recent methodological advances in modified virus trans-synaptic tracing. In addition,

optogenetic approaches in transgenic rodents have revealed discrete VTA neuronal phenotypes that seem to have distinct roles in reward, aversion, motivation and learning. We also discuss recent evidence that suggests that specific circuits, including both dopamine-mediated and non-dopamine-mediated outputs from the VTA, are sufficient to produce either reward- or aversion-related behaviour.

2.2. Neuroanatomy of the ventral tegmental area

The VTA is formed by heterogeneous nuclei with undefined borders and includes several neuron types, functions and outputs. This region corresponds to the A10 group of monoaminergic neurons and was described in detail using Tyrosine Hydroxylase (TH) immunostaining (Phillipson, 1979), and contains several nuclei that will not be discussed in the context of this thesis (reviewed in (Morales and Margolis, 2017)).

Despite being described as one of the main dopaminergic nucleus (60-65%) in the brain (Nair-Roberts et al., 2008), the VTA has GABA- and glutamate-releasing neurons as well (Nair-Roberts et al., 2008; Swanson, 1982).

Additional to being regulated by inputs from different brain regions, VTA dopamine neurons are regulated by local GABAergic and glutamatergic neurons and, apart from this local microcircuit, VTA GABA or glutamate neurons provide long-range projections to several brain regions that are also innervated by VTA dopaminergic neurons (Fig. 2).

Dopaminergic neurons

VTA dopaminergic neurons are often identified as being TH+ (tyrosine hydroxylase), considering the function of this enzyme in the conversion of l-tyrosine to l-DOPA (3,4-dihydroxyphenylalanine), which is then converted to dopamine. However, VTA dopaminergic neurons are highly heterogeneous and may present distinct electrophysiological properties and project to different target areas (Lammel et al., 2008, 2014). One subpopulation, located in the lateral part of the VTA, adjacent to the substantia nigra pars compacta (SNc), presents classical physiological properties of dopamine neurons: 1) presence of a large hyperpolarization-activated cation current (I_h) (Lammel et al., 2008; Margolis et al., 2006); 2) long action potential duration and firing rate <10 Hz (Grace and Bunney, 1983, 1984) and 3) the ability to be inhibited by dopamine receptor D2 (D2R) agonists. Contrastingly, another subpopulation of dopamine neurons

shows a higher degree of I_h diversity in magnitude, fast firing properties and different pharmacological responses, considering that they lack D2R agonist auto-inhibition (Lammel et al., 2008; Margolis et al., 2008). These differences seem to be well correlated with cell location and projection to target areas (Ford et al., 2006; Lammel et al., 2008; Margolis et al., 2008; Roeper, 2013; Sarti et al., 2007).

GABA neurons

GABA neurons are distributed throughout the VTA and represent around 30-35% of VTA neurons (González-Hernández et al., 2001; Margolis et al., 2012; Nair-Roberts et al., 2008; Olson and Nestler, 2007). There are two general populations of GABA neurons: interneurons, providing local inhibition of dopaminergic neurons and projection neurons, which provide long-range inhibition of multiple brain regions. VTA GABA neurons present a short action potential duration and do not respond to $GABA_B$ receptor agonists, oppositely to VTA dopamine neurons. However, they are also physiologically and pharmacologically heterogeneous: around 50% of GABA neurons were shown to be inhibited by a selective μ-opioid or by D2R agonist (Margolis et al., 2012).

Using *in vivo* extracellular recordings, it was shown that driving activity of these GABA neurons inhibited the spontaneous firing rate of dopaminergic neurons (Tan et al., 2012), whereas inhibiting the activity of GABA neurons induced or disinhibited the activity of dopaminergic cells (Bocklisch et al., 2013).

Glutamate neurons

In the VTA, a population of neurons was described to express mRNA encoding the vesicular glutamate transporter 2 (VGLUT2) (Kawano et al., 2006; Yamaguchi et al., 2007, 2011) and were distributed in the rostral and medial portions of the VTA, outnumbering TH-expressing neurons (Yamaguchi et al., 2007, 2011, 2015). VTA glutamate neurons present relatively short action potential duration and a smaller I_h. In addition, electrical or optical stimulation of the VTA was shown to produce rapid excitatory signalling in the medial PFC (mPFC) (Lavin et al., 2005; Mercuri et al., 1985) or the NAc and ventral pallidum (VP) (Hnasko et al., 2012), respectively. *In vivo* studies showed that VGLUT2-expressing neurons are able to establish asymmetrical excitatory synapses (Wang et al., 2015; Zhang et al., 2015), releasing glutamate (Hnasko et al., 2012; Root et al., 2014a; Wang et al., 2015; Zhang et al., 2015) and highlighting the possible role of VTA glutamate neurons in behaviour.

Combinatorial neurons

Interestingly, some VTA neurons present combinatorial neurotransmitter characteristics, with studies showing that VTA neurons co−release dopamine and GABA, dopamine and glutamate or glutamate and GABA (Berrios et al., 2016; Root et al., 2014a; Zhang et al., 2015). *In vitro* work from Tritsch and colleagues showed that TH-positive neurons were able to release GABA in the striatum, through the vesicular monoamine transporter 2 (VMAT2), in the same pool of vesicles (Tritsch et al., 2012). However, VTA dopaminergic neurons do not express conventional GABA synthesizing enzymes, such as glutamate decarboxylase 1 (GAD1) or 2 (GAD2), suggesting an active re-uptake of GABA in the terminals, mediated by GABA transporter-1 (GAT1) (Kim et al., 2015; Tritsch et al., 2012). The presence of combinatorial dopamine–glutamate neurons were shown in *in vitro* electrophysiological studies that demonstrated glutamate signalling in rat dopamine neuron cultures (Dal Bo et al., 2004; Sulzer et al., 1998) and also *in vivo,* where some NAc projecting TH-positive neurons, showed mRNA expression of the vesicular glutamate transporter-2 (VGLUT2) (Kawano et al., 2006; Yamaguchi et al., 2007, 2011, 2015). Oppositely of what is believed to occur in dopamine-GABA neurons, combinatorial VTA TH- and VGLUT2-expressing neurons were shown to present distinct pools of vesicles for glutamate and dopamine in the axon (Zhang et al., 2015). Glutamate vesicles were located in axon terminals, establishing asymmetric synapses, commonly related with excitatory transmission. In this way, a single dopamine-glutamate axon has the ability to provide fast excitatory glutamate signalling and slower modulatory signalling via dopamine. In addition, a group of non-dopamine neurons within the VTA expresses VGLUT2 together with GAD1, GAD2 and the vesicular GABA transporter (VGAT). A population of these neurons were shown to establish asymmetric and symmetric synapses onto LHb neurons (Root et al., 2014a) in rodents and activation of these combinatorial cells may evoke either fast inhibition that may be followed by excitation or fast excitation that may be followed by inhibition providing pronounced temporal specificity mediated by either glutamate and GABA (Root et al., 2014a).

This heterogeneity in the VTA increased the need to determine the organization of connectivity in the VTA and the functional nature of the neurons in downstream brain regions.

2.3. Connectivity of the VTA

Initial focus on the connectivity of the VTA was put on the brain regions that innervate the VTA. The use of tract tracers enabled the analysis of specific afferents projecting to dopamine neurons and the distribution of VTA dopamine efferents (Morales and Pickel, 2012; Yetnikoff et al., 2014, 2015). Recently, advances in viral vector approaches and the use of transgenic mice facilitated mapping afferents and efferents to and from VTA neurons, together with unveiling the interactions in the microcircuitry of the VTA between dopamine, GABA and glutamate neurons.

2.3.1. Inputs and outputs of VTA neurons

Several studies in either rodents or primates have shown the presence of excitatory (Geisler et al., 2007) and inhibitory connections to VTA neurons (Fig. 2) .

Anatomical electron microscopy and functional electrophysiology studies showed that VTA dopamine neurons receive glutamatergic inputs from the mPFC, the PPT, the LDT, the LHb, the periaqueductal gray (PAG), the bed nucleus of stria terminalis (BNST) and the dorsal raphe nucleus (DRN) (Carr and Sesack, 2000; Charara et al., 1996; Georges and Aston-Jones, 2001; Omelchenko and Sesack, 2005, 2010; Omelchenko et al., 2009; Qi et al., 2014). GABAergic inputs onto VTA dopamine neurons arise from RMT/the "tail" of the VTA, the PAG, the DRN, the lateral hypothalamus (LHT) and the VP (Beier et al., 2015; Hjelmstad et al., 2013; Jhou et al., 2009; Kaufling et al., 2010; Nieh et al., 2015; Omelchenko and Sesack, 2010). Furthermore, local glutamatergic and GABAergic synapses to dopamine neurons were found within the VTA (Dobi et al., 2010; Omelchenko and Sesack, 2009; Tan et al., 2012; van Zessen et al., 2012; Wang et al., 2015). GABA neurons of the VTA receive glutamatergic inputs from the LHb, the mPFC, the PAG, the DRN, the LHT and the BNST (Beier et al., 2015; Bocklisch et al., 2013; Carr and Sesack, 2000; Kudo et al., 2012; Nieh et al., 2015; Omelchenko and Sesack, 2009, 2010). Dominant GABAergic inputs onto VTA GABA neurons arise from NAc medium spiny neurons (MSNs) expressing the D1R (D1-MSN) (Bocklisch et al., 2013; Xia et al., 2011), even though it was initially thought that the NAc provided an extensive input onto VTA dopamine neurons (Beier et al., 2015; Faget et al., 2016; Watabe-Uchida et al., 2012). Also, the DRN, BNST, the PAG and LHT send GABAergic projections to VTA GABA neurons (Beier et al., 2015; Bocklisch et al., 2013; Kudo et

al., 2012; Nieh et al., 2015; Omelchenko and Sesack, 2009, 2010). Cholinergic inputs from the PPT and LDT also target VTA dopamine and GABA neurons (Dautan et al., 2016a; Oakman et al., 1995), where acetylcholine (ACh) was shown to produce an excitatory effect (Mameli-Engvall et al., 2006; Tolu et al., 2013).

Apart from studies of VTA inputs, viral vector tracer circuit-mapping techniques have provided evidence that subpopulations of VTA neurons innervate more that one brain region (Aransay et al., 2015). One of the major targets of VTA dopamine neurons is the NAc (Zhang et al., 2015), specifically the MSNs (a more detailed description on the NAc cell composition is provided ahead). These neurons express DAT, VMAT2 and D2R (Morales and Pickel, 2012; Pickel et al., 1988), and also target the amygdala, cortex, hippocampus, the VP, the PAG, the BNST, the locus coeruleus (LC) and the olfactory tubercle. Combinatorial dopamine-glutamate neurons also target NAc MSNs (Stuber et al., 2010; Tecuapetla et al., 2010; Yamaguchi et al., 2011; Zhang et al., 2015) and cholinergic interneurons (Chuhma et al., 2014; Kabanova et al., 2015) co-releasing dopamine (Zhang et al., 2015); and mPFC parvalbumin (PV)-expressing interneurons (Kabanova et al., 2015; Yamaguchi et al., 2015), resulting in the inhibition of cortical neurons (Kabanova et al., 2015). Dual releasing dopamine-GABA neurons target NAc MSNs as well (Berrios et al., 2016; Kim et al., 2015).

Ultrastructural and electrophysiological findings show the presence of inputs of VTA GABA neurons onto cholinergic interneurons of the NAc (Brown et al., 2012) and onto glutamate neurons of the LHb (Root et al., 2014a), establishing inhibitory synapses. Single axons from glutamate-expressing neurons establish excitatory synapses on glutamate neurons of the LHb (Root et al., 2014a) and PV-expressing interneurons in the NAc (Qi et al., 2016).

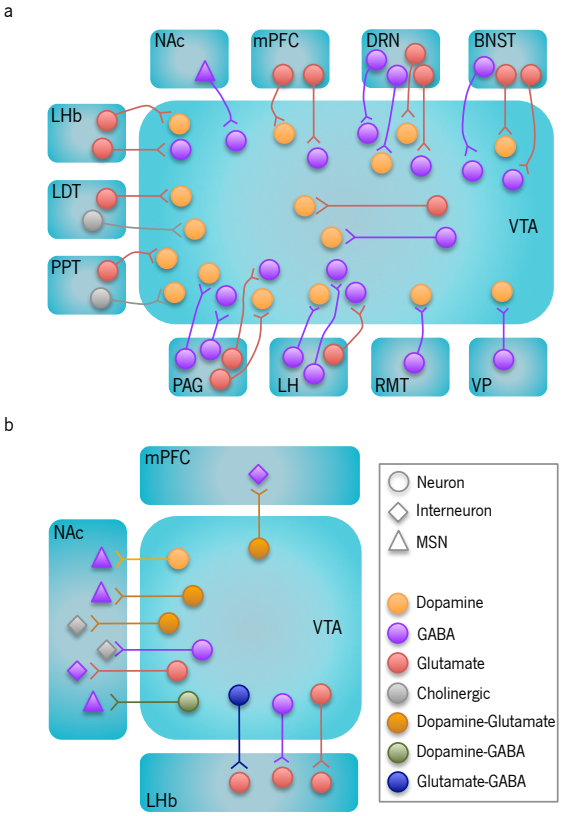

Figure 2- Inputs and outputs from ventral tegmental area neurons. a) VTA dopamine neurons receive glutamatergic inputs from the mPFC, DRN, BNST, LHb, LDT, PPT and PAG. GABAergic inputs from the VP, RMT, LH, PAG and DRN also innervate VTA dopamine neurons. Local glutamate and GABA neurons synapse onto VTA dopamine neurons. Glutamatergic inputs from the LHb and mPFC and GABAergic inputs from the NAc medium spiny neurons (MSNs) project to VTA GABA neurons. In addition, glutamatergic and GABAergic neurons from the PAG, DRN, LHT and BNST project to VTA GABA neurons. b) VTA dopamine neurons establish symmetric synapses on MSNs in the NAc. The combinatorial dopamine–glutamate neurons target NAc MSNs, NAc cholinergic interneurons and mPFC GABAergic interneurons. The combinatorial dopamine–GABA neurons target NAc MSNs. Glutamate neurons in the VTA target glutamate neurons of the LHb and NAc interneurons. VTA GABA neurons target cholinergic interneurons of the NAc and glutamatergic neurons of the LHb, as well. VTA combinatorial glutamate–GABA neurons target glutamate neurons of the LHb.

Amy: amygdala; BNST: bed nucleus of the stria terminalis; DRN: dorsal raphe nucleus; Hipp: hippocampus; LDT: laterodorsal tegmentum ; LHb: lateral habenula; LH: lateral hypothalamus; mPFC: medial prefrontal cortex; NAc: nucleus accumbens; PAG: periaqueductal grey; PPT: pedunculopontine tegmentum; RMT: rostromedial tegmental nucleus; VP: ventral pallidum; VTA: ventral tegmental area.

2.4. Role of VTA circuitry in behaviour

The majority of studies have focused on the VTA dopamine neurons, considering that stimulation of these neurons and the release of dopamine in projection sites, most notably the NAc, is known to produce reward (Schultz, 2015; Schultz et al., 1997). In fact, VTA dopamine neurons play a role in reward-related learning for natural outcomes, such as food, or addictive substances, such as alcohol or drugs of abuse (Liu et al., 2012; Schultz, 1986). In a classical set of studies Schultz and colleagues showed that putative dopamine neurons are activated in response to unpredicted rewards during reward delivery in a cue-reward association task (CS). Following training, dopamine neurons increase firing rate, shifting activation, following the CS and not the reward and transiently decrease firing when an expected reward is omitted (Bromberg-Martin et al., 2010; Cohen et al., 2012; Eshel et al., 2016; Matsumoto and Hikosaka, 2009; Schultz, 2007; Schultz et al., 1997). Dopamine neurons also increase their firing when a surprising unexpected reward is presented. This type of response is termed reward prediction error signal (Lerner et al., 2015; Schultz, 2007; Schultz et al., 1997). In addition, it has been shown that dopamine is involved in learning not only for rewards, but also punishments or aversion (Brischoux et al., 2009; Joshua et al., 2008), since they can be excited or inhibited by aversive stimuli or by cues that predict an aversive outcome (Brischoux et al., 2009; Matsumoto and Hikosaka, 2009; Mileykovskiy and Morales, 2011; Schultz, 2007) and have also been implicated in the processing of stressful events (Chaudhury et al., 2013; Tye et al., 2013). However, the response of putative dopamine neurons to either a reward or punishment is not always consistent. Application of an aversive stimulus, like hind paw pinches, induced a significant increase in the firing rate on the majority of TH-expressing neurons, whereas 20% of these neurons decreased their activity (Brischoux et al., 2009). Additionally, depending on the intensity of punishment (pinch or electric shock) or even reward (sugar pellet or sucrose solution), the same neuron can respond differently (Joshua et al., 2008; Lak et al., 2014; Stauffer et al., 2016).

Recent optogenetic studies have made it possible to determine the role of specific neuronal cell types in reward-related behaviours. Optogenetic tools allow direct control of a defined subset of neurons in freely moving animals through the expression of light-activated channels that increase (channelrhodopsin, ChR2) or inhibit (archaerhodopsin; hallorhodopsin) neuronal activity (Deisseroth, 2011). This technique was shown to be essential in identifying circuits necessary for

various aspects of reward and addiction (Stuber et al., 2012). General local activation of VTA dopamine neurons was shown to be rewarding (Ilango et al., 2014; Kim et al., 2012; Tsai et al., 2009; Witten et al., 2011). In an earlier study, phasic optical stimulation of VTA dopamine neurons alone was sufficient to drive intracranial self-stimulation in rodents (Witten et al., 2011). The rewarding effects of optogenetic stimulation of these neurons were further confirmed by inducing preference in both contingent and non-contingent conditioned place preference paradigma, and increased dopamine release in the NAc (Tsai et al., 2009). Additionally, in a food-seeking operant task, phasic activation of VTA dopamine neurons causally enhances positive reinforcing actions in freely moving mice (Adamantidis et al., 2011). The role of activation of these neurons in reward behaviours relevant to addiction was also assessed (Pascoli et al., 2015). Pascoli and colleagues showed that mice that acquired self-stimulation for burst firing of VTA dopamine neurons, decreased self-stimulation upon injection of cocaine, in a dose-dependent manner, suggesting that the reinforcing effects of self-stimulation rely on the same circuits as drugs of abuse. Interestingly, in a follow-up study, after a period of abstinence, mice developed cue-induced seeking behaviour and changes in the NAc synaptic plasticity similar to the ones observed in the same period after cocaine self-administration (Pascoli et al., 2014; Wolf, 2016).

Of the various inputs onto VTA dopamine neurons, excitatory inputs from LDT neurons onto VTA dopamine neurons projecting to the lateral shell of the NAc (Lammel et al., 2012) seem to be particularly involved in motivated behaviour. General optogenetic stimulation of LDT inputs to mesoaccumbens-projecting VTA dopamine neurons (Lammel et al., 2012) or specific optical stimulation of glutamatergic or cholinergic inputs from VGLUT2-expressing or ChAT-expressing LDT neurons to the VTA, respectively (Steidl et al., 2017a), produces a conditioned placed preference. Contrastingly, optogenetic activation of LHb inputs to mPFC-projecting VTA dopamine neurons induces conditioned place aversion (Lammel et al., 2012).

Table 1. Outcome of VTA circuits on motivated behaviour (reward determined by place preference or instrumental behaviour; aversion determined by place avoidance)

Input	VTA neuronal population	Output	Behavioural outcome	Reference
Dopaminergic source				
-	Dopamine	NAc	Reward	Kim et al. (2012) Owesson-White et al. (2016) Steinberg et al. (2014) Witten et al. (2011) Tsai et al. (2009)
Glutamatergic source				
LHb	Dopamine	mPFC	Aversion	Lammel et al. (2012)
LHT	General	Unknown	Aversion	Nieh et al. (2016)
BNST	General	Unknown	Aversion	Jennings et al. (2013)
LDT	Dopamine	NAc (shell sub-region)	Reward	Lammel et al. (2012) Xiao et al. (2016) Dautan et al. (2016b)
PPT	Dopamine Non-Dopamine	NAc -	Reward	Yoo et al. (2017) Yau et al. (2016
DRN	Dopamine	NAc	Reward	Geisler et al. (2007) Qi et al. (2014)
Anterior cortex	Dopamine	NAc (shell sub-region)	Reward	Beier et al. (2015)
-	Glutamate	LHb (VGLUT-neurons)	Aversion	Root et al. (2014) Lammel et al. (2015)
-	Glutamate	NAc (PV-interneurons)	Aversion	Qi et al. (2016)
VTA	Dopamine	NAc	Reward	Wang et al. (2015)
GABAergic source				
LHT	GABA	VTA (Dopamine neurons)	Reward	Barbano et al. (2016) Nieh et al. (2016)
-	GABA	NAc	No disruption	Brown et al. (2012)
-	GABA	VTA (Dopamine neurons)	Aversion	Tan et al. (2012) Van Zessen et al. (2012)

Table 1 (continuation). Outcome of VTA circuits on motivated behaviour (reward determined by place preference or instrumental behaviour; aversion determined by place avoidance)

Input	VTA neuronal population	Output	Behavioural outcome	Reference
GABAergic source				
BNST	General	Unknown	Reward	Jennings et al. (2013)
-	TH-GABA Combinatorial neurons	NAc	Reward	Berrios et al. (2016)
-	TH-GABA Combinatorial neurons	LHb (VGLUT-neurons)	Reward	Stamatakis et al. (2013)
Cholinergic source				
LDT	Dopamine	NAc (shell sub-region)	Reward	Lammel et al. (2012) Steidl et al. (2016) Xiao et al. (2016) Dautan et al. (2016b)
PPT	Dopamine Non-Dopamine	NAc -	Reward	Yoo et al. (2016) Yau et al. (2016)

Glutamatergic inputs from VGLUT3-expressing neurons of the DRN establish monosynaptic connections onto mesoaccumbens-projecting dopamine neurons (Qi et al., 2014) and selective activation of these neurons elicits glutamate release, increasing the firing of mesoaccumbens-projecting dopamine neurons, leading to an increase in dopamine release in the NAc. This activation is able to induce optical intracranial self-stimulation in mice. In addition, cortical glutamatergic inputs onto mesoaccumbens-projecting VTA dopamine neurons are sufficient to produce reward in mice, since activation of these inputs also induces self-stimulation (Beier et al., 2015).

Oppositely, stimulation of VTA GABA neurons disrupted reward consumption and promoted conditioned place aversion and inhibition of these neurons produces preference (Tan et al., 2012; van Zessen et al., 2012) . The role of these GABA neurons in reward and aversion is thought to induce inhibition of VTA dopamine neuronal activity and, consequently, produce a concomitant decrease in dopamine release in the NAc (van Zessen et al., 2012). Regarding the outputs of VTA GABA neurons, recent studies have shown that activation of mesoaccumbens-projecting VTA GABA

neurons elicits GABA release without producing reward or aversion (Brown et al., 2012). Interestingly, suggesting a role in associative learning, a population of VTA GABA neurons synapse onto NAc cholinergic interneurons and brief optical activation pauses the spontaneous activity of cholinergic interneurons and enhance discrimination of a stimulus associated with an aversive outcome (Brown et al., 2012). The inhibitory inputs from the LHT onto VTA GABA neurons was shown to induce conditioned place preference, decreasing the activity of these neurons and increasing dopamine release in the NAc (Barbano et al., 2016; Nieh et al., 2016). Conversely, activation of excitatory LHT inputs to the VTA elicits conditioned place avoidance (Nieh et al., 2016). Similar to what occurs from general activation and inhibition of VTA GABA neurons, optical activation of glutamatergic inputs from the BNST to the VTA also induces aversion and optical activation of VTA-projecting BNST GABA neurons elicits reward in mice (Jennings et al., 2013). Altogether, this seems to suggest that VTA GABA neurons also present functional heterogeneity, especially dependent on the neuronal connectivity, and play a role in various behaviours.

Selective activation of VTA glutamate interneurons synapsing onto VTA dopamine neurons was shown to induce conditioned place preference and also reinforce instrumental behaviour (Wang et al., 2015). However, activation of mesoaccumbens-projecting glutamate neurons induces aversion, by activating PV-expressing interneurons and increasing GABA release onto NAc MSNs (Qi et al., 2016). Additionally, the same effect is seen when activating VTA glutamate neurons projecting onto the LHb (Lammel et al., 2015; Root et al., 2014b).

Studies have also shown the importance of VTA combinatorial neurons in reward-related behaviours. Interestingly, mice depleted of VGLUT2 in DAT-expressing neurons displayed blunted locomotor responses to amphetamine (Birgner et al., 2010) or cocaine (Hnasko et al., 2010) injections. Additionally, these animals were shown to self-administer both cocaine and high sucrose food and increased cocaine seeking maintained by drug-associated cues (Alsiö et al., 2011). Loss of combinatorial VTA dopamine- and glutamate-releasing neurons projecting to the mPFC was shown to induce cortical desinhibition and increase perseverative behaviour (Kabanova et al., 2015).

Altogether, these studies show the importance of the VTA and its circuits and how it is involved in many functionally distinct phases that are involved in reinforcement, from working memory to outcome evaluation (Morales and Margolis, 2017).

2.5. Neuroanatomy of the nucleus accumbens

As described above, a major reward player and output of VTA dopaminergic neurons is the NAc that together with the olfactory tubercle comprises the ventral striatum. In primates, the striatum (Str) is easily divisible into the caudate nucleus and putamen, separated by a white matter tract, the internal capsule. However, in rodents, such clear anatomical divisions have not been observed. With a combination of antibodies (calretinin, calbindin and ChAT), the dorsal and the ventral Str, the NAc core and shell, can be clearly separated (Cragg et al., 2002). The NAc shell was described as a complex region with higher neuroanatomical diversity than the core, and also receiving greater inputs from the thalamus and the dopaminergic midbrain (Bossert et al., 2007; Voorn et al., 2004). At a molecular level, core and shell show differences in the distribution of a number of substances and receptors, including serotonin (Deutch and Cameron, 1992), dopamine (Patel et al., 1995) and calretinin (Prensa et al., 2003), with these substances being preferentially located in the shell. Additionally, evidences seem to suggest that the core is the main dopaminergic target of the NAc, since it receives more dense inputs from the VTA and the SN (Mogenson et al., 1980; Phillipson and Griffiths, 1985).

The NAc is a structure with a heterogeneous neural composition, with complex interconnections and a wide variety of receptors. In adult rats, the Str contains approximately 1,500,000 neurons in each hemisphere (Fentress et al., 1981).

Medium spiny neurons

The primary population of striatal neurons is the small to medium sized GABAergic MSNs, with less than 20 μm in diameter (Mehler, 1981; Ramón y Cajal, 1909). These projection neurons, also known as spiny projection neurons, are the only output (Bolam, 1984) of the Str and represent up to 95% of the total population. MSNs are traditionally subdivided into two distinct subtypes, D1-MSNs and D2-MSNs, depending on the molecules they express; MSNs that express D1R, substance P and dynorphin, and MSNs that express D2R, adenosine receptor 2a (A2aR), and enkephalin (Gerfen, 1992; Heiman et al., 2008; Kawaguchi, 1997; Lobo et al., 2006) (nicely reviewed in (Soares-Cunha et al., 2016a). In rats, approximately 6% of all MSNs in the Str express both D1R and D2R, allowing the potential to modulate the basal ganglia network bi-directionally (Perreault et al., 2012).

MSNs bear a large number of spines on their dendrites and receive synaptic input from several structures, such as cortex, thalamus, hippocampus and dopaminergic neurons of the midbrain (Chuhma et al., 2011; Wall et al., 2013). Cortical inputs to the Str activate both D1- and D2-MSNs resulting in different behaviours (DeLong and Wichmann, 2007; Huerta-Ocampo et al., 2014).

Classical subdivision of MSNs arise from their axonal targets from the dorsal Str: striatonigral MSNs that directly project to different output nuclei of the basal ganglia, as the globus pallidus internal (GPi), SN and VTA and is considered to be the direct pathway; and the striatopallidal MSNs, that indirectly project to those output nuclei via projections to the globus pallidus external (GPe) and STN, appropriately named the indirect pathway (Gerfen, 1992; Nicola, 2007).

However, in the NAc, this separation of D1-MSNs and D2-MSNs in direct/indirect pathway is not as accurate: the direct pathway is comprised by projections to the ventral mesencephalon (SN and VTA) and then to the mediodorsal thalamus (MDT) and is mediated by D1-MSNs only; whereas the indirect circuit encompasses NAc projecting neurons to the VP and STN before reaching the ventral mesencephalon and have contributions of both D1- and D2-MSNs (Kupchik et al., 2015; Lu et al., 1997). The core part of the NAc projects to the dorsolateral VP and SN and the shell to the ventromedial VP (vmVP) and VTA (Zahm and Heimer, 1990). In the NAc, considering these projections it seems reasonable to believe that both D1- and D2-expressing neurons can inhibit/disinhibit thalamic activity, contrary to dorsal Str (Kupchik et al., 2015).

The remaining 5% of Str neurons are interneurons (Graveland and DiFiglia, 1985) which play an important role in basal ganglia function and regulation, by directly or indirectly controlling MSN excitability, and affecting behaviour (Gittis and Kreitzer, 2012; Tepper et al., 2010; Tritsch and Sabatini, 2012).

GABAergic interneurons

The second biggest population of striatal neurons is the GABAergic interneurons, which consist of several subpopulations based on expression or co-expression of molecular markers (Tepper et al., 2010). This population can be subdivided into three distinct subtypes of GABAergic in the Str: 1) fast spiking interneurons (FSI) expressing the calcium-binding protein PV; 2) low-threshold spiking interneurons expressing the neuropeptide Y (NPY), the neuropeptide somatostatin

(SOM) and nitric oxide synthase (NOS) and 3) low-threshold calcium spike interneurons expressing the calcium channel marker calretinin (CR) (Bennett and Bolam, 1994; Kawaguchi, 1997; Kubota et al., 1993; Vincent et al., 1983).

PV-expressing interneurons display a very fast phasic firing rate with short spike duration (Bean, 2007; Sharott et al., 2012). A single FSI can establish synapses onto a large population of MSNs, exerting a strong control over striatal output neurons (Kita, 1993).

Low-threshold spiking interneurons present a medium-sized soma (English et al., 2012; Hope et al., 1991) with three to five aspiny dendrites, which extend over 600 µm diameter (Aoki and Pickel, 1988).

The last main groups of GABAergic interneurons are the CR-expressing interneurons. This population consists of medium sized aspiny neurons, with thin processes and a diameter between 12-20 µm (Bennett and Bolam, 1993). Other striatal interneurons have been observed and recorded recently, such as TH+ interneurons (Busceti et al., 2008; Dubach et al., 1987), cholecystokinin (CCK) or vasoactive intestinal polypeptide (VIP) expressing interneurons (Adams and Fisher, 1990; Takagi et al., 1984). However, these represent a very small amount of neurons and little information is available about them.

Cholinergic interneurons (CINs)

The third striatal cell population is the large aspiny CINs often referred to in humans/monkeys as tonically active neurons (TANs). Originally described as the only cholinergic source of ACh in the Str (Woolf and Butcher, 1986), the CINs constitute up to 2% of the striatal neurons (Bolam, 1984) and can innervate the entire Str (Contant et al., 1996; Oorschot, 1996; Woolf and Butcher, 1981). CINs present a large soma of up to 40 µm, making them anatomically easily distinguishable from other striatal neurons. CINs establish synaptic contacts with MSNs and other cholinergic interneurons (English et al., 2012) and display a tonic firing rate of 3 to 10Hz (Wilson et al., 1990; Witten et al., 2011), a I_h current (Deng et al., 2007) and a long action potential duration (Threlfell et al., 2012). Additionally, pre-synaptic contacts have been observed on dopaminergic and glutamatergic synapses in the Str (Nelson et al., 2014; Oldenburg and Ding, 2011).

Despite their sparse number, interneurons can exert a very significant role in the control of striatal circuit activity and establishment of behavioural conditioning in vivo (Pakhotin and Bracci, 2007; Tepper and Bolam, 2004; Tepper et al., 2010; Threlfell et al., 2012).

2.6. Inputs and outputs of the NAc

The major neural input to the striatum is excitatory. Retrograde tracer studies suggested that all regions of the cortex target direct and indirect pathway neurons (Wall et al., 2013) as well as interneurons (Ding et al., 2010; Reynolds et al., 2004). Cortical regions projecting to the NAc are the sensory cortex, the motor cortex and the parietal cortex (Goldman and Nauta, 1977; McGeorge and Faull, 1989). Glutamatergic projections from all cortical areas, from the thalamus, hippocampus and from the amygdala have been observed (Kelley, 1982). Other inputs arise from the SN/VTA, providing a strong dopaminergic input, the DRN, locus coeruleus or the brainstem (Beier et al., 2015; Flaherty and Graybiel, 1994; Pan et al., 2010) and hypothalamus. Additionally, despite what was initially thought, the NAc receives a major cholinergic input from the LDT (Dautan et al., 2014, 2016b). Cholinergic neurons arising in the LDT were shown to preferentially innervate the medial Str and the NAc, forming asymmetric excitatory synapses. This may suggest that the difference between the activity of cholinergic interneurons and cholinergic neurons of the brainstem during reward-related paradigms may contribute differently in the processing of information in the NAc.

MSNs constitute the only output of the NAc and target the GPe, the GPi and the SN and VTA.

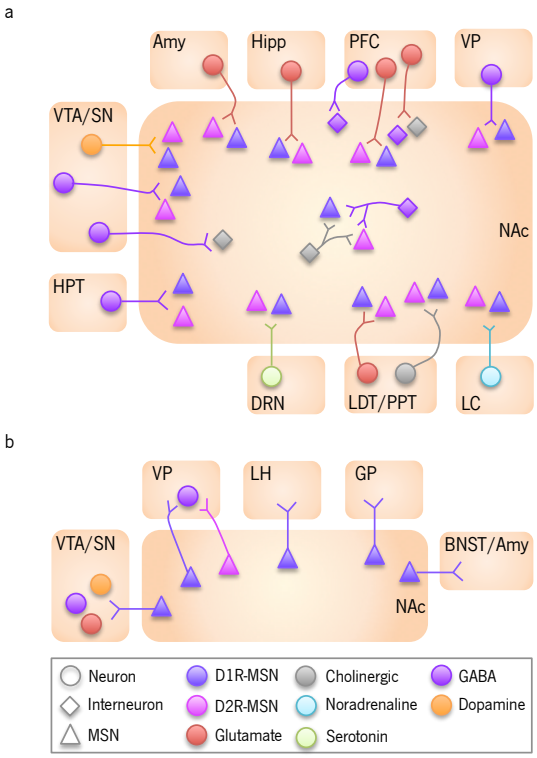

Figure 3 - Connectivity of the nucleus accumbens. a) Inputs: NAc dopamine 1 receptor (D1R) and dopamine 2 receptor (D2R) MSNs receive dopaminergic inputs from the VTA and glutamatergic inputs from the PFC, Hipp, Amy, LDT and PPT. GABAergic inputs from the VP, PFC, VTA, SN and HPT innervate NAc neurons. In addition, noradrenergic and serotonergic neurons project to NAc MSNs from the DRN and LC, respectively. NAc cholinergic or GABAergic interneurons are able to modulate MSN activity and impact behaviour outcome. b) NAc MSNs are the only output neurons: D1R-MSNs establish direct connections with VTA, VP, LH, GP, BNST and Amy; however NAc D2R-MSNs only project to the VP and, together with D1R-MSNs, indirectly modulate the reward system.

Amy: amygdala; BNST: bed nucleus of the stria terminalis; DRN: dorsal raphe nucleus; GP: globus pallidus; Hipp: hippocampus; HPT: hypothalamus; LC: locus coeruleus; LDT: laterodorsal tegmentum ; LHb: lateral habenula; LH: lateral hypothalamus; MSN: medium spiny neuron; NAc: nucleus accumbens; PFC: prefrontal cortex; PPT: pedunculopontine tegmentum; RMT: rostromedial tegmental nucleus; SN: substantia nigra; VP: ventral pallidum; VTA: ventral tegmental area.

2.7. Role of NAc in reward-related behaviours

The NAc is a subcortical brain structure crucial for motivation (Da Cunha et al., 2012) that appears to be highly conserved across the evolution of several species (Stephenson-Jones et al., 2011), indicating a fundamental functional role. Dysfunctions of the NAc have been described in a wide variety of human neurological and psychological disorders, such as addiction (Koob, 1992; Koob and Volkow, 2010), obsessive-compulsive disorder (Chudasama and Robbins, 2006), depression (Nestler and Carlezon, 2006) or eating disorders (Berridge et al., 2010; Norgren et al., 2006).

General activation of the direct pathway in the NAc MSNs modulates reward-related learning (Kravitz et al., 2012; Lobo et al., 2010), whereas activation of the indirect pathway increases aversive association (Danjo et al., 2014; Hikida et al., 2013).

It has been reported that MSNs in the NAc core seem to be critical for conveying motivational value to discrete stimuli associated with reward or aversion, as well as updating these values when a change occurs. Meanwhile, in the NAc shell, MSNs drive behavioural responses to repeated exposure to rewarding experiences, such as chronic drug administration (Meredith et al., 2008).

In the context of aversive stimuli, the NAc core seems to be involved in learning to identify the cues of aversive stimuli in order to avoid them, whereas the NAc shell is responsible to define or signal safety periods between aversive cues (Feja et al., 2014; Fernando et al., 2013). Therefore, this dissociable functionality can contribute for avoidance and approach towards an intended goal.

Besides anatomical compartmentalization, the two classes of MSNs also seem to play different roles in reward and aversion. Pioneering studies have proposed that D1-MSNs encode reward and D2-MSNs aversion.

The reinforcing properties of D1-MSN activation has been elegantly described using natural rewards and drugs of abuse: rodents showed increased motivation to work for food and higher sensitization for cocaine (Lobo et al., 2010; Soares-Cunha et al., 2016b). Activation of D1-MSNs during cocaine exposure was sufficient to induce CPP to a sub-threshold dose of cocaine, while D2-MSN activation produced the opposite result, decreasing CPP (Chandra et al., 2013; Lobo et al., 2010). In line with this, Chandra and colleagues suppressed cocaine sensitization by optogenetically inactivating D1-MSNs (Chandra et al., 2013). Conflicting results arise when it

comes to the role of D2-MSNs. Stimulation of NAc D2-MSNs resulted in attenuation of cocaine conditioned place preference and suppression of cocaine self-administration (Bock et al., 2013; Lobo et al., 2010). Contradicting this function, Song and colleagues showed that acquisition and cocaine sensitization was not affected by activation of D2-MSNs (Song et al., 2014). The differential effects observed in cocaine-related behaviour may be explained by drug-induced synaptic changes that can be cell type-specific, as are their outputs (Smith et al., 2013).

Most of the available studies focused on the role of the two MSNs subpopulations in a drug context. Yet, in naive conditions, the role of D1- and D2-MSNs appears to be remarkably more complex. For example, optogenetic activation of D1- or D2-MSN did not generate place preference *per se*, as opposing VTA dopamine neuron optical activation (Lobo et al., 2010). In addition, our group has shown that both D1- and D2-MSN activation strongly enhanced motivation to work for food in rodents, suggesting a concurrent role rather than opposing (Soares-Cunha et al., 2016b).

Furthermore, cocaine exposure simultaneously potentiated D1-MSN NAc to VP output, leading to sensitization of cocaine-induced locomotion, but depressed D2-MSN NAc to VP output (Creed et al., 2016), leading to anhedonia caused by drug withdrawal. This suggests that NAc D1- versus D2-MSN efferents to VP may regulate distinct behavioural states (Creed et al., 2016), highlighting the differential impact of D1- versus D2-MSN circuits. Interestingly, in non-cocaine exposed animals, potentiation of D2-MSN to VP synapses lead to better performance on a PR task and to more time spent in the reward zone of a free access task. This may imply that D2-MSN to VP synapses modulates the affective state and motivation for natural reward.

Table 2. Behavioural outcomes of optogenetic manipulation of NAc circuits

Region	Cell manipulation	NAc cell subtype	Behavioural outcome	Reference
VTA	Dopaminergic Activation	General	Promotes reward	Adamantidis et al. (2011) Kim et al. (2012) Owesson-White et al. (2016) Steinberg et al. (2014) Witten et al. (2011) Tsai et al. (2009)
VTA	Glutamatergic Activation	General	Reward	Wang et al. (2015)
VTA	GABAergic Activation	Cholinergic interneurons	Increased discrimination of motivational stimuli	Brown et al. (2012)
mPFC	Non-specific Activation	General	Decreased cocaine seeking reinstatement	Stefanik et al. (2013)
Hipp	Glutamatergic Activation	D1-MSNs	Promotes reward	Britt et al. (2012) Pascoli et al. (2014)
Hipp	Glutamatergic Inhibition	General	Decreased cocaine-induced locomotion	Britt et al. (2012)
Amy	Glutamatergic Activation	General	Promotes self-stimulation	Stuber et al. (2011) Britt et al (2012)
Amy	Glutamatergic Inhibition	General	Reduces responding to a sucrose reward Inhibits cocaine reinstatement	Stuber et al. (2011) Stefanik and Kalivas (2013)
Amy	Non-specific Activation	General	Promotes and increases motivation	Robinson et al. (2014)
NAc	Non-specific Inhibition	General	Attenuates reinstatement	Stefanik et al. (2013b)
NAc	Cholinergic Inhibition	Cholinergic interneurons	Reduced drug conditioned place preference	Witten et al. (2010)
NAc	GABAergic Activation	D2R-MSNs	Blocks reward, inhibiting cocaine preference and attenuating sensitization during withdrawal	Lobo et al. (2010) Chandra et al. (2013) Song et al. (2014)
NAc	GABAergic Activation	D1R-MSNs	Promotes reward, enhancing cocaine preference and induced locomotion	Lobo et al. (2010) Chandra et al. (2013)

2.8. Neuroanatomy of LDT

Comparative studies of the anatomy of the cholinergic brainstem show that it has a similar construction and pattern of connections in all vertebrate species. This region in humans, non-human primates, and rodents contain neurons expressing the neurotransmitters ACh (Hirsch et al., 1987), GABA, glutamate (Barroso-Chinea and Bezard, 2010; Martinez-Gonzalez et al., 2011, 2012; Mena-Segovia et al., 2009; Wang and Morales, 2009), and glycine (Pienaar et al., 2013). Classically defined by the outline of the cholinergic neuron population, comprising the cholinergic group Ch6, the LDT has similar characteristics with respect to cellular composition and connectivity with the PPT, the cholinergic group Ch5, forming the mesopontine tegmentum (Armstrong et al., 1983; Mesulam et al., 1983; Satoh and Fibiger, 1986). Both areas, although in different proportions, contain the three main neuronal populations, cholinergic, GABAergic and glutamatergic neurons (Ford et al., 1995; Lavoie and Parent, 1994; Mena-Segovia et al., 2009; Wang and Morales, 2009). Distribution of the neuronal subtypes is not homogeneous in either region and it is estimated that cholinergic neurons account for approximately 23% of all neurons in the PPT and 32% in the LDT (Wang and Morales, 2009).

The LDT is a small nucleus bordered dorsally by the fourth ventricle (V4) and ventrally by the cerebropontine formation and is positioned caudally of the PPT (Cornwall et al., 1990; Maley et al., 1988; Mesulam et al., 1986; Woolf, 1991). The LDT contains a ventral part with acetylcholine esterase (AChE) -positive cells that extend into the fibrous tegmentum ventral to the periaqueductal gray. The medial portion area presents the most concentrated cholinergic presence (32% of total neurons), although the number of the other two cell types was found to be similar in this sub-region (Wang and Morales, 2009). Curiously, despite considering cholinergic neurons the principal cell type within the LDT, they do not represent the predominant cell type. Glutamatergic and GABAergic neurons represent the majority of cells in the LDT (Ford et al., 1995; Wang and Morales, 2009) with the highest percentage present in the caudal LDT. Oppositely, the highest percentage of glutamate containing neurons is present in the rostral LDT. Despite initial studies claiming that GABA or glutamate could co-express in a subset of the population of cholinergic LDT cells (Clements et al., 1991; Jia et al., 2003; Lavoie and Parent, 1994), this did not necessarily implied co-release (Stamatakis et al., 2013). More recent data has questioned this and it is now believed that the GABAergic, glutamatergic and cholinergic neurons represent distinct populations (Wang and Morales, 2009).

2.9. Functional connectivity of the LDT

The cholinergic brainstem topographically innervates the midbrain, the striatal complex and the thalamus. Whole cell recordings of cholinergic neurons in the LDT performed *in vitro* showed two types of cholinergic firing patterns: after a current injection, a major group of cholinergic cells presented regular activity and another group a bursting activity (Kamii et al., 2015). Different reports *in vivo* showed that putative cholinergic neurons have a short spike duration (<1ms) and a large range of firing rates (0.08 to 5Hz), while non-cholinergic neurons have a long spike duration (±2ms) and a regular firing frequency (2Hz) (Koyama et al., 1999; el Mansari et al., 1989, 1990). In fact, many studies explore the firing activity of LDT neurons and their role in controlling states of arousal, wakefulness and rapid eye movement (REM) sleep, specially, during cortical activation.

2.9.1. Inputs of the LDT

Using retrograde and anterograde tracing techniques, major efferents have been identified originating in the mPFC, the reticular formation, the medial preoptic nucleus, the hypothalamus, basal forebrain, locus coeruleus, dorsal raphe and lateral habenula projecting to the LDT (Fig.4) (Satoh and Fibiger, 1986; Semba and Fibiger, 1992). Additionally, LDT cholinergic neurons receive sensory input from the superior colliculus and respond to sensory stimuli (Koyama et al., 1994; Satoh and Fibiger, 1986).

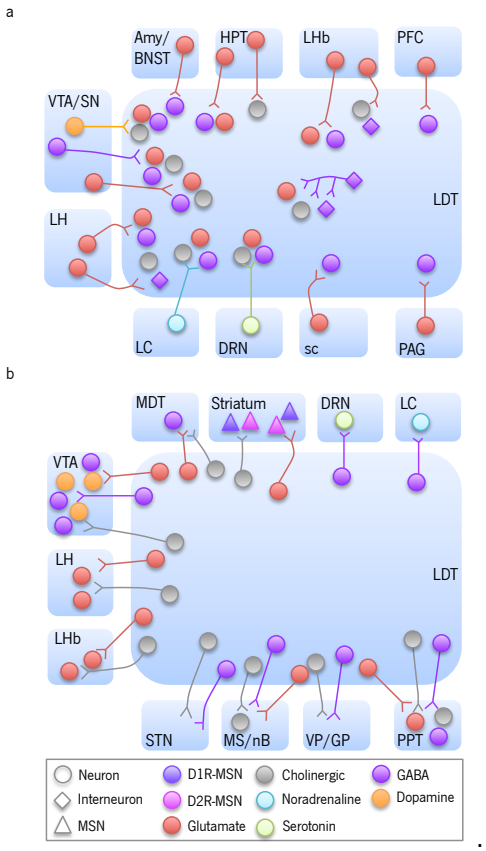

Figure 4 - Inputs and outputs to and from the laterodorsal tegmental nucleus. a) LDT neurons receive excitatory inputs from the PFC, LHb, LH, HPT, Amy/BNST, sc, VTA and SN. In addition, the LC and DRN project to LDT neurons. Local GABAergic SOM- or PV- expressing interneurons are able to modulate LDT activity. VTA GABA neurons also synapse onto LDT neurons. LHb and LH glutamate neurons establish connections with LDT GABAergic interneurons. b) LDT GABA neurons target the PPT, VP, GP, MS, nB, VTA, DRN and LC. Cholinergic neurons send projections to the regions mentioned as well as the LH, LHb, MDT and more recently reported, the striatum (dorsal and ventral parts). LDT glutamate neurons project to the PPT, LHb, LH, VTA, MS, nB, MDT and the striatum.

Amy: amygdala; BNST: bed nucleus of the stria terminalis; DRN: dorsal raphe nucleus; GP: globus pallidus; LDT: laterodorsal tegmentum; LHb: lateral habenula; LH: lateral hypothalamus; MDT: medialdorsal thalamus; MS: medial septum; nB: nucleus of Broca; PAG: periaqueductal grey; PFC: prefrontal cortex; PPT: pedunculopontine tegmentum; sc: superior colliculus; STN: subthalamic nucleus; VP: ventral pallidum; VTA: ventral tegmental area.

2.9.2. Outputs of the LDT

The LDT preferentially targets the VTA dopaminergic mesoaccumbens neurons and GABAergic mesoaccumbens or mesocortical projections (Fig. 4) (Gould et al., 1989; Oakman et al., 1995; Omelchenko and Sesack, 2005; Woolf and Butcher, 1986). This indicates that neurons from this region may be involved in dopamine processing of reward, goal-directed approach behaviours and development of motivation underlying drug abuse (Winn, 2006, 2008). Interestingly, although apparently similar in physiology and transmitter content, detailed studies have revealed some divergent projection patterns arising from the LDT when compared to those of the PPN. Such divergent projections may underlie differing neurophysiological roles of these nuclei in control of diverse behaviours (Clements et al., 1991; Ford et al., 1995; Jia et al., 2003; Kayama et al., 1992; Lavoie and Parent, 1994; el Mansari et al., 1989; Satoh et al., 1983; Semba and Fibiger, 1992; Steriade and McCarley, 1990).

Apart from targeting the VTA mesoaccumbal and mesocortical neurons (PBN and Rli) (Lammel et al., 2012), the LDT also innervates the dorsomedial and ventral striatum (Dautan et al., 2014) and the medial thalamic groups (Cornwall et al., 1990). A significant number of glutamatergic and cholinergic projecting neurons in the LDT target the VTA, leading to an excitatory response and increase in the dopaminergic cell activity (Clements and Grant, 1990; Lammel et al., 2012; Lodge and Grace, 2006; Oakman et al., 1995). Interestingly, cholinergic neurons have been described to innervate multiple target structures through divergent axonal branches (Bolton et al., 1993; Cornwall et al., 1990; Jourdain et al., 1989; Losier and Semba, 1993; Semba and Fibiger, 1992). However, only a small percentage of cholinergic LDT neurons collateralize to ventral thalamic nuclei and the VTA, with the majority of cholinergic neurons sending projections to one nucleus or another (Holmstrand and Sesack, 2011).

Additionally, LDT terminals are present, specifically, in the cholinergic basal forebrain including the VP, the medial septum, the nucleus of Broca, the cingulate cortex, the thalamus, the LHb, the globus pallidus and the STN (Bevan et al., 1995; Cornwall and Phillipson, 1989; Eid et al., 2016; Gonzalo-Ruiz et al., 1995; Holmstrand and Sesack, 2011; Holmstrand et al., 2010; Semba and Fibiger, 1992).

Altogether, the pattern of connectivity seems to point the LDT to preferentially target circuits associated with limbic functions

2.10. Neuromodulatory role of LDT in reward and aversion

With the exception from the role in sleep and wake functions, the LDT is described as having an important role in limbic functions, including reward behaviours (Kamii et al., 2015; Lammel et al., 2012; Lester et al., 2008; Nelson et al., 2014). Lesions of the LDT have been shown to induce changes in the neurochemical responding within the mesocorticolimbic reward pathway. Blaha and colleagues showed that neostigmine-induced, an AChE blocker, increase of NAc dopamine levels were attenuated in LDT-lesioned animals (Blaha et al., 1996). Lesions of the LDT were shown to decrease locomotion in basal conditions (Dobbs and Mark, 2012), as well as attenuate drug-induced locomotion, like nicotine- and amphetamine-induced locomotion (Alderson and Winn, 2005; Dobbs and Cunningham, 2014; Laviolette et al., 2000). Stereotypical behaviours were increased in LDT-lesioned animals after exposure to nicotine (Forster and Blaha, 2000; Ishibashi et al., 2009) and amphetamine (Forster et al., 2002). Oppositely, bilateral lesions of the LDT were shown to decrease stereotypy in response to morphine (Forster et al., 2002), suggesting a role in mediating (either enhancing or attenuating) the effects of different drugs of abuse, depending on the pharmacology of the drug. LDT lesion studies focusing on natural reward-related behaviours are scarcer.

Pharmacological blocking of cholinergic neurons of the LDT results in motor (Dobbs and Cunningham, 2014), learning (Shinohara et al., 2014) and behavioural state deficit (Kohlmeier and Kristiansen, 2010). On the other hand, self-administration of cholinergic agonists directly into the VTA provides evidence for a role of Ach input in addiction and learning (David et al., 2006; Ikemoto and Wise, 2002). In a self-administration paradigm, systemic cocaine was shown to induce LDT-stimulated levels of dopamine in the NAc that were blocked by microinjection of scopolamine into the VTA (Lester et al., 2010). Intra-LDT administration of a muscarinic agonist was able to decrease intravenous cocaine self-administration in a fixed ratio (FR) schedule and breakpoint for cocaine responding under a progressive ratio schedule of reinforcement (Shabani et al., 2010). Additionally, this agonist was able to attenuate food self-administration. Furthermore, nicotine administered to the VTA enhances drug-seeking behaviour during self-administration paradigms and is able to produce changes in plasticity in dopamine neurons that may underlie addiction (Volkow and Morales, 2015). This suggests that the LDT-to-VTA cholinergic projection is important in modulating the rewarding effects of drugs of abuse and natural rewards, and on NAc dopamine release.

Recent reports using an optogenetic modulation approach of the brainstem confirmed the role of cholinergic neurons in REM sleep (Van Dort et al., 2015) and in operant behaviour (Steidl and Veverka, 2015). Specifically, optogenetic stimulation of both glutamatergic and cholinergic LDT projections to the VTA is sufficient to promote reward-related behaviours in rodents, specifically, inducing place preference and delaying extinction in a lever-pressing task, where sugar pellets were replaced by laser stimulation (Dautan et al., 2016a; Lammel et al., 2012; Steidl and Veverka, 2015; Xiao et al., 2016). In a more recent study, Steidl and colleagues showed that specific optogenetic stimulation of cholinergic but not glutamatergic neurons is able to induce place preference in a non-contingent paradigm (Steidl et al., 2017a). Interestingly it was shown that PPT neurons, on the other hand, appear to preferentially influence motor control via cholinergic projections to the SN, but not VTA (Xiao et al., 2016), whereas glutamatergic PPT neurons target non-dopamine VTA neurons, which have been demonstrated to be necessary for the acquisition of stimulus-reward associations (Yau et al., 2016).

Optical stimulation in the VTA of either PPT or LDT axons was shown to increase motor activity, either during PPT axon stimulation or during cumulative LDT axon stimulation (i.e., repeated pulses over the entire 30-min trial session) (Dautan et al., 2016a). Intriguingly, the two types of cholinergic inputs exert distinct modulations on particular neurons: NAc-projecting dopaminergic neurons are preferentially excited, whereas the NAc-projecting non-dopaminergic neurons are selectively inhibited by LDT cholinergic activation, with PPT stimulation having no effect (Dautan et al., 2016a).

Overall, this seems to suggest that the ascending cholinergic projections may promote goal-directed locomotion by increasing dopamine neuron activity and subsequent dopamine release in the Str. Nevertheless, further studies are necessary to characterize the opposing nature of cholinergic and glutamatergic signals originating in the mesopontine tegmentum, more specifically the LDT, and whether their interaction forms the basis of a more complex mechanism of motor and limbic function regulation.

Altogether, the LDT appears to be uniquely positioned to exercise control of not only burst firing and increase in dopamine release via their direct excitatory projections to mesoaccumbens-projecting VTA dopamine neurons, but possibly controlling the NAc directly, considering the anatomical link between these regions.

3. Stress, glucocorticoids and impact on the reward circuit

Stress is a major risk factor for the development of several psychiatric disorders, including depression, anxiety, and addiction (Chrousos, 2009; Cottrell and Seckl, 2009; Lupien et al., 2009; Rodrigues et al., 2011; Teicher et al., 2003). Stress activates the hypothalamus-pituitary-adrenal (HPA) axis and culminates with the release of GC by the adrenal glands. Briefly, upon exposure to a stressor, the hypothalamus produces corticotropin-releasing hormone (CRH), binding to specific receptors of the pituitary and inducing the production of the adrenocorticotropic hormone (ACTH). The ACTH will then induce the production and release of GCs in the adrenal glands onto the circulating blood. Circulating GCs will then act through a negative feedback in the hypothalamus and the pituitary to end further release of GCs and avoid prolonged exposure that might lead to detrimental effects.

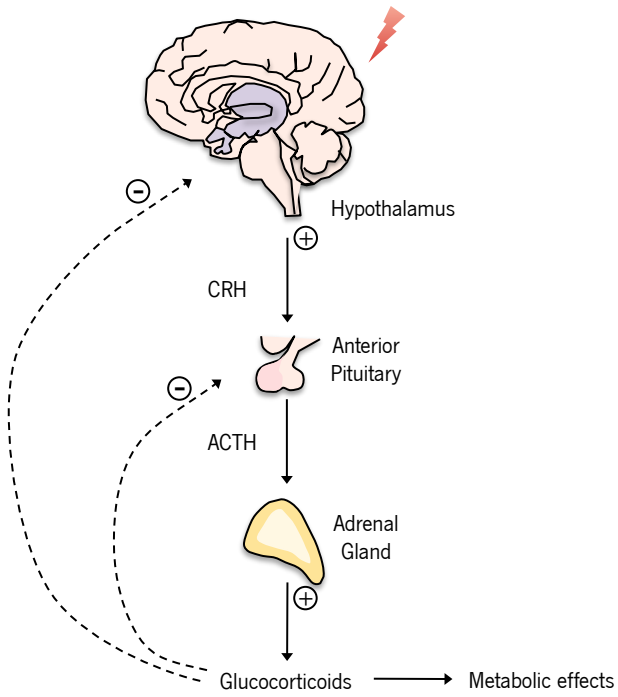

Figure 5 - The major components of the stress response mediated by the hypothalamus-pituitary-adrenal (HPA) axis. Stress can activate cells in the hypothalamus leading to the release of CRH. This hormone is transported to the pituitary gland triggering the secretion of ACTH, which stimulates the production of glucocorticoids (GCs) in the adrenal glands, releasing them into blood circulation. Through GCs, a negative feedback inhibition occurs in the pituitary and hypothalamus, regulating ACTH and CRH release.

ACTH: adrenocorticotropic hormone; CRH: corticotropin releasing hormone.

GCs display affinity to two receptors: mineralocorticoid receptors (MR) or glucocorticoid receptors (GR) that, through signalling cascades, will produce genomic and non-genomic effects. GCs present a very important modulatory function in metabolic (Andrews and Walker, 1999; Peckett et al., 2011; Vegiopoulos and Herzig, 2007; Wang, 2005), cardiovascular (Rosmond and Björntorp, 2000; Walker, 2007) and immune (Chrousos, 2009; Dhabhar and McEwen, 1999; Sapolsky et al., 2000) processes. GCs mainly activate MR, while increased GR receptor-binding is observed when the levels of GCs increase (Reul et al., 1987). Due to their pleiotropic and potent effects, GCs secretion de-regulation has been strongly implicated in the pathogenesis of numerous stress-related diseases, increasing vulnerability to anxiety, depression, addiction and eating disorders in animal models and humans (Lupien et al., 2009).

The brain reward circuitry is known to be affected by GCs action. Stress or GCs exposure has been described to induce changes in volume and dendritic architecture of different brain regions, hippocampal neurogenesis, electrophysiological changes and induce depressive- or anxious-like behaviour in rodents. Many of the regions affected are also key players in the brain reward circuit. For example, numerous stress paradigms have been shown to induce morphological changes in the PFC and hippocampus, as well as in the mesolimbic dopaminergic system (Akirav and Maroun, 2007; Berton et al., 2006; Chaudhury et al., 2013; Radley et al., 2006; Russo and Nestler, 2013; Surget et al., 2011; Tye et al., 2011, 2013, Vialou et al., 2010, 2014).

Adverse events occurring during early prenatal or postnatal life, can have major outcomes later in life, increasing the risk for development of several neuropsychiatric disorders, discussed in more detail next (Cottrell and Seckl, 2009; Lupien et al., 2009; Rodrigues et al., 2011).

Reprogramming of the HPA axis can occur during critical developmental periods, when excessive exposure to GCs and consequential maladaptive GC secretion ensues (Koehl et al., 1999). In rodents, the circadian rhythm of the HPA axis is altered by prenatal stress modifications along with hippocampal corticosteroid receptor expression. In humans, evidence shows that reprogramming of the HPA axis responsiveness can occur after maternal stress in infants (Haley et al., 2006; Harris and Seckl, 2011). The fetal period seems to be particularly sensitive to GC effects. In part, the presence of the 11 Beta-hydroxysteroid dehydrogenase (11 β-HSD), protects the foetus by converting cortisol/corticosterone to its inactive form (cortisone/11 dehydrocorticosterone). However, several studies have shown that increased prenatal cortisol is able to predict and negatively impact regional brain volumes and connectivity leading to cognitive

and motor impairments and affective disorders (Buss et al., 2010; Davis and Sandman, 2012; Huizink et al., 2002, 2003; Kim et al.).

Synthetic GCs are widely used in medical care for a broad range of disorders, due to their wide range of effects. Dexamethasone (DEX), a synthetic glucocorticoid commonly administered to pregnant women in case of premature delivery, promotes fetal lung maturation, ensuring the efficacy and safety against disorders, such as respiratory distress syndrome, reducing neonatal morbidity and mortality (Crowley, 1995). These synthetic GCs differ from their endogenous equivalent in their chemical structure, pharmacokinetics and target specificity properties, exhibiting an intensified affinity to the GR than to the MR (Tegethoff et al., 2009) and possess an ability to cross the placenta easily (Brown et al., 1996; Seckl and Meaney, 2004). This early and high exposure to GC can, potentially and persistently, affect the developing brain, programming it in a way that may promote a maladaptive physiology leading or predisposing the system to disease (Cottrell and Seckl, 2009; Harris and Seckl, 2011; Rodrigues et al., 2011).

Our laboratory has developed an animal model of prenatal GC exposure (iuGC model), which consists of subcutaneous administration of DEX 1mg kg^{-1} on gestation days 18 and 19. This model presents a long-lasting hyperanxious state, depressive-like behaviour, deficits in social behaviours (Borges et al., 2013; Leão et al., 2007; Oliveira et al., 2006, 2012; Roque et al., 2011) and proneness for addictive behaviour and natural reward deficits (Rodrigues et al., 2012; Soares-Cunha et al., 2014). This model is especially important to study, since it presents anatomical changes in several regions of the reward system, as shown by a significant decrease in the number of TH^+ fibbers in the VTA, coupled with a decrease in dopamine innervation of the NAc (Leão et al., 2007). Additionally, an overall decrease in NAc volume is observed, along with an increase in the number of immature spines and in the expression of the D2R, specifically. iuGC animals also present a significant decrease in dopamine levels in the NAc and amygdala. Normalization of dopamine levels by peripheral administration of dopamine precursor levodopa (L-DOPA) improved the reward deficits observed in these animals (Borges et al., 2013; Leão et al., 2007; Oliveira et al., 2006, 2012; Roque et al., 2011).

3.1. Impact of stress in the LDT circuitry

Anatomical studies suggest that the ascending cholinergic projections from the LDT to the forebrain and diencephalon form a pathway associated with aversive emotional states, equivalent

to the mesocorticolimbic dopamine pathway that is important for motivation and positively valenced states (Brudzynski, 2014). Aversive distress vocalizations occurred if those projections were stimulated, suggesting that this pathway acts as a "physiological, psychological, and social arousing and alarming system". In accordance, acute stress was shown to transiently increase the cholinergic tone and induce a phase of enhanced neuronal excitability. Additionally, stress was able to specifically modulate expression of genes that regulate ACh availability (Kaufer et al., 1998). However, one cannot exclude that the LDT, as mentioned, projects extensively to the VTA and has a major role in reward processing through its effects on the dopaminergic pathways (Steidl et al., 2017b; Xiao et al., 2016).

Different studies have demonstrated the importance of the VTA in the stress response via modulation of dopaminergic transmission (Peña et al., 2017). Interestingly, nicotine and social stress seem to induce a bidirectional effect in the activity of VTA dopaminergic neurons, via nicotinic receptors and implicating VTA cholinergic tone in stress-related disorders (Morel et al., 2017). Additionally, a causal link between VTA dopaminergic cells activities and pro-depressive/antidepressant effects has been shown. In a model of social defeat, mice presented aberrant firing of VTA dopamine neurons, compared to normal mice (Chaudhury et al., 2013; Friedman et al., 2014). Considering how the activity of these cells can be regulated by cholinergic afferents from the LDT, this pathway may also be a target of GCs and ultimately be impaired in different stress-related neuropsychiatric disorders. Indeed, immobility time in the forced swimming test is increased after intracranial infusion of AChE inhibitors or AChR agonists into the VTA. Contrariwise, AChR antagonists effectively decreased the immobility time, suggesting a reliable cholinergic effect on the depressive reaction in the VTA (Addy et al., 2015; Small et al., 2016).

Stress can also have several effects on behaviours associated with drugs of abuse. Stress during adulthood can reinstate extinguished addictive behaviours related to cocaine, such as cocaine self-administration (Capriles et al., 2003; Mantsch et al., 2016), cocaine conditioned place preference in rodents (Mantsch et al., 2010; Redila and Chavkin, 2008) and potentiates the expression of rewarding memory of cocaine through the activation of LDT-VTA circuitry (Shinohara et al., 2018).

Cholinergic signalling can alter several processes in neuronal development. This signalling by nAChRs was shown to regulate the timing of expression of the chloride transporter, important for the ability of GABA to hyperpolarize, and therefore inhibit, central neurons (Liu et al., 2006). Thus, impairments in nAChR signalling could alter the switch from GABA-mediated excitation to

inhibition. Additionally, nAChRs contribute to the maturation of GABAergic (Kawai et al., 2002; Zago et al., 2006) and glutamatergic (Lozada et al., 2012a, 2012b) synapses, highlighting an important role for ACh signalling in synaptic development (reviewed in (Role and Berg, 1996)).

Taking advantage of the iuGC model from our lab, it was possible to assess the impact of early life exposure to increased levels of GCs in specific time windows during development in the LDT. As mentioned in the previous section, these animals present hyperanxiety and an increased response to negative stimuli associated with increased fear behaviour (Borges et al., 2013). This was accompanied with an increase in the number of 22kHz ultrasonic vocalizations in response to aversive cues, as suggested by the role of the LDT cholinergic pathway in the control of emotional arousal and aversive state. Curiously, exposure to iuGC induced an increase in the number of cholinergic cells in this region and a higher activation of these cells upon exposure to an aversive cue, however, the impact of this exposure was not explored in rewarding and motivation related behaviours. iuGC animals present prominent reward deficits that partially occur due to impairments in the dopaminergic innervation to the NAc and the upstream cholinergic innervation by LDT inputs, providing evidence towards an imbalance in these two neuronal systems.

Understanding the organization and functional properties of afferents from the LDT to the VTA or NAc will be beneficial for understanding the neural substrates of physiological and pathological phenomena mediated by these regions.

5. Objectives

The LDT is a brainstem nucleus interconnected with several nuclei of the basal ganglia, including the NAc, and the VTA.

The LDT has been recently proposed to influence striatal activity in two ways: by innervation of midbrain dopaminergic neurons, which in turn project to striatum, or by direct innervation of the striatum. However, to date, no studies about the nature and function of LDT-NAc neurons have been performed. Besides analysing the role of LDT-VTA-NAc projections in physiological conditions, we are also interested in understanding the impact of stress/GCs in this network, and how this affects reward-related behaviours.

The main objectives of this thesis were:

1) Assess the impact of *in utero* exposure to high levels of GC (iuGC model) on the LDT region, performing anatomical, molecular and electrophysiological measurements;

2) Define the behavioural contribution of the LDT-VTA circuit in reward-dependent tasks in control and iuGC animals;

3) Characterize the nature of LDT-NAc projections and evaluate their role in motivation and in reward-related tasks;

4) Assess the impact of selective optogenetic manipulation of LDT-NAc cholinergic neurons in reward-related tasks.

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Chapter 2

Optogenetic modulation of Ventral Tegmental Area-projecting neurons of the Laterodorsal Tegmentum during reward-dependent behaviour

Chapter 2.1

Impairments in laterodorsal tegmentum to VTA projections underlie glucocorticoid-triggered reward deficits

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RESEARCH ARTICLE

Impairments in laterodorsal tegmentum to VTA projections underlie glucocorticoid-triggered reward deficits

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Abstract Ventral tegmental area (VTA) activity is critical for reward/reinforcement and is tightly modulated by the laterodorsal tegmentum (LDT). *In utero* exposure to glucocorticoids (iuGC) triggers prominent motivation deficits but nothing is known about the impact of this exposure in the LDT-VTA circuit. We show that iuGC-rats have long-lasting changes in cholinergic markers in the LDT, together with a decrease in LDT basal neuronal activity. Interestingly, upon LDT stimulation, iuGC animals present a decrease in the magnitude of excitation and an increase in VTA inhibition, as a result of a shift in the type of cells that respond to the stimulus. In agreement with LDT-VTA dysfunction, we show that iuGC animals present motivational deficits that are rescued by selective optogenetic activation of this pathway. Importantly, we also show that LDT-VTA optogenetic stimulation is reinforcing, and that iuGC animals are more susceptible to the reinforcing properties of LDT-VTA stimulation.
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Introduction

The ventral tegmental area (VTA) is an heterogeneous brain region containing distinctive neuronal populations essential for the expression of motivated behaviors and reinforcement (***Berridge and Robinson, 1998***; ***Wise, 2004***; ***Bayer and Glimcher, 2005***; ***Fields et al., 2007***; ***Berridge, 2007***; ***van Zessen et al., 2012***). The VTA comprises dopaminergic (~65%), GABAergic (~30%), and glutamatergic neurons (~5%) (***Nair-Roberts et al., 2008***; ***Yamaguchi et al., 2011***) that receive inputs from diverse brain regions, including the laterodorsal tegmentum (LDT) (***Woolf and Butcher, 1986***; ***Cornwall et al., 1990***; ***Oakman et al., 1995***; ***Oakman et al., 1999***).

Several studies have shown that exposure to unexpected rewards, or cues that predict rewards, can activate VTA dopaminergic neurons culminating in the release of dopamine in the nucleus accumbens (NAc) (***Roitman et al., 2004***; ***Stuber et al., 2005***; ***Stuber et al., 2008***; ***Schultz et al., 1997***; ***Bromberg-Martin et al., 2010***). Importantly, this activity is tightly modulated by cholinergic projections (***Omelchenko and Sesack, 2005***; ***Omelchenko and Sesack, 2006***), with an additional contribution of glutamatergic projections, arising from the LDT (***Cornwall et al., 1990***; ***Oakman et al., 1999***; ***Lammel et al., 2012***). This input is vital for the activity of dopaminergic cells in the VTA, facilitating dopamine-related behaviors involved in reward signaling or encoding reward prediction signals (***Lodge and Grace, 2006***). In agreement, 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 responses in rodents (***Steidl and Veverka, 2015***).

Notably, different labs have shown that the mesolimbic system is particularly vulnerable to the effects of prenatal stress/high levels of glucocorticoids (GCs) (***Matthews, 2000***; ***Boksa and El-***

Khodor, 2003; *McArthur et al., 2005*; *Leão et al., 2007*; *Rodrigues et al., 2011*; *Borges et al., 2013a*; *Soares-Cunha et al., 2014*). These changes may increase the risk to develop different neuropsychiatric disorders in adulthood, namely depression, anxiety and addiction (*Seckl, 2008*; *Rodrigues et al., 2012*). Surprisingly, very few studies have focused on the impact of stress/GCs in the cholinergic system. This is particularly intriguing because GCs can induce acetylcholine release (*Finkelstein et al., 1985*; *Gilad et al., 1985*; *Imperato et al., 1989*) and bind to GC-responsive elements of cholinergic enzymes, namely choline acetyltransferase (ChAT) and acetylcholine esterase (AChE) to control their expression (*Berse and Blusztajn, 1997*). In accordance, we have previously shown that prenatal GC exposure induces a long-lasting hyperanxious state associated with an increase in the recruitment of cholinergic cells from the LDT (*Borges et al., 2013b*), suggesting that GCs are able to *program* the LDT, which prompted us to evaluate the impact of prenatal GC in the LDT-VTA circuitry and its impact in reward-related behaviors.

Results

Sustained cholinergic dysfunction in iuGC animals

Previous data from our team suggested that LDT cholinergic cells were differentially recruited in response to an adverse stimulus (*Borges et al., 2013b*) in a model of *in utero* GC (iuGC) exposure at gestation days 18 and 19 (*Blaha and Winn, 1993*). Considering this, we first evaluated the impact of GCs on the cholinergic circuitry of iuGC animals. We quantified $ChAT^+$ cells in the LDT of 3, 30 and 90 days old animals (*Figure 1a–c*) and observed an effect of iuGC treatment (Two-way ANOVA; $F_{(1,25)} = 19.31$, p=0.0002). iuGC animals had a significant increase in the density of the cholinergic population of the LDT at 30 days of age (*post-hoc* Bonferroni; $CTR_{(30\ days)}$ vs. $iuGC_{(30\ days)}$: $t_{(25)} = 2.616$, p=0.0446) that persisted until adulthood (*post-hoc* Bonferroni; $CTR_{(90\ days)}$ vs. $iuGC_{(90\ days)}$: $t_{(25)} = 3.971$, p=0.0016). Other brain regions containing cholinergic neurons such as the nucleus basalis of Meynert or the NAc remained unaltered (*Figure 1—figure supplement 1*).

We next evaluated gene and protein expression levels of ChAT and AChE in the LDT (*Figure 1d–h*). We found a significant effect of iuGC treatment in ChAT (Two-way ANOVA; $F_{(1,24)} = 26.27$, p<0.0001) and AChE (Two-way ANOVA; $F_{(1,23)} = 15.71$, p=0.0006) gene expression. ChAT gene expression levels were increased at 3 and 30 days of age in iuGC animals (*Figure 1d*; *post-hoc* Bonferroni; $CTR_{(3\ days)}$ vs. $iuGC_{(3\ days)}$: $t_{(24)} = 3.383$, p=0.0074; $CTR_{(30\ days)}$ vs. $iuGC_{(30\ days)}$: $t_{(24)} = 3.053$, p=0.0164; $CTR_{(90\ days)}$ vs. $iuGC_{(90\ days)}$: $t_{(24)} = 2.494$, p=0.059). Decreased AChE levels were found in adult iuGC animals (*Figure 1e*; *post-hoc* Bonferroni; $CTR_{(3\ days)}$ vs. $iuGC_{(3\ days)}$: $t_{(23)} = 1.24$, p=0.6827; $CTR_{(30\ days)}$ vs. $iuGC_{(30\ days)}$: $t_{(23)} = 2.244$, p=0.1043; $CTR_{(90\ days)}$ vs. $iuGC_{(90\ days)}$: $t_{(23)} = 3.298$, p=0.0094). We also evaluated the levels of another cholinergic marker, the vesicular acetylcholine transporter (VAChT) and found that mRNA levels were unchanged between groups in the LDT (*Figure 1—figure supplement 2*).

Two-way ANOVA showed a significant effect of iuGC treatment in ChAT ($F_{(1,23)} = 32.82$, p<0.0001) and AChE protein expression ($F_{(1,18)} = 425.08$, p<0.0001). Western blot analysis confirmed the upregulation of ChAT (*Figure 1f–g*; *post-hoc* Bonferroni; $CTR_{(3\ days)}$ vs. $iuGC_{(3\ days)}$: $t_{(23)} = 4.401$, p=0.0006; $CTR_{(30\ days)}$ vs. $iuGC_{(30\ days)}$: $t_{(23)} = 2.762$, p=0.0333; $CTR_{(90\ days)}$ vs. $iuGC_{(90\ days)}$: $t_{(23)} = 2.712$, p=0.0373); and downregulation of AChE in the iuGC group at all ages tested (*Figure 1f and h*; *post-hoc* Bonferroni; $CTR_{(3\ days)}$ vs. $iuGC_{(3\ days)}$: $t_{(18)} = 4.73$, p=0.0005; $CTR_{(30\ days)}$ vs. $iuGC_{(30\ days)}$: $t_{(18)} = 3.157$, p=0.0164; *post-hoc* Bonferroni $CTR_{(90\ days)}$ vs. $iuGC_{(90\ days)}$: $t_{(18)} = 3.349$, p=0.0107).

Considering the heterogeneous nature of LDT inputs to the VTA, we also assessed the impact of iuGC exposure on glutamatergic and GABAergic markers (*Figure 1—figure supplement 3a–c,e–g*). Gene and protein expression levels of glutamate transporter EAAC1 and GAD1/67 + GAD2/65 were not significantly affected by iuGC exposure.

We also decided to evaluate the expression levels of glucocorticoid receptor (GR) since early life adversity has been shown to change GR epigenetic status. We found no differences between groups regarding GR expression (*Figure 1—figure supplement 3d,h*).

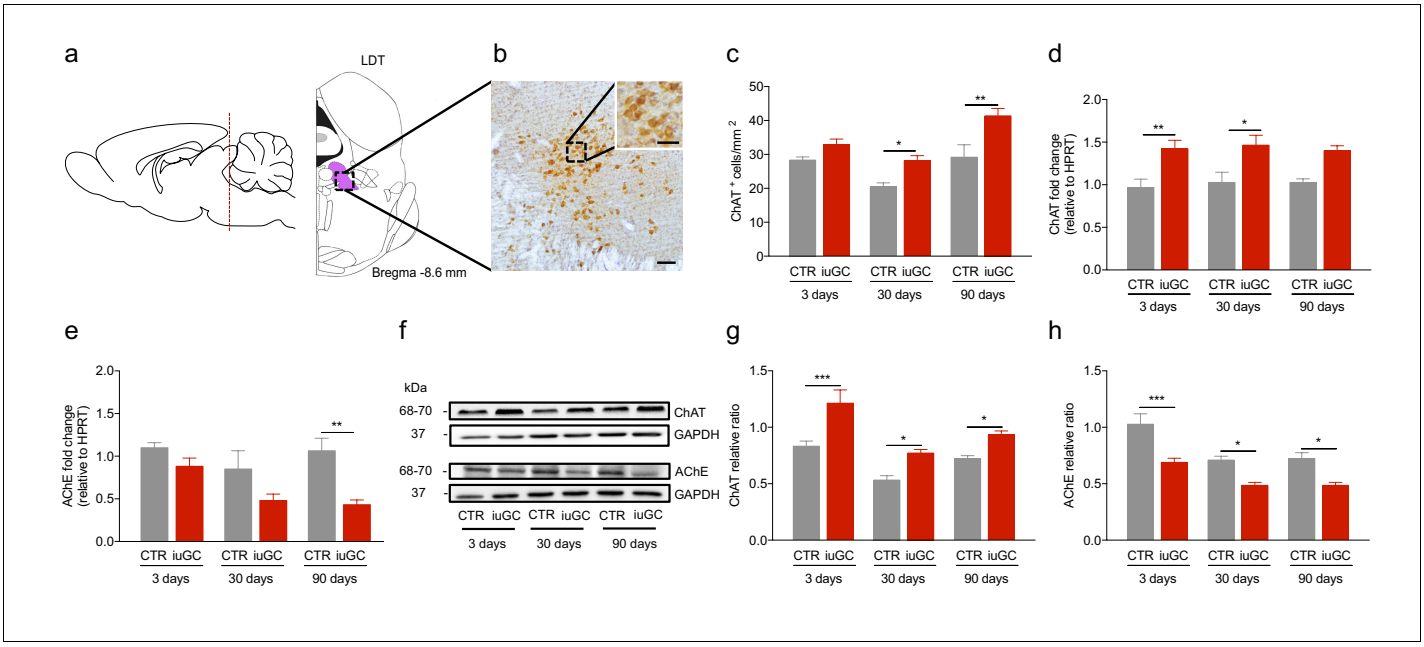

Figure 1. Prenatal exposure to glucocorticoids alters LDT cholinergic system. (**a**) Schematic representation of the LDT. (**b**) Coronal section of the LDT showing ChAT immunohistochemistry. (**c**) iuGC animals present increased number of ChAT$^+$ cells in the LDT at postnatal day 30 and 90. (**d**) Real-time PCR analysis revealed that ChAT mRNA levels are increased in the LDT of iuGC animals from postnatal day 3. (**e**) Conversely, AChE mRNA levels are decreased at postnatal day 90 (n_{CTR} = 4; n_{iuGC} = 5). (**f**) Representative immunoblot of ChAT and AChE in the LDT of 3, 30 and 90 days old animals. (**g**) Protein quantification confirmed the upregulation of ChAT and (**h**) downregulation of AChE in the LDT from postnatal day 3 until adulthood (n_{CTR} = 4; n_{iuGC} = 5). Data represented as mean ±s.e.m. *p<0.05, **p<0.001, ***p<0.0001. Scale bars in b: 100 µm and inset - 50 µm. Additional data is depicted in ***Figure 1—figure supplements 1***, ***2*** and ***3***.

DOI: https://doi.org/10.7554/eLife.25843.002

The following figure supplements are available for figure 1:

Figure supplement 1. iuGC animals do not present changes in the number of cholinergic cells in other regions.

DOI: https://doi.org/10.7554/eLife.25843.003

Figure supplement 2. iuGC exposure does not change the expression levels of VAChT.

DOI: https://doi.org/10.7554/eLife.25843.004

Figure supplement 3. iuGC exposure does not change the expression levels of GABAergic and glutamatergic markers in the LDT.

DOI: https://doi.org/10.7554/eLife.25843.005

iuGC treatment impairs the LDT-VTA circuitry

Previous work from our group showed that iuGC animals presented a VTA-NAc hypodopaminergic state (***Leão et al., 2007***; ***Borges et al., 2013a***; ***Soares-Cunha et al., 2014***). Since the LDT innervates the VTA and can influence NAc dopamine release (***Blaha and Winn, 1993***; ***Blaha et al., 1996***; ***Forster and Blaha, 2000***; ***Forster et al., 2002***; ***Miller et al., 2002***; ***Forster and Blaha, 2003***), we decided to characterize the LDT-VTA circuit using *in vivo* single cell electrophysiology in anesthetized animals (***Figure 2***).

iuGC treatment significantly decreased the spontaneous activity of LDT neurons (***Figure 2a–b***; $t_{(128)}$ = 4.674, p<0.0001, total number of cells: CTR = 36, iuGC = 94; n_{CTR} = 8, n_{iuGC} = 11 animals). No differences were found in the basal activity of VTA (***Figure 2c–d***; $t_{(70)}$ = 0.0576, p=0.9542, total number of cells: CTR = 40, iuGC = 32; animals: CTR = 8, iuGC = 11).

Electrical stimulation at 0.5 Hz of the LDT evoked both excitatory and inhibitory responses in VTA neurons in contrasting percentages (***Figure 2e***; CTR: 50% excitatory and 30% inhibitory; iuGC: 31% excitatory and 44% inhibitory). We also used defined criteria based on extracellular waveforms and firing rate (***Ungless et al., 2004***; ***Ungless and Grace, 2012***; ***Totah et al., 2013***) to divide recorded cells into putative DAergic (pDAergic) or GABAergic (pGABAergic) neurons (***Figure 2f–h***). Cells that did not match these criteria were considered to be 'other' types of neurons.

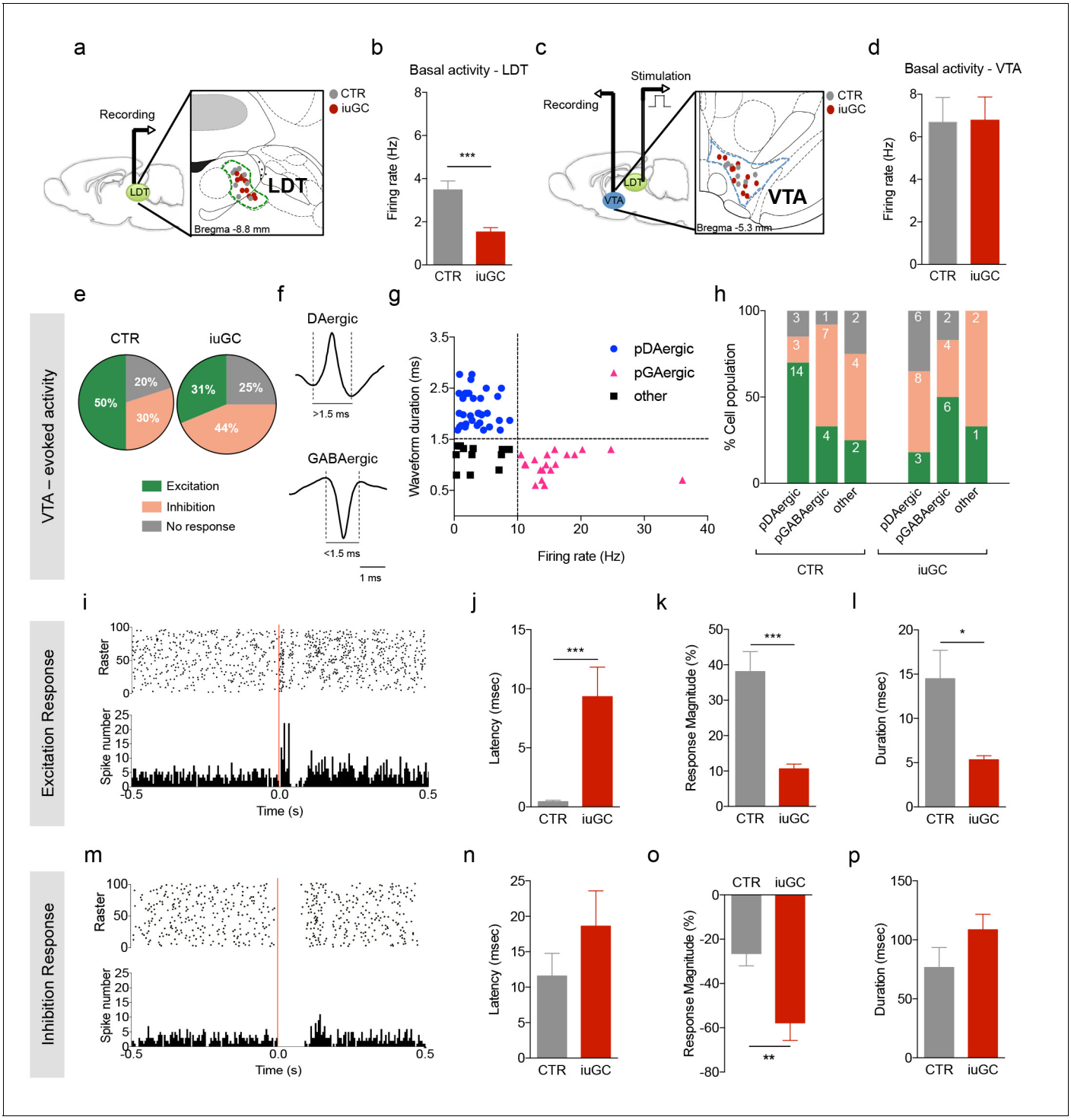

Figure 2. Distinct VTA neuronal response to LDT electrical stimulation in iuGC animals. (**a**) Schematic representation of the *in vivo* single-cell electrophysiological recording experiments and electrode placement in the LDT in anesthetized animals. (**b**) iuGC animals present decreased basal activity of LDT neurons in comparison to CTR ($n_{LDT\text{-}CTR}$ = 36 cells; $n_{LDT\text{-}iuGC}$ = 94 cells). (**c**) Schematic representation of the *in vivo* single-cell electrophysiological recording experiments in the VTA with electric stimulation performed in the LDT; and recording electrode placement. (**d**) The basal activity of the VTA is similar between groups ($n_{VTA\text{-}CTR}$ = 40 cells; $n_{VTA\text{-}iuGC}$=32 cells). (**e**) Electrical stimulation of the LDT (0.5 Hz) induces excitatory and inhibitory responses in VTA neurons. Pie plots represent the percentage of excitatory, inhibitory and no responses of VTA neurons. (**e–g**) In CTR animals, 50% of neurons present an excitatory response (70% pDAergic, 20% pGABAergic) and 30% present an inhibitory response (21% pDAergic, 50% pGABAergic). iuGC group shows a different profile, with 31% of recorded cells presenting an excitatory response (30% pDAergic, 60% pGABAergic) versus 44% with inhibitory response (57% pDAergic, 29% pGABAergic). (**f**) Representative examples of rat VTA pDAergic and pGABAergic neuronal

Figure 2 continued on next page

Figure 2 continued

waveforms. (**g**) Firing rate and waveform duration were used to classify single units into 3 types of neurons. (**h**) Percentage of each putative neuronal population presenting excitation, inhibition or with no response to LDT stimulation. There is a shift in the percentage of putative DAergic and GABAergic neurons presenting excitatory and inhibitory responses. Numbers in bars represent number of cells in each category. (**i, m**) Peristimulus time histograms (PSTHs) show LDT-evoked responses of VTA dopamine neurons; (**i**) excitation; (**m**) inhibition. (**j**) VTA neurons that display an excitatory profile in response to LDT electrical stimulus present increased latency to fire in iuGC animals. (**k**) The magnitude and (**l**) duration of response of VTA neurons is reduced in iuGC animals. (**n**) VTA neurons that display an inhibitory response in response to LDT electrical stimulus do not show differences in the latency to fire in both groups. (**o**) The magnitude of response of inhibited neurons of the VTA is increased in iuGC animals, with no differences in (**p**) the duration of inhibition in VTA neurons upon LDT stimulation. pDAergic: putative dopaminergic neurons; pGABAergic: putative GABAergic neurons. Data is represented as mean ±s.e.m. *p<0.05, **p<0.001, ***p<0.0001.

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Interestingly, iuGC animals present a shift in the type of neurons that present excitatory responses, with an increase in pGABAergic neurons and concomitant decrease in pDAergic neurons. Excitatory responses were observed in 70% of pDAergic and 20% of pGABAergic neurons in CTR animals versus 30% of pDAergic and 60% of pGABAergic neurons in iuGC animals.

Regarding inhibition, the iuGC group presented an increase in pDAergic neurons together with a decrease in pGABAergic neurons (***Figure 2h***). Briefly, inhibitory responses were observed in 21% of pDAergic and 50% of pGABAergic neurons in CTR animals versus 57% of pDAergic and 28% of pGABAergic neurons in iuGC animals.

The onset of excitation was significantly increased in iuGC group in comparison to control group (***Figure 2j***; CTR: 0.45 ± 0.114 ms vs. iuGC: 9.33 ± 2.49 ms; $t_{(30)}$ = 4.637, p<0.0001). Interestingly, iuGC treatment reduced the magnitude and duration of the excitatory response (***Figure 2k–l***; magnitude: $t_{(30)}$ = 3.723, p=0.0008; duration: $t_{(30)}$ = 2.196, p=0.0360).

LDT stimulation did not affect the latency of inhibition, although there was a trend for increased latency in iuGC animals (***Figure 2n***; CTR: 11.58 ± 3.175 ms vs. iuGC: 18.61 ± 4.979 ms; $t_{(28)}$ = 1.057, p=0.2997). Also, the duration of response was not affected (***Figure 2p***; $t_{(28)}$ = 1.510, p=0.1422). However, the response magnitude of inhibitory responses in the VTA evoked by LDT electrical stimulation was significantly higher in iuGC animals (***Figure 2o***; $t_{(28)}$ = 2.905, p=0.0071).

Altogether, this data demonstrated an imbalance in the excitatory and inhibitory inputs to the VTA when electrically stimulating the LDT.

Optogenetic activation of LDT terminals in the VTA elicits distinct responses in control and iuGC animals

We next used a combined viral approach to specifically modulate LDT direct inputs to the VTA and exclude the effects of indirect activation of other regions to where LDT projects to. We decided to activate all types of LDT-VTA inputs (and not only cholinergic) because we observed an effect of iuGC exposure in both excitatory and inhibitory VTA responses elicited by LDT activation.

To do so, we injected a viral vector containing a WGA–Cre fusion construct (AAV5–EF1a–WGA–Cre–mCherry) in the VTA, and a cre-dependent ChR2 vector in the LDT (AAV5-EF1a-DIO-hChR2-eYFP). The WGA–Cre fusion protein is retrogradely transported (***Gradinaru et al., 2010***), inducing the expression of cre-dependent ChR2-YFP only in LDT neurons that directly project to the VTA (***Figure 3a–c***). Four weeks post-injection, we observed YFP staining in axonal terminals of LDT neurons in the VTA (***Figure 3b***) and in cell bodies in the LDT (***Figure 3c***).

We performed *in vivo* single cell electrophysiology in the VTA while stimulating LDT terminals in this region, in order to activate the LDT-VTA circuit specifically. As depicted in ***Figure 3d***, optogenetic stimulation of LDT terminals (30 pulses of 15 ms at 20 Hz) induced an increase in firing rate of VTA neurons (***Figure 3d***, *post-hoc* Bonferroni test CTR-ChR2: baseline vs. stimulus - $t_{(118)}$ = 5.883, p<0.0001, stimulus vs. post-stimulus - $t_{(118)}$ = 3.749, p=0.0008; iuGC-ChR2: baseline vs. stimulus - $t_{(118)}$ = 10.99, p<0.0001; stimulus vs. post-stimulus - $t_{(118)}$ = 10.88, p<0.0001), eliciting a response in 69% and 71% of recorded cells of CTR and iuGC animals, respectively (***Figure 3e***). Optic stimulation did not affect the latency of stimulus response for the groups (***Figure 3—figure supplement 1***).

In CTR animals, 49% of cells presented increased firing rate upon stimulation, and of these, 69% were pDAergic, 17% pGABAergic and 14% were categorized as 'other' neuronal subtypes. Moreover, 87% of cells that presented inhibitory responses were pGABAergic neurons (***Figure 3f–g***). In

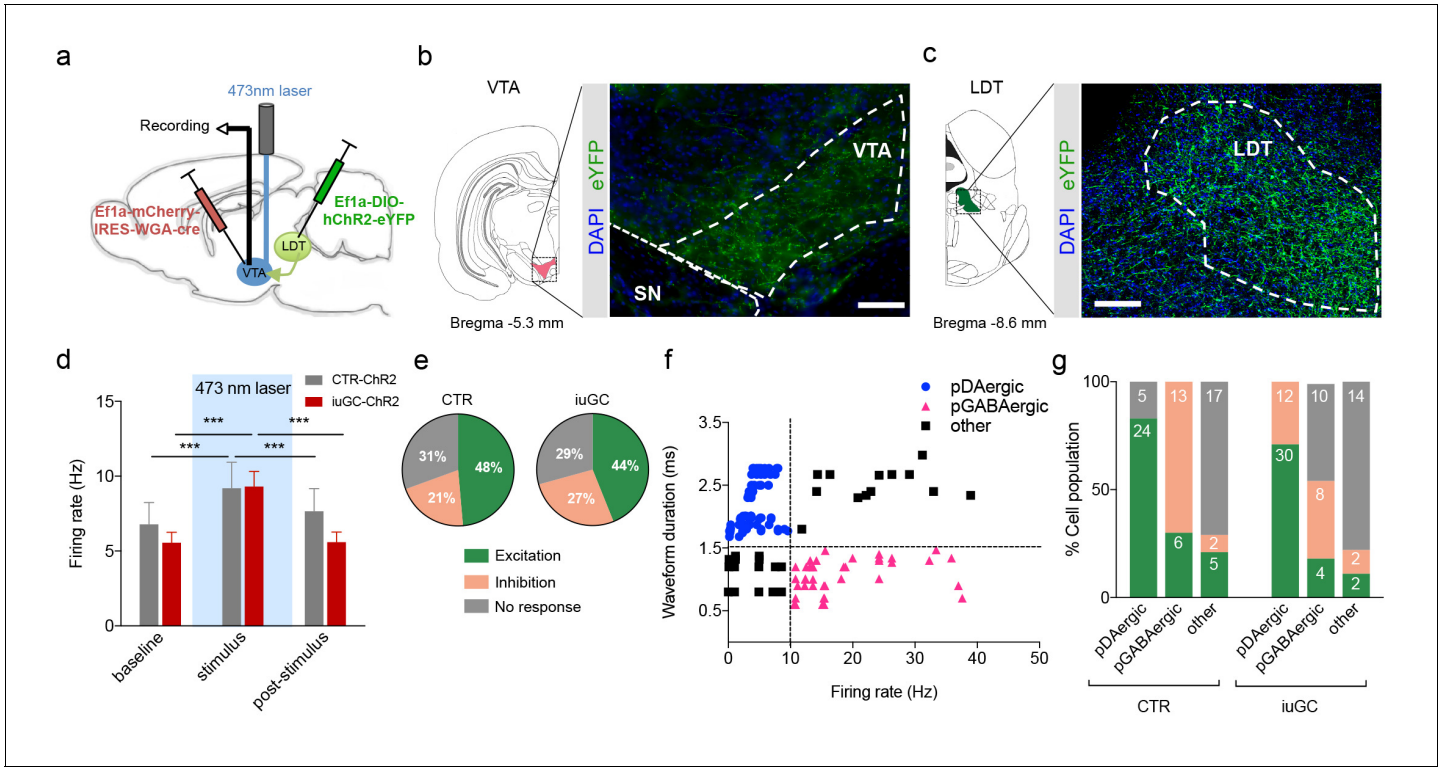

Figure 3. Optogenetic activation of LDT terminals in VTA elicits a differential electrophysiological response in iuGC animals. (**a**) Strategy used for optogenetic activation of LDT projecting neurons in the VTA. An AAV5–EF1a–WGA–Cre–mCherry virus construct was injected unilaterally in the VTA, and a cre-dependent ChR2 vector (AAV5-EF1a-DIO-hChR2-eYFP) in the LDT. WGA-Cre will retrogradely migrate and induce the expression of ChR2 in LDT neurons that directly project to the VTA. (**b**) Representative image of immunofluorescence for GFP showing LDT axon terminals in the VTA and (**c**) cell bodies in the LDT; scale bar: 200 µm. (**d**) Optogenetic stimulation of LDT terminals in the VTA (blue rectangle; 30 pulses of 15 ms at 20 Hz) increases the firing rate of VTA neurons in both groups (n_{CTR} = 72 cells; n_{iuGC} = 82 cells). (**e**) In CTR, upon LDT terminal stimulation, 48% of recorded VTA cells present an increase in firing rate (of those 69% pDAergic, 17% pGABAergic), 21% decrease activity (0% pDAergic, 87% pGABAergic) and 31% presented no change. In iuGC animals, upon LDT terminal stimulation, 44% of recorded VTA cells present an increase in firing rate (83% pDAergic; 11% GABAergic) 27% decrease activity (55% pGABAergic, 36% DAergic) and 29% presented no change. (**f**) Firing rate and waveform duration were used to classify single units into 3 types of neurons. (**g**) Percentage of each putative neuronal population presenting excitation, inhibition or with no response to LDT terminals optogenetic stimulation. Numbers in bars represent number of cells in each category. pDAergic: putative dopaminergic neurons; pGABAergic: putative GABAergic neurons. Data represented as mean ±s.e.m. ***p<0.001. Additional data is depicted in ***Figure 1—figure supplement 1***.

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The following figure supplement is available for figure 3:

Figure supplement 1. iuGC treatment has no effect on the response latency after optical stimulation of the LDT-VTA circuit.

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iuGC animals, 44% of cells presented increased firing rate upon stimulation, and the majority were pDAergic neurons (83%). Surprisingly, and clearly different from CTR animals, 55% of cells that presented inhibitory responses were pDAergic neurons and 36% were considered to be pGABAergic neurons (***Figure 3g***). Again, and in accordance with the electrical stimulation data, our optogenetic results suggest an imbalance in the excitatory and inhibitory inputs from the LDT to the VTA.

Activation of LDT terminals in the VTA rescues motivational deficits of iuGC animals

Since the LDT-VTA circuitry has been described to contribute for positive reinforcement (***Lammel et al., 2012***; ***Steidl and Veverka, 2015***; ***Lammel et al., 2011***), we evaluated the motivational drive by testing willingness to work for food in a progressive ratio (PR) schedule of reinforcement. This test measures the breakpoint or maximum effort rats are willing to perform for an outcome, when the demand grows progressively over a session.

Training was similar between CTR, CTR-YFP, CTR-ChR2 and iuGC-ChR2 groups across days either in the continuous reinforcement (CRF) or fixed ratio (FR) sessions (***Figure 4—figure supplement 1a–b***). In the test day, iuGC-ChR2 rats presented a significant decrease in breakpoint in comparison to CTR, CTR-YFP and CTR-ChR2 animals (***Figure 4a***; 48,9% decrease; *post-hoc* Bonferroni test CTR vs. iuGC-ChR2: $t_{(68)}$ = 2.882, p=0.0317; CTR-YFP vs. iuGC-ChR2: $t_{(68)}$ = 2.78, p=0.0421; CTR-ChR2 vs. iuGC-ChR2: $t_{(68)}$ = 4.141, p=0.0006), with no differences in the number of pellets earned during the test (***Figure 4—figure supplement 1c***).

We next assessed if selective optogenetic activation of the LDT-VTA pathway was sufficient to enhance motivation during the PR test session. We decided to stimulate animals during cue exposure period since previous work from our group suggested that iuGC animals presented deficits in the Pavlovian-to-Instrumental Transfer test (PIT) (***Soares-Cunha et al., 2014***; ***Soares-Cunha et al., 2016***), which measures the ability of a Pavlovian conditioned stimulus that is associated with a reward to invigorate instrumental responding for that (or other) reward (***Corbit and Balleine, 2005***; ***Corbit and Janak, 2007***; ***Holmes et al., 2010***).

Activation of LDT terminals in the VTA during cue exposure period (30 pulses of 15 ms at 20 Hz; around 15 stimulations per session) reverted the breakpoint of iuGC animals but had no effect in CTR, CTR-eYFP and CTR-ChR2 animals (***Figure 4a–c***; *post-hoc* Bonferroni test CTR: $t_{(34)}$ = 0.1836, p>0.9999; CTR-ChR2: $t_{(34)}$ = 1.203, p=0.9498; CTR-eYFP: $t_{(34)}$ = 0.5099, p>0.9999; iuGC-ChR2: $t_{(34)}$ = 5.007, p<0.0001;). No effect was observed in the number of pellets earned (***Figure 4—figure supplement 1c***). Moreover, no effects in locomotion or free feeding consumption were observed using the same stimulation parameters in either group (***Figure 4—figure supplement 1e–f***).

Importantly, if the LDT-VTA optogenetic activation occurred during the inter-trial interval (ITI) period of the test session, it did not revert iuGC-ChR2 motivational deficits (***Figure 4b–c***; *post-hoc* Bonferroni: $t_{(34)}$ = 0.5138, p>0.9999), suggesting that LDT-VTA activation elicits a positive behavioral response only when it occurs during specific periods of the test.

Because the results of control animals were surprising in the light of previous evidence showing that LDT-VTA stimulation was reinforcing (***Lammel et al., 2012***; ***Xiao et al., 2016***), we performed another stimulation protocol in a new set of animals to test this hypothesis (80 pulses of 15 ms at 20 Hz) (***Figure 4—figure supplement 2a–b***). Importantly, when we increased the number of pulses, we observed an increase in the breakpoint of CTR-ChR2 animals (*post-hoc* Bonferroni: $t_{(22)}$ = 3.666, p=0.0054) when compared to breakpoint of CTR or CTR-eYFP animals (*post-hoc* Bonferroni CTR-ChR2 vs. CTR: $t_{(44)}$ = 3.075, p=0.0217; CTR-ChR2 vs. CTR-eYFP: $t_{(44)}$ = 3.194, p=0.0156). Additionally, we observed that this stimulation also increased the breakpoint of iuGC animals (*post-hoc* Bonferroni: $t_{(22)}$ = 4.641, p=0.0005), with no effect in CTR and CTR-eYFP groups (*post-hoc* Bonferroni CTR: $t_{(22)}$ = 0.116, p>0.9999; CTR-eYFP: $t_{(22)}$ = 0.4163, p>0.9999).

Stimulation of LDT-VTA terminals induces place preference

To get further insight on the role of the LDT-VTA circuit in behavior, we also evaluated the impact of the stimulation of LDT-VTA terminals in the conditioned place preference (CPP) test, which measures the reinforcing capacities of a particular stimulus (***Figure 4d***). LDT-VTA stimulation (30 pulses of 15 ms at 20 Hz every 60 s) elicited conditioning in iuGC group, given by increased preference for the stimulus-associated chamber, ON side, (***Figure 4e–f***; *post-hoc* Bonferroni: $t_{(20)}$ = 5.892, p<0.0001), whereas it did not shift preference in CTR and CTR-eYFP animals.

However, if we increase the number of stimulus (80 pulses of 15 ms every 15 s), we were able to induce conditioning in CTR-ChR2 animals (***Figure 4—figure supplement 2c***; $t_{(8)}$ = 4.737, p=0.0015). Additionally, we observe the same effect in the iuGC-ChR2 group (***Figure 4—figure supplement 2d***; *post-hoc* Bonferroni CTR-ChR2: $t_{(23)}$ = 3.576, p=0.0064; iuGC-ChR2: $t_{(23)}$ = 2.761, p=0.044). No effect was found in CTR and CTR-eYFP animals, as expected (*post-hoc* Bonferroni CTR: $t_{(23)}$ = 0.04378, p>0.9999; CTR-eYFP: $t_{(23)}$ = 0.1521, p>0.9999).

In order to further explore the reinforcing feature of LDT-VTA stimulation, we performed the real-time place preference test (RTPP; ***Figure 4g***), where one chamber is paired with optical stimulation and the other is not. Every time the animal was in the designated stimulation box (ON side), it received optical stimulation (15 ms pulses at 20 Hz) that only ended when the animal crossed to the no-stimulation box (OFF side). This test is different from a classic CPP since the animal is able to choose the chamber throughout the test.

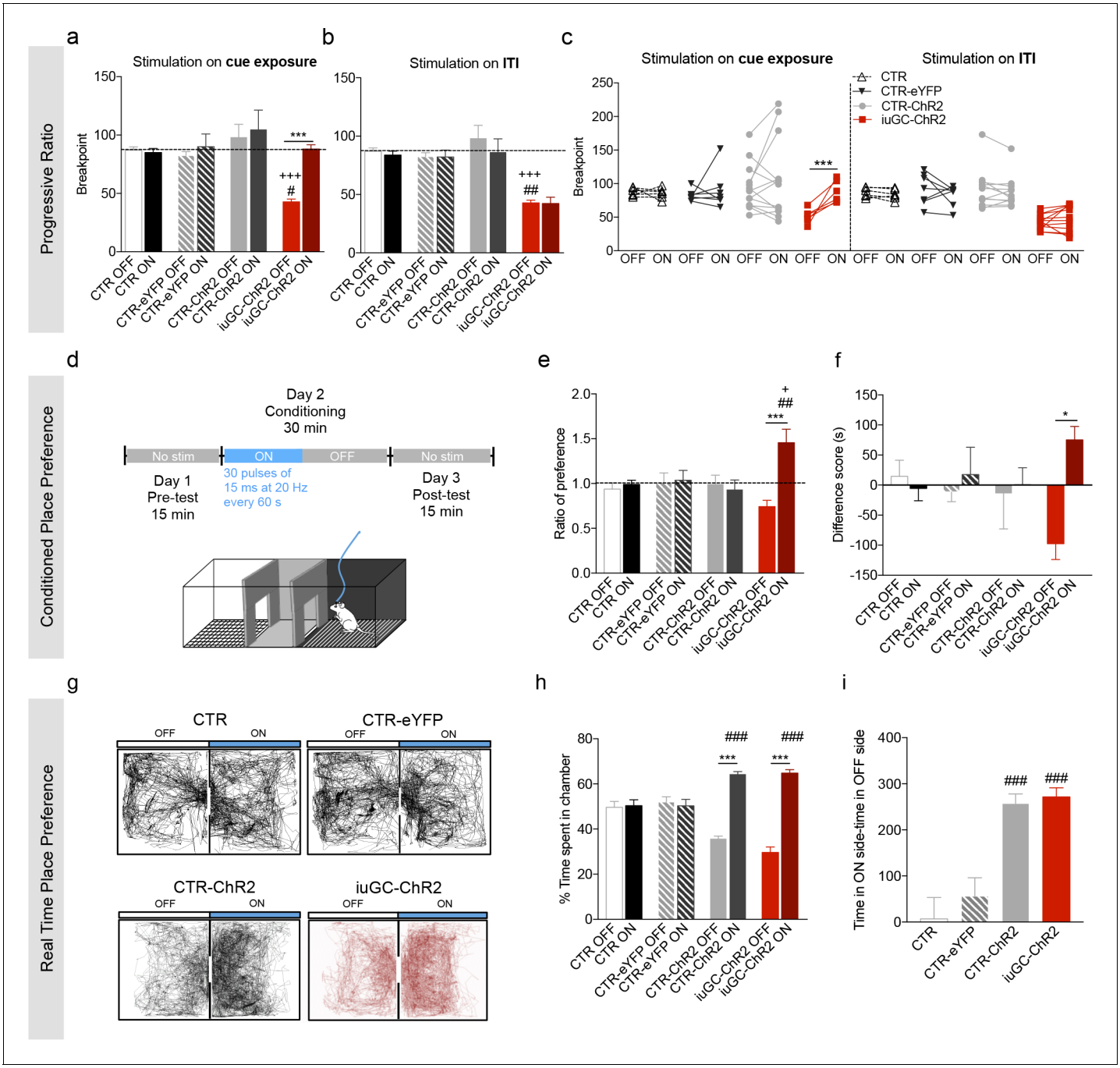

Figure 4. Optogenetic activation of LDT-VTA rescues motivational deficits of iuGC-ChR2 animals and induces conditioning. (**a**) Optogenetic stimulation of LDT terminals in the VTA during cue exposure (30 pulses of 15 ms at 20 Hz) rescues the breakpoint deficits in the PR test of iuGC-ChR2 animals, with no effect in other groups (n_{CTR} = 6; $n_{CTR\text{-}eYFP}$ = 7; $n_{CTR\text{-}ChR2}$ = 13; $n_{iuGC\text{-}ChR2}$ = 12). (**b**) Activation of LDT terminals in the VTA in an *irrelevant* period, such as for example during inter-trial interval (ITI) does not change breakpoint of iuGC-ChR2 animals. (**c**) Individual performance in the PR test. All iuGC-ChR2 animals increase their breakpoint when stimulation is associated with the cue but not during the ITI. (**d**) Schematic representation of the CPP protocol. Laser stimulation (30 pulses of 15 ms at 20 Hz, every 60 s) is associated to one chamber. (**e**) Optogenetic stimulation of LDT terminals in the VTA increases preference for the stimulation-paired box (ON) in iuGC-ChR2 but not in CTR-eYFP nor CTR-ChR2 animals (n_{CTR} = 6; $n_{CTR\text{-}eYFP}$ = 7; $n_{CTR\text{-}ChR2}$ = 5; $n_{iuGC\text{-}ChR2}$ = 6). (**f**) Difference score of CPP protocol shown as the difference in time spent in pre- and post-test. iuGC-ChR2 animals present a shift in preference for the ON chamber. (**g**) Real Time Place Preference (RTPP) protocol: animals were placed in a box with two identical chambers for 15 min and allowed to freely explore. When animals crossed to the ON side, optical stimulation was given until exiting the chamber. Shown are representative tracks from a CTR, CTR-eYFP, CTR-ChR2 and an iuGC-ChR2 animal. (**h**) CTR-ChR2 and iuGC-ChR2 rats spend a significantly higher percentage of time in the stimulation-associated box (ON side) (n_{CTR} = 6; $n_{CTR\text{-}eYFP}$ = 7; $n_{CTR\text{-}ChR2}$ = 8; $n_{iuGC\text{-}ChR2}$ = 6). (**i**) Difference between time spent

Figure 4 continued on next page

Figure 4 continued

in the ON versus OFF side. Data represented as mean ±s.e.m. *p<0.05, **p<0.001, ***p<0.0001. #: comparison with CTR-eYFP; +: comparison with CTR-ChR2. Additional data is depicted in ***Figure 4—figure supplements 1–3***.

DOI: https://doi.org/10.7554/eLife.25843.009

The following figure supplements are available for figure 4:

Figure supplement 1. Effects of iuGC treatment or optogenetic activation of the LDT-VTA circuit in operant learning, food consumption and locomotion.

DOI: https://doi.org/10.7554/eLife.25843.010

Figure supplement 2. Behavioral effects of higher stimulation of LDT-VTA terminals.

DOI: https://doi.org/10.7554/eLife.25843.011

Figure supplement 3. Optic fiber placement of animals used for behavioral experiments.

DOI: https://doi.org/10.7554/eLife.25843.012

We observed that stimulation of LDT terminals in the VTA was sufficient to elicit preference for the stimulation-paired chamber in both CTR-ChR2 and iuGC-ChR2 groups (***Figure 4h***; *post-hoc* Bonferroni CTR-ChR2: $t_{(23)}$ = 8.212, p<0.0001; iuGC-ChR2 $t_{(23)}$ = 8.748, p<0.0001) with no effect on control groups (CTR and CTR-eYFP) (*post-hoc* Bonferroni CTR: $t_{(23)}$ = 0.1981, p>0.9999; CTR-eYFP: $t_{(23)}$ = 1.784, p>0.9999). Both CTR-ChR2 and iuGC-ChR2 groups spent significantly more time in the ON side as assessed by the difference of total time spent in each chamber (***Figure 4i***; *post-hoc* Bonferroni CTR-ChR2 vs. CTR: $t_{(23)}$ = 5.451, p=0.0001; CTR-ChR2 vs. CTR-eYFP: $t_{(23)}$ = 4.562, p=0.0008; iuGC-ChR2 vs. CTR: $t_{(23)}$ = 5.387, p<0.0001; iuGC-ChR2 vs. CTR-eYFP: $t_{(23)}$ = 4.543, p=0.0009).

Discussion

Here we show that prenatal exposure to GCs alters the number of $ChAT^+$ cells and induces long-lasting expression changes on cholinergic markers (ChAT and AChE) in the LDT, but had no effect on glutamatergic or GABAergic markers. These findings are particularly interesting because both ChAT and AChE contain a glucocorticoid response element (GRE) in their gene *loci*, pinpointing a direct transcriptional regulation by GCs (***Finkelstein et al., 1985***; ***Gilad et al., 1985***; ***Imperato et al., 1989***; ***Berse and Blusztajn, 1997***; ***Battaglia and Ogliari, 2005***), although this remains to be confirmed. In fact, it has been shown that stress changes cholinergic enzymes expression (***Finkelstein et al., 1985***; ***Kaufer et al., 1998***), either by inducing an alternative splicing of AChE gene (***Nijholt et al., 2004***), or by modulating the epigenetic status of its promoter regions (***Sailaja et al., 2012***). These cholinergic changes observed in iuGC group are more likely to derive from a new equilibrium set *in utero* by GC exposure rather than by changes in the hypothalamic–pituitary–adrenal (HPA) axis, since in adulthood iuGC animals present normal basal levels of corticosterone (***Blaha and Winn, 1993***).

Importantly, this GC *programming effect* in gene expression has been previously demonstrated for other circuits. For example, GC-exposed animals displayed differential methylation status of dopamine receptor D2 promoter region, accompanied by long-lasting gene/protein expression changes in the NAc (***Rodrigues et al., 2012***). These results show that a brief exposure to GC during critical developmental periods may induce persistent effects in specific genes, which may contribute for the increased vulnerability for emotional disorders observed in early life stress models (***Borges et al., 2013a***; ***Borges et al., 2013b***; ***Piazza and Le Moal, 1996***; ***Murgatroyd et al., 2009***).

We also found that the LDT presents decreased basal neuronal activity, and that LDT electrical stimulation produces a differential response in the VTA of iuGC animals. Indeed, iuGC group presented a decrease in the magnitude and duration of excitatory responses in the VTA, and inversely, the inhibitory response was increased, suggesting that the function of the LDT-VTA pathway was compromised. To our knowledge, this is the first report showing electrophysiological differences in the LDT-VTA circuitry induced by GCs. The latency of excitatory responses in the VTA upon LDT electrical (or optogenetic) stimulation in control animals was remarkably low, but we were unable to find any electrophysiological studies to compare to.

Importantly, the latency of VTA excitatory responses to LDT electrical stimulation was substantially increased in iuGC animals. A combination of pre- and post-synaptic iuGC-induced changes may contribute for this phenomenon, however, because this delay is not observed upon optical

excitation of LDT-VTA terminals, it pinpoints to changes in axonal conductivity. Additional studies are now needed in order to understand how GC induces these long-lasting electrophysiological changes.

The VTA contains around 65% of DAergic neurons and 35% of non-DAergic neurons (**Nair-Roberts et al., 2008**), being the latter mainly GABAergic, though subpopulations of glutamatergic neurons as well as dopamine/glutamate co-releasing neurons have been identified (**Yamaguchi et al., 2011**; **Hnasko et al., 2012**). Considering this, we sub-divided the VTA recorded cells into putative DAergic and GABAergic neurons based on their waveform pattern (**Ungless et al., 2004**; **Ungless and Grace, 2012**; **Totah et al., 2013**), yet, it is important to refer that there is still some controversy regarding this categorization (**Margolis et al., 2006**). Importantly, Omelchenko and colleagues suggested that the LDT mediates a divergent excitation/inhibition influence on mesoaccumbens neurons that is likely to excite DAergic cells and inhibit GABA neurons of this region (**Omelchenko and Sesack, 2005**; **Omelchenko and Sesack, 2006**); which is in accordance with our data in control animals.

The LDT provides the tonic input necessary for maintaining burst firing of DAergic neurons (**Lodge and Grace, 2006**) and dopamine release to the NAc (**Blaha et al., 1996**; **Forster and Blaha, 2000**), contributing to reward behaviors (**Lammel et al., 2012**; **Steidl and Veverka, 2015**; **Xiao et al., 2016**). In fact, phasic activation of VTA DAergic neurons can induce behavioral conditioning (**Tsai et al., 2009**) and facilitate positive reinforcement (**Adamantidis et al., 2011**; **Witten et al., 2011**). Conversely, VTA GABAergic neurons provide local inhibition of DAergic neurons (but also long-range inhibition of projection regions, including the NAc), and their activation disrupts reward consummatory behavior (**van Zessen et al., 2012**). Surprisingly, in iuGC animals we observe a shift in the LDT-VTA evoked responses: an increase of inhibition of DAergic neurons and simultaneous decrease of inhibition of GABAergic neurons. This suggests that VTA DAergic neurons are less active, which is in accordance with the observed decreased basal levels of dopamine in the NAc of iuGC animals (**Leão et al., 2007**; **Rodrigues et al., 2012**).

Confirming the functional relevance of the abovementioned electrophysiological data, we have previously shown that iuGC animals exhibited impaired cue-driven motivational drive (**Soares-Cunha et al., 2014**; **Soares-Cunha et al., 2016**). In line with this, we found that iuGC animals presented significant motivational deficits in the PR test. Remarkably, brief optogenetic activation of LDT-VTA terminals during cue exposure was sufficient to rescue the motivation of iuGC animals, with no major impact on control animals, proving that iuGC exposure induces changes in this circuit that are translated into motivational deficits. However, when LDT-VTA stimulation was done during the time-out period of the test, it did not induce any behavioral effect, reinforcing the importance of specific time windows for the stimulation. To our knowledge, this is the first report showing a role of the LDT-VTA circuit in the control of cue-induced motivation. It is important to refer that we decided to use a strategy that activates all LDT inputs because although the majority of its neurons are cholinergic, there are also glutamatergic and GABAergic neurons in the LDT (**Xiao et al., 2016**; **Wang and Morales, 2009**), and each provide parallel sources of input to the VTA. Certainly, additional studies are needed to dissect and evaluate the contribution of each LDT neuronal population for this type of behaviors.

To further understand the role of LDT-VTA in reward behaviors, we tested the animals in two different conditioning paradigms, the non-contingent CPP and the contingent RTPP. Activation of LDT-VTA specific projections shifts animal's preference for the stimulus-associated chamber in both tests. However, iuGC animals seem more susceptible to these reinforcing effects because a *lower stimulation* protocol (30 pulses of 15 ms at 20 Hz) was able to shift iuGC group preference but had no effect in control animals. Importantly, this vulnerability to rewarding/reinforcing stimulus is in accordance with previous data from our team showing that iuGC animals presented increased morphine-associated CPP in comparison to controls (**Rodrigues et al., 2012**). It is thus tempting to speculate that the increased vulnerability of iuGC animals to the effects of LDT-VTA stimulation is due to an imbalance in the excitation-inhibition responses in the VTA triggered by LDT inputs.

In summary, iuGC exposure leads to long-lasting molecular and physiological alterations in the LDT-VTA circuit in parallel with prominent motivational deficits, which were rescued by optogenetic activation of the LDT-VTA terminals. Moreover, we showed that activation of LDT-VTA inputs is reinforcing and that iuGC animals appear to be more vulnerable to the reinforcing properties of this

stimulus. Further studies are now needed to identify how GCs lead to functional changes in vulnerable regions such as the LDT and how this translates into altered behavior.

Materials and methods

Animals and treatments

Pregnant Wistar rats were individually housed under standard laboratory conditions (light/dark cycle of 12/12 hr; 22°C); food and water *ad libitum*. Subcutaneous injections of a synthetic GC, dexamethasone (DEX, Sigma, Germany) at 1 mg kg^{-1} (iuGC animals) or vehicle (sesame oil, Sigma, Germany; CTR- control animals) were administered on gestation days 18 and 19 (details of the model can be found in ***Leão et al., 2007***; ***Borges et al., 2013a***; ***Soares-Cunha et al., 2014***; ***Rodrigues et al., 2012***; ***Borges et al., 2013b***; ***Blaha and Winn, 1993***). This model, named iuGC (from *in utero* exposure to GCs) partially mimics the clinical administration of GCs on women in risk of preterm labour (~8% of pregnancies) to promote fetal lung maturation or to manage congenital adrenal hyperplasia during pregnancy. On postnatal day 21, progeny was weaned according to prenatal treatment and gender. Male offspring derived from at least 4 different litters were used.

All manipulations were conducted in strict 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 – DGAV. All the experiments were approved by the Ethics Committee of the University of Minho (SECVS protocol #107/2015). The experiments were also authorized by the national competent entity DGAV (#19074).

Macrodissection and molecular analysis

Rats were anaesthetized with sodium pentobarbitone (Eutasil, Sanofi, CEVA, Algés, Portugal), decapitated, and heads were immediately snap-frozen in liquid nitrogen. Brain areas of interest were rapidly dissected on ice under a magnifier following specific anatomical landmarks (***Paxinos and Watson, 2007***).

For real-time PCR analysis, total RNA was isolated from samples using Trizol (Invitrogen, Carlsbad, CA, USA) and treated using DNase (Fermentas, Burlington, Canada) according to the manufacturer's instructions. cDNA was synthetized using the iSCRIPT kit (Biorad, Hercules, CA, USA). PCR was performed using EVAGreen SMX (Biorad, Hercules, CA, USA) and the Biorad q-PCR CFX96 apparatus (Biorad, Hercules, CA, USA). *Hprt* was used as housekeeping gene. Relative quantification was used to determine fold changes (control vs. iuGC), using the ΔΔCT method.

Primer sequences used for transgene expression quantification were:

ChAT: Forward, 5'-TCATTAATTTCCGCCGTCTC-3', Reverse, 5'-CCGGTTGGTGGAGTCTTTTA-3'; AChE: Forward, 5'-CCAGAGACAGAGGACATTCTGA-3', Reverse, 5'-GCGTTCCTGCTTGCTATAG TG-3'; VAChT: Forward, 5'-AGTGCCTACTTGGCCAACAC-3', Reverse, 5'-GTCGTAGCTCATGCGA TCAA-3'; EAAC1; Forward, 5'-CATCCCTCATCCCACATCCG-3', Reverse, 5'-CTACCACGA TGCCCAGTACC-3'; GAD1/Gad67: Forward, 5'- GCTCCCTGTGGCTGAATCG-3', Reverse, 5'- GTCC TTTGCAAGAAACCACAG-3'; GAD2/Gad65: Forward, 5'-CTGGCTTTTGGTCCTTCGGA-3', Reverse, 5'-AGCAGAGCGCATAGCTTGTT-3'; GR: Forward, 5'- AGGCCGGTCAGTGTTTTCT-3', Reverse, 5'-CAATCGTTTCTTCCAGCACA-3'.

For western blotting analysis, samples were prepared as previously described (***Rodrigues et al., 2012***). 30 µg of the protein was run in SDS-polyacrylamide gel and then transferred to nitrocellulose membranes. Membranes were incubated with one of the primary antibodies: goat anti-choline acetyltransferase (ChAT, 1:500, Millipore, MA, USA), goat anti-acetylcholine esterase (AChE, 1:500, Abcam, Cambridge, UK), mouse anti-glutamate transporter, excitatory amino-acid transporter, EAAC1 (EAAC1, 1:200, Millipore, MA, USA), rabbit anti-glutamic acid decarboxylase (GAD) 65 + GAD 67 (GAD65/67, 1:10000, Abcam, Cambridge, UK), rabbit anti-glucocorticoid receptor (GR, 1:500, Santa Cruz, CA, USA) and mouse anti-GAPDH (1:200, Iowa, USA) or mouse anti-beta actin (1:2500, Abcam, Cambridge, UK) were used as loading controls for ChAT or AChE, and EAAC1, GAD65/67 or GR, respectively. The secondary antibodies were incubated at a 1:10000 (anti-mouse), 1:5000 (anti-rabbit) and 1:7500 (anti-goat) dilution (Santa Cruz Biotechnologies, Santa Cruz, CA, USA). Membranes were stripped for 15 min at room temperature in stripping buffer (Restore PLUS Western Blot Stripping Buffer, Thermo Scentific, IL, USA) and re-blocked and re-incubated.

Detection was performed using ECL kit (Biorad, Hercules, CA, USA) and bands were quantified using ImageJ (http://rsbweb.nih.gov/ij/).

Immunohistochemistry (IHC)

Animals were anaesthetized with sodium pentobarbitone (Eutasil, Lisbon, Portugal) and transcardially perfused with saline followed by 4% paraformaldehyde. Brains were removed and sectioned coronally at a thickness of 50 µm, on a vibrating microtome (VT1000S, Leica, Germany).

Free-floating sections were pre-treated with 3% H_2O_2 in PBS for 30 min. After blocking using 2.5% fetal bovine serum (FBS) in PBS-Triton 0.3% for 2 hr at room temperature, sections were incubated overnight at 4˚C with primary antibody anti-ChAT (1:1000; Millipore, MA, USA). Afterwards, sections were washed and incubated with the secondary polyclonal swine anti-goat biotinylated antibody (1:200, DAKO, Denmark) for 1 hr, and processed with an avidin-biotin complex solution (ABC-Elite Vectastain reagent; Vector Lab., USA) and detected with 0.5 mg ml^{-1} 3,3´-diaminobenzidine (Sigma, Germany) including 12.5 µl of 30% H_2O_2 as a substrate in Tris-HCL solution. Sections were washed and mounted on glass slides, air-dried, counterstained with Hematoxilin and coverslipped with Entellan (Merck, NJ, USA). Cell density estimation was obtained by normalizing $ChAT^+$ cells in the corresponding area, determined using an Olympus BX51 optical microscope and the StereoInvestigator software (Microbrightfield). For each animal, 5 slices containing the LDT were used - coordinates according to Paxinos and Watson (***Blaha and Winn, 1993***). The distance of the LDT region analyzed from bregma ranged from: −8.16 mm to −9.48 mm.

In vivo electrophysiology recordings and stimulation

Animals were anesthetized and submitted to a stereotaxic surgery for the placement of the stimulating and recording electrodes, following anatomical coordinates (***Paxinos and Watson, 2007***). Surgeries were performed under sodium pentobarbitone anaesthesia (induction: 60 mg kg^{-1}; maintenance: 15–20 mg kg^{-1}, intraperitoneal, Eutasil, Sanofi, CEVA, Algés, Portugal); body temperature was maintained at approximately 37˚C with a homoeothermic heat pad system (DC temperature controller, FHC, ME, USA). Anaesthesia level was assessed by observation of pupil size, general muscle tone and by assessing withdrawal responses to noxious pinching.

Stimulating and recording electrodes were placed in the following coordinates: LDT: −8.5 from bregma, 0.8 lateral from midline, −5.5 to −7.9 ventral to brain surface; VTA: −5.4 from bregma, 0.6 lateral from midline, −7.5 to −8.2 ventral to brain surface. A reference electrode was fixed in the skull, in contact with the dura.

Extracellular neural activity from the LDT and the VTA was recorded using a recording electrode (3–7 MΩ at 1 kHz). Recordings were amplified and filtered by the Neurolog amplifier (NL900D, Digitimer Ltd, UK) (low-pass filter at 500 Hz and high-pass filter at 5 kHz). Bi-polar concentric electrode (0.05–0.1 MΩ, Science Products) was inserted in the LDT region. Spontaneous activity of single neurons was recorded to establish baseline for at least 100 s. The stimulation was administered using a square pulse stimulator and a stimulus isolator (DS3, Digitimer, UK). The stimulation consisted of 100 pulses of 0.5 Hz with 0.5 ms duration with intensity from 0.2 to 1 mA. Spikes of a single neuron were discriminated, and data sampling was performed using a CED micro 1401 interface and SPIKE 2 software (Cambridge Electronic Design, Cambridge, UK). Single pulses were delivered to the specific brain region every 2 s. At least 100 trials were administered per cell.

For data analysis, peristimulus time histograms (PSTHs; 5 ms bin width) of neuronal activity were generated during electrical stimulation of the LDT, for each neuron recorded in the VTA. PSTHs were analysed 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). The onset of inhibition was defined as the first of 5 bins whose mean value were below 30% of the baseline activity and the response offset when the activity of the neurons was consistently above 30% of the baseline activity. The total duration of the inhibition was determined for each neuron. We classified single units in the VTA into three separated

groups of putative neurons: putative dopamine (DA), putative GABA, and 'other' neurons. This classification was based on firing rate and waveform duration (calculated from average spike waveform) (*Ungless et al., 2004*; *Ungless and Grace, 2012*; *Totah et al., 2013*). Cells presenting a firing rate <10.0 Hz and a duration of >1.5 ms were considered putative DAergic (pDAergic) neurons. If the firing rate was >10.0 Hz and waveform duration <1.5 ms, cells were assigned to putative GABAergic (pGABAergic) neuron group. Other single units were assigned to the 'other' neuron group. This group likely contains units from both DA and GABA groups.

Regarding the experiments with optical stimulation, a recording electrode coupled with a fiber optic patch cable (Thorlabs) was placed in the VTA or LDT. The DPSS 473 nm laser system (CNI), controlled by a stimulator (Master-8, AMPI), was used for intracranial light delivery and fiber optic output was pre-calibrated to 10–15 mW. Spontaneous activity was recorded for 60 s to establish baseline activity. Optical stimulation consisted of 30 pulses of 15 ms at 20 Hz and 80 pulses of 15 ms at 20 Hz. Firing rate was calculated for the baseline, stimulation period and post stimulation period (60 s after the end of stimulation). Neurons showing a firing rate increase or decrease by more than 20% from the mean frequency of the baseline period were considered as responsive, as previously reported by Benazzouz and colleagues (*Benazzouz et al., 2000*).

At the end of each electrophysiological experiment, all brains were collected and processed to identify recording region.

Optogenetics constructs

AAV5–EF1a–WGA–Cre–mCherry, AAV5–EF1a–DIO–hChR2–YFP and AAV5–EF1a–DIO–YFP were obtained directly from the Gene Therapy Center Vector Core (UNC) center (vectors kindly provided by Karl Deisseroth, Stanford University). AAV5 vector titers were 2.1–6.6 $\times$ 10^{12} virus molecules ml^{-1}.

Surgery and cannula implantation

Rats designated for behavioral experiments were anesthetized with 75 mg kg^{-1} ketamine (Imalgene, Merial) plus 0.5 mg kg^{-1} medetomidine (Dorbene, Cymedica). One µl of AAV5–EF1a–WGA–Cre–mCherry was unilaterally injected into the VTA (coordinates from bregma, according to Paxinos and Watson: −5.4 mm anteroposterior, +0.6 mm mediolateral, and −7.8 mm dorsoventral) and 1 µl of AAV5–EF1a–DIO–hChR2–YFP was injected in the LDT (coordinates from bregma: −8.5 mm anteroposterior, +0.9 mm mediolateral, and −6.5 mm dorsoventral) in both CTR and iuGC groups (CTR-ChR2 and iuGC-ChR2). We had two additional groups: a control group (CTR) that was injected only with 1 µl AAV5–EF1a–DIO–hChR2–YFP in the LDT; and CTR-YFP animals which were injected with 1 µl AAV5–EF1a–WGA–Cre–mCherry in the VTA and 1 µl AAV5–EF1a–DIO–YFP in the LDT. Rats were then implanted with an optic fiber (200 µm core fiber optic; Thorlabs, NJ, USA) with 2.5 mm stainless steel ferrule (Thorlabs, NJ, USA) using the injection coordinates for the VTA (with the exception of dorsoventral: −7.7 mm) that were secured to the skull using 2.4 mm screws (Bilaney, Germany) and dental cement (C and B kit, Sun Medical). Rats were removed from the stereotaxic frame and sutured. Anaesthesia was reverted by administration of atipamezole (1 mg/kg). After surgery animals were given anti-inflammatory (Carprofeno, 5 mg/kg) for one day, analgesic (butorphanol, 5 mg/kg) for 3 days, and were let to fully recover before initiation of behavior. Optic fiber placement was confirmed for all animals after behavioral experiments (*Figure 4—figure supplement 3*). Animals that were assigned for electrophysiological experiments were not implanted with an optic fiber.

Behavior

Progressive ratio schedule of reinforcement

Rats were placed and maintained on food restriction (≈7 g/day of standard lab chow) to maintain 90% free-feeding weight. Behavioral sessions were performed in operant chambers (Med Associates, IL, USA) containing a central magazine that provided access to 45 mg food pellets (Bio-Serve), two retractable levers located on each side of the magazine with cue lights above them. A 2.8W, 100mA house light positioned at the top-centre of the wall opposite to the magazine provided illumination. A computer equipped with Med-PC software (Med Associates, IL, USA) controlled the equipment and recorded the data.

The behavioral protocol was previously described (*Soares-Cunha et al., 2016*; *Wanat et al., 2013*). Animals were first trained on continuous reinforcement (CRF) schedule: a single lever press

yields one pellet. Side of the active lever was alternated between sessions. Rats were then trained in a fixed ratio (FR) schedule comprising 50 trials with both levers presented, but the active lever signalled by the illumination of the above cue light. When achieving the correct number of lever presses, a pellet was delivered, levers retracted and the cue light turned off for a 20 s inter-trial interval (ITI). Following up, rats were trained using an FR4 reinforcement schedule for 4 days and a FR8 for one day, for both levers. Rats were then exposed to the following schedule: day 1 – FR4 (left lever); day 2- PR (left lever); day 3- FR4 (left lever); day 4 – FR4 (right lever); day 5 – PR (right lever). Food rewards were earned on an FR4 reinforcement schedule during FR sessions. PR sessions were similar to FR4 sessions except the operant requirement on each trial (T) was the integer (rounded down) of $1.4^{(T-1)}$ lever presses, starting at 1 lever press. PR sessions ended after 15 min without completion of the response requirement in a trial.

Before the PR session began, rats were connected to an opaque optical fiber in the VTA through previously implanted fiber optic cannula. The optical fiber was connected to a 473 nm DPSS laser (CNI Laser), controlled using a pulse generator (Master-8; AMPI). At the beginning of each trial of the PR session – when the cue light was turned on – animals received an optical stimulation, which consisted in 30 pulses of 15 ms at 20 Hz (473 nm; 10 mW of light at the tip of the optic fiber). In a second set of animals, the number of pulses was increased to 80 pulses of 15 ms at 20 Hz during each cue exposure. CTR, CTR-YFP, CTR-ChR2 and iuGC-ChR2 received this optical stimulation.

Conditioned place preference – CPP

The CPP protocol was adapted from a previously published report (***Lammel et al., 2012***; ***Ungless and Grace, 2012***). Briefly, on day 1, individual rats were placed in the centre chamber and allowed to freely explore the entire apparatus for 15 min (pre-test). On day 2, rats were confined to one of the side chambers for 30 min and paired with optical stimulation, ON side; in the second session, rats were confined to the other side chamber for 30 min with no stimulation, OFF side. Conditioning sessions were counterbalanced. On day 3 rats were allowed to freely explore the entire apparatus for 15 min (post-test). Optical stimulation consisted of 30 pulses of 15 ms at 20 Hz, every 60 s. In a second set of animals optical stimulation was increased to 80 pulses of 15 ms at 20 Hz, every 15 s.

Real-time place preference – RTPP

RTPP test was performed in a custom-made black plastic arena (60 × 60 × 40 cm) comprised by two indistinguishable chambers, for 15 min. One chamber was paired with light stimulation of 15 ms pulses at 20 Hz during the entire period that the animal stayed in the stimulus-paired side. The choice of paired chamber was counterbalanced across rats. Animals were placed in the no-stimulation chamber at the start of the session and light stimulation started at every entry into the paired chamber. Animal activity was recorded using a video camera and time spent in each chamber was manually assessed. Results are presented as total time spent in each chamber.

Immunofluorescence (IF)

Animals were anaesthetized with sodium pentobarbitone (Eutasil, Lisbon, Portugal) and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde. Brains were removed and sectioned coronally at a thickness of 50 µm, on a vibrating microtome (VT1000S, Leica, Germany). Sections were incubated overnight, with the primary antibody goat anti-GFP (1:500, Abcam, Cambridge, UK), followed by secondary fluorescent antibody (1:1000, Invitrogen, MA, USA). All sections were stained with 4',6-diamidino-2-phenylindole (DAPI; 1 mg ml-1) and mounted using mounting media (Permafluor, Invitrogen, MA, USA).

Statistical analysis

Statistical analysis was performed in GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA) and SPSS Statistics v19.0 (IBM corp., USA). Parametric tests were used whenever Shapiro-Wilk normality test SW >0.05. Two-way analysis of variance (ANOVA) was used when appropriate. Bonferroni's post hoc multiple comparison tests were used for group differences determination. Statistical analysis between two groups was made using Student's t-test. Results are presented as mean ±SEM. Statistical significance was accepted for $p<0.05$.

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Author contributions

Bárbara Coimbra, Data curation, Formal analysis, Validation, Investigation, Visualization, Methodology, Writing—original draft, Writing—review and editing; Carina Soares-Cunha, Data curation, Formal analysis, Investigation, Methodology; Sónia Borges, Formal analysis, Visualization; Nivaldo AP Vasconcelos, Data curation, Formal analysis, Investigation; Nuno Sousa, Conceptualization, Supervision, Funding acquisition, Project administration, Writing—review and editing; AJR, Conceptualization, Supervision, Funding acquisition, Investigation, Methodology, Writing—original draft, Project administration, Writing—review and editing

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Animal experimentation: All manipulations were conducted in strict accordance with European Regulations (European Union Directive 2010/63/EU). Animal facilities and the people directly involved in

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Supplementary Information

Impairments in laterodorsal tegmentum to VTA projections underlie glucocorticoid-triggered reward deficits

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RESEARCH ARTICLE

Figures and figure supplements

Impairments in laterodorsal tegmentum to VTA projections underlie glucocorticoid-triggered reward deficits

Bárbara Coimbra *et al*

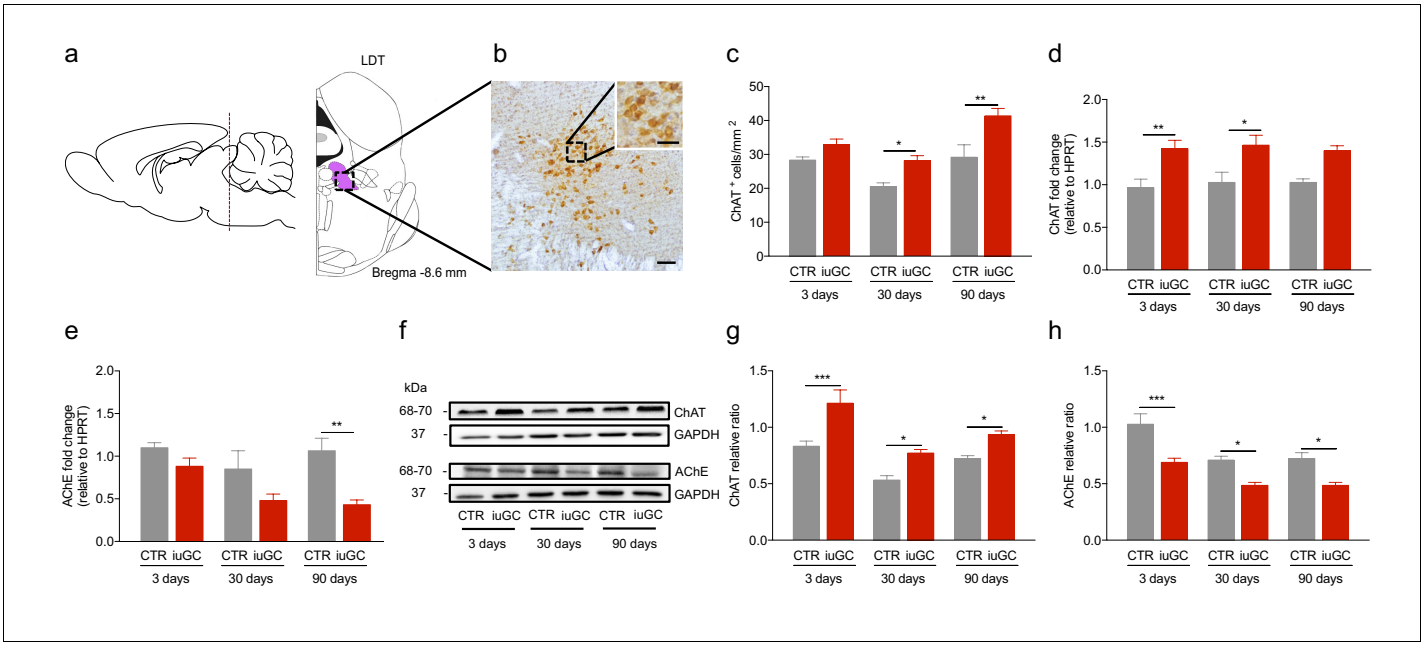

Figure 1. Prenatal exposure to glucocorticoids alters LDT cholinergic system. (**a**) Schematic representation of the LDT. (**b**) Coronal section of the LDT showing ChAT immunohistochemistry. (**c**) iuGC animals present increased number of ChAT^{+} cells in the LDT at postnatal day 30 and 90. (**d**) Real-time PCR analysis revealed that ChAT mRNA levels are increased in the LDT of iuGC animals from postnatal day 3. (**e**) Conversely, AChE mRNA levels are decreased at postnatal day 90 (n_{CTR} = 4; n_{iuGC} = 5). (**f**) Representative immunoblot of ChAT and AChE in the LDT of 3, 30 and 90 days old animals. (**g**) Protein quantification confirmed the upregulation of ChAT and (**h**) downregulation of AChE in the LDT from postnatal day 3 until adulthood (n_{CTR} = 4; n_{iuGC} = 5). Data represented as mean ±s.e.m. *p<0.05, **p<0.001, ***p<0.0001. Scale bars in b: 100 µm and inset - 50 µm. Additional data is depicted in ***Figure 1—figure supplements 1***, ***2*** and ***3***.

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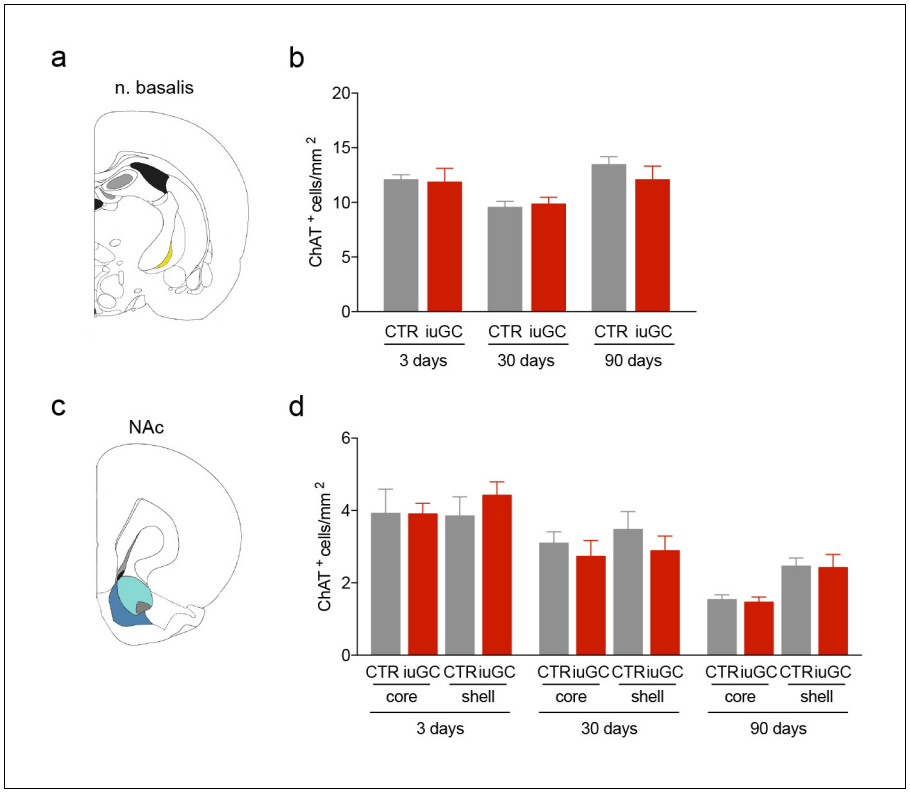

Figure 1—figure supplement 1. iuGC animals do not present changes in the number of cholinergic cells in other regions. (**a**) Schematic representation of the nucleus basalis of Meynert (n. basalis) and (**d**) nucleus accumbens (NAc). (**b-d**; **f–h**) No changes between groups were observed in the number of ChAT^{+} cells. Data represented as mean ±s.e.m.

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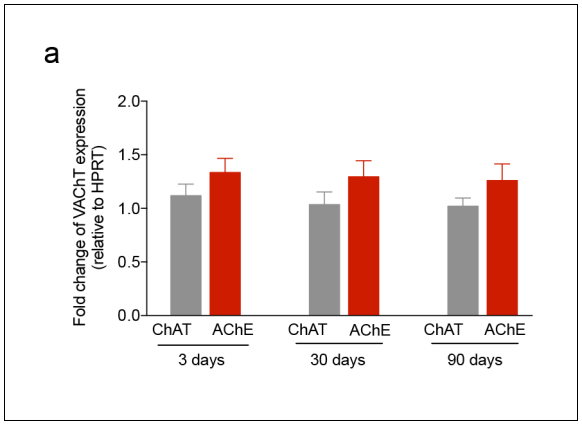

Figure 1—figure supplement 2. iuGC exposure does not change the expression levels of VAChT. (**a–c**) Real-time PCR analysis showed no changes in the levels of VAChT in the LDT for all ages. Data represented as mean ±s.e.m.

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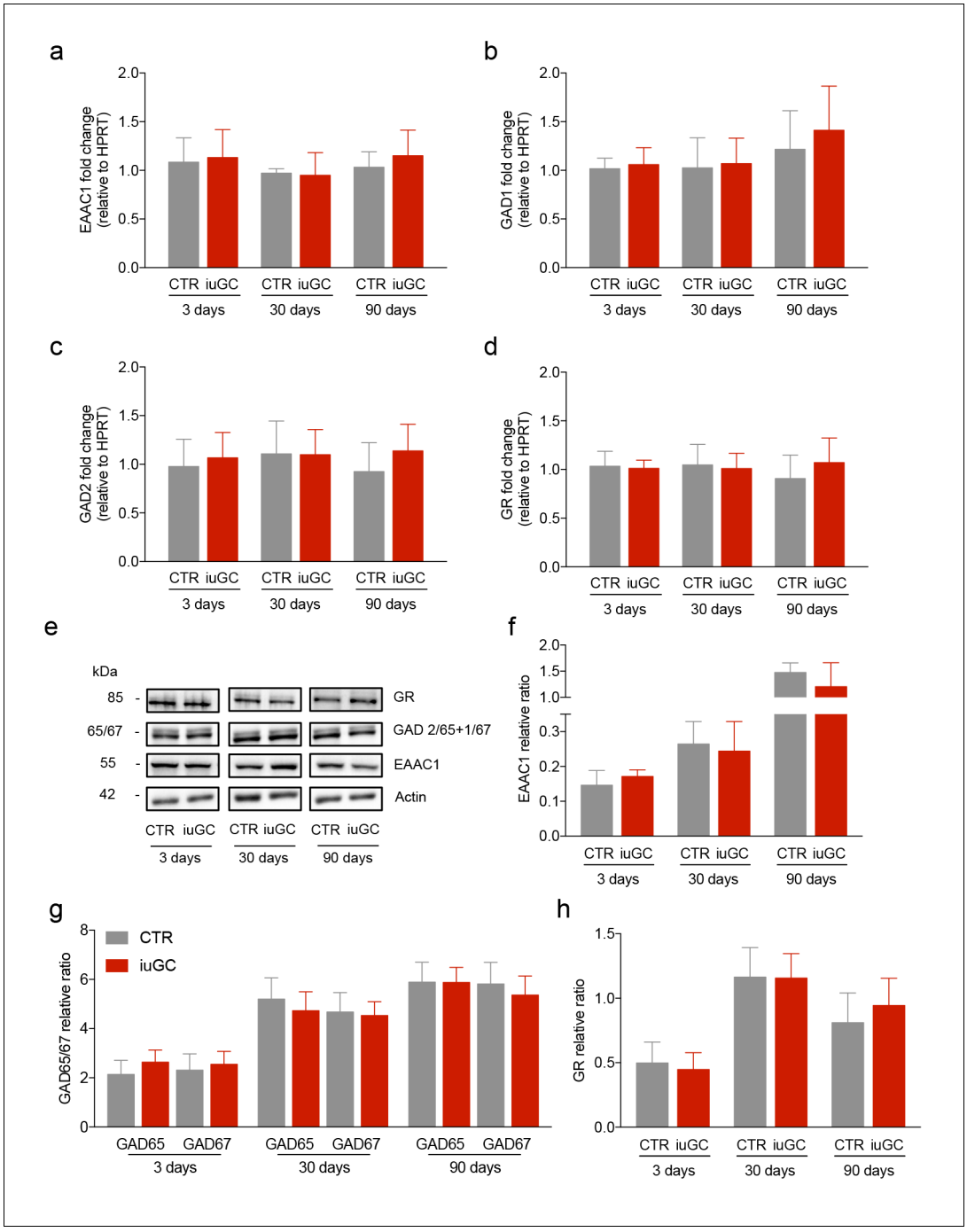

Figure 1—figure supplement 3. iuGC exposure does not change the expression levels of GABAergic and glutamatergic markers in the LDT. Real-time PCR analysis showed no changes in the mRNA levels of (**a**) EAAC1, (**b**) GAD1, (**c**) GAD2 and (**d**) GR in the LDT for all ages. (**e**) Representative immunoblot of EAAC1, GAD67/GAD65, GR and loading controls in the LDT of 3, 30 and 90 days old animals. Band quantification of (**f**) EAAC1, (**g**) GAD67 +GAD65, (**h**) GR in the LDT of 3, 30 and 90 days old animals. (n_{CTR} = 4; n_{iuGC} = 5). Data represented as mean ±s.e.m.

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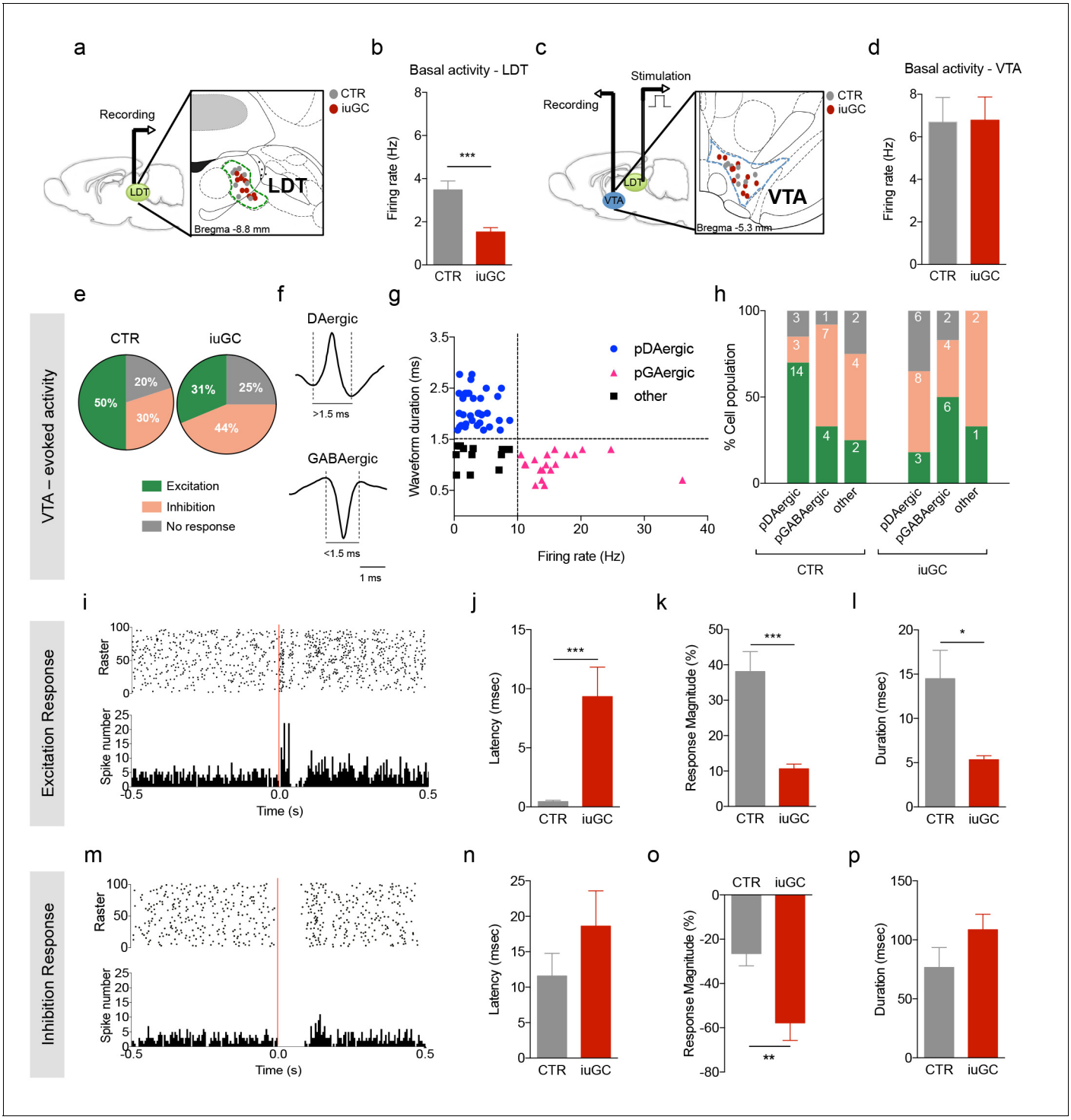

Figure 2. Distinct VTA neuronal response to LDT electrical stimulation in iuGC animals. (**a**) Schematic representation of the *in vivo* single-cell electrophysiological recording experiments and electrode placement in the LDT in anesthetized animals. (**b**) iuGC animals present decreased basal activity of LDT neurons in comparison to CTR ($n_{LDT\text{-}CTR}$ = 36 cells; $n_{LDT\text{-}iuGC}$ = 94 cells). (**c**) Schematic representation of the *in vivo* single-cell electrophysiological recording experiments in the VTA with electric stimulation performed in the LDT; and recording electrode placement. (**d**) The basal activity of the VTA is similar between groups ($n_{VTA\text{-}CTR}$ = 40 cells; $n_{VTA\text{-}iuGC}$=32 cells). (**e**) Electrical stimulation of the LDT (0.5 Hz) induces excitatory and inhibitory responses in VTA neurons. Pie plots represent the percentage of excitatory, inhibitory and no responses of VTA neurons. (**e–g**) In CTR animals, 50% of neurons present an excitatory response (70% pDAergic, 20% pGABAergic) and 30% present an inhibitory response (21% pDAergic, 50% pGABAergic). iuGC group shows a different profile, with 31% of recorded cells presenting an excitatory response (30% pDAergic, 60% pGABAergic)

Figure 2 continued on next page

Figure 2 continued

versus 44% with inhibitory response (57% pDAergic, 29% pGABAergic). (**f**) Representative examples of rat VTA pDAergic and pGABAergic neuronal waveforms. (**g**) Firing rate and waveform duration were used to classify single units into 3 types of neurons. (**h**) Percentage of each putative neuronal population presenting excitation, inhibition or with no response to LDT stimulation. There is a shift in the percentage of putative DAergic and GABAergic neurons presenting excitatory and inhibitory responses. Numbers in bars represent number of cells in each category. (**i, m**) Peristimulus time histograms (PSTHs) show LDT-evoked responses of VTA dopamine neurons; (**i**) excitation; (**m**) inhibition. (**j**) VTA neurons that display an excitatory profile in response to LDT electrical stimulus present increased latency to fire in iuGC animals. (**k**) The magnitude and (**l**) duration of response of VTA neurons is reduced in iuGC animals. (**n**) VTA neurons that display an inhibitory response in response to LDT electrical stimulus do not show differences in the latency to fire in both groups. (**o**) The magnitude of response of inhibited neurons of the VTA is increased in iuGC animals, with no differences in (**p**) the duration of inhibition in VTA neurons upon LDT stimulation. pDAergic: putative dopaminergic neurons; pGABAergic: putative GABAergic neurons. Data is represented as mean ±s.e.m. *p<0.05, **p<0.001, ***p<0.0001.

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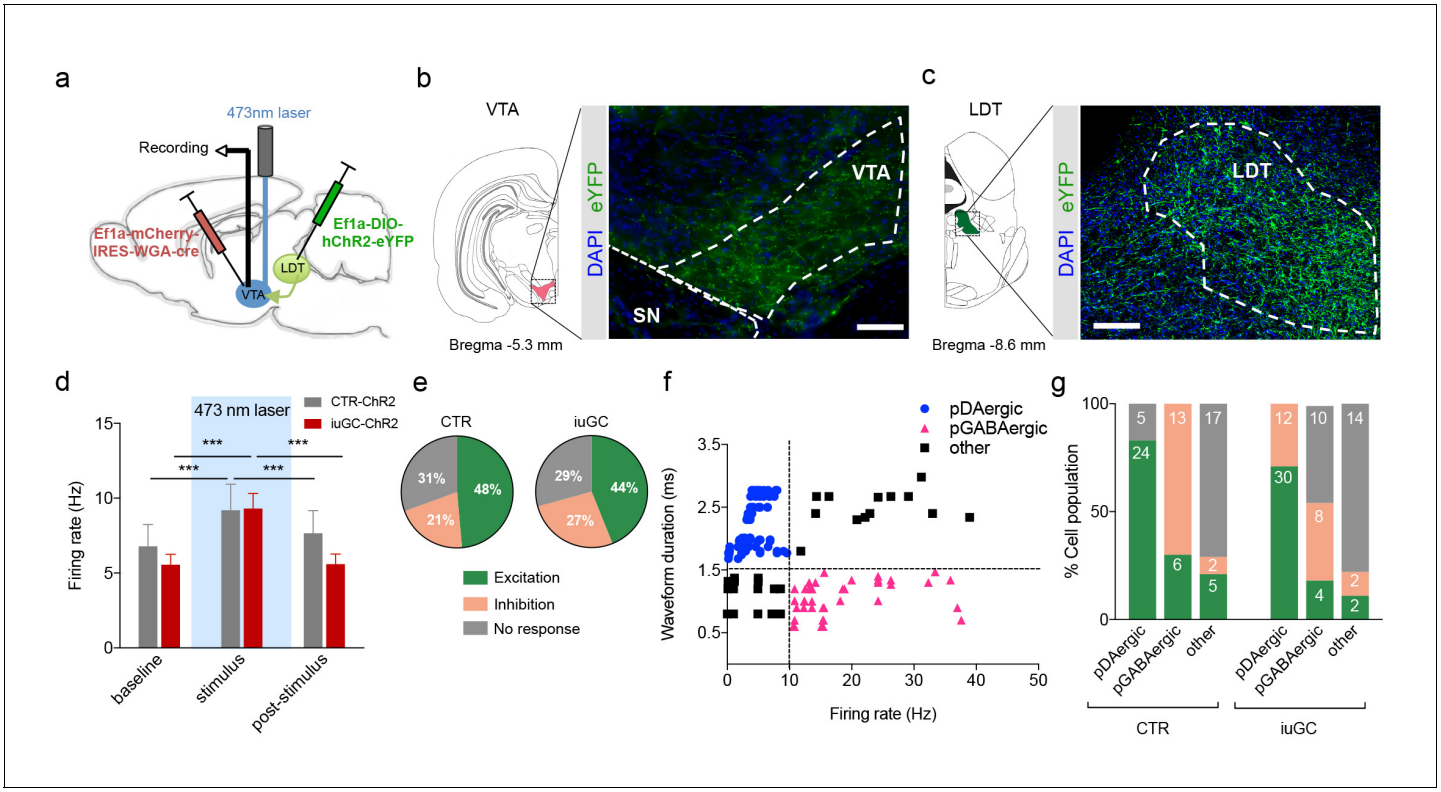

Figure 3. Optogenetic activation of LDT terminals in VTA elicits a differential electrophysiological response in iuGC animals. (**a**) Strategy used for optogenetic activation of LDT projecting neurons in the VTA. An AAV5–EF1a–WGA–Cre–mCherry virus construct was injected unilaterally in the VTA, and a cre-dependent ChR2 vector (AAV5-EF1a-DIO-hChR2-eYFP) in the LDT. WGA-Cre will retrogradely migrate and induce the expression of ChR2 in LDT neurons that directly project to the VTA. (**b**) Representative image of immunofluorescence for GFP showing LDT axon terminals in the VTA and (**c**) cell bodies in the LDT; scale bar: 200 µm. (**d**) Optogenetic stimulation of LDT terminals in the VTA (blue rectangle; 30 pulses of 15 ms at 20 Hz) increases the firing rate of VTA neurons in both groups (n_{CTR} = 72 cells; n_{iuGC} = 82 cells). (**e**) In CTR, upon LDT terminal stimulation, 48% of recorded VTA cells present an increase in firing rate (of those 69% pDAergic, 17% pGABAergic), 21% decrease activity (0% pDAergic, 87% pGABAergic) and 31% presented no change. In iuGC animals, upon LDT terminal stimulation, 44% of recorded VTA cells present an increase in firing rate (83% pDAergic; 11% GABAergic) 27% decrease activity (55% pGABAergic, 36% DAergic) and 29% presented no change. (**f**) Firing rate and waveform duration were used to classify single units into 3 types of neurons. (**g**) Percentage of each putative neuronal population presenting excitation, inhibition or with no response to LDT terminals optogenetic stimulation. Numbers in bars represent number of cells in each category. pDAergic: putative dopaminergic neurons; pGABAergic: putative GABAergic neurons. Data represented as mean ±s.e.m. ***p<0.001. Additional data is depicted in ***Figure 1—figure supplement 1***.

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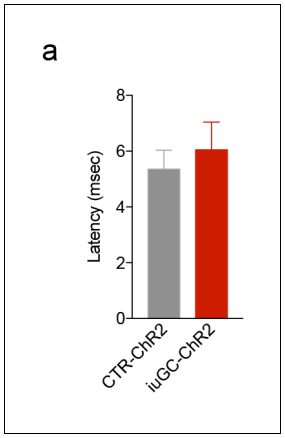

Figure 3—figure supplement 1. iuGC treatment has no effect on the response latency after optical stimulation of the LDT-VTA circuit. (**a**) Latency of neuronal response to LDT-VTA terminals optical stimulation (30 pulses of 15 ms at 20 Hz) from cells depicted in ***Figure 3***. Data represented as mean ±s.e.m.

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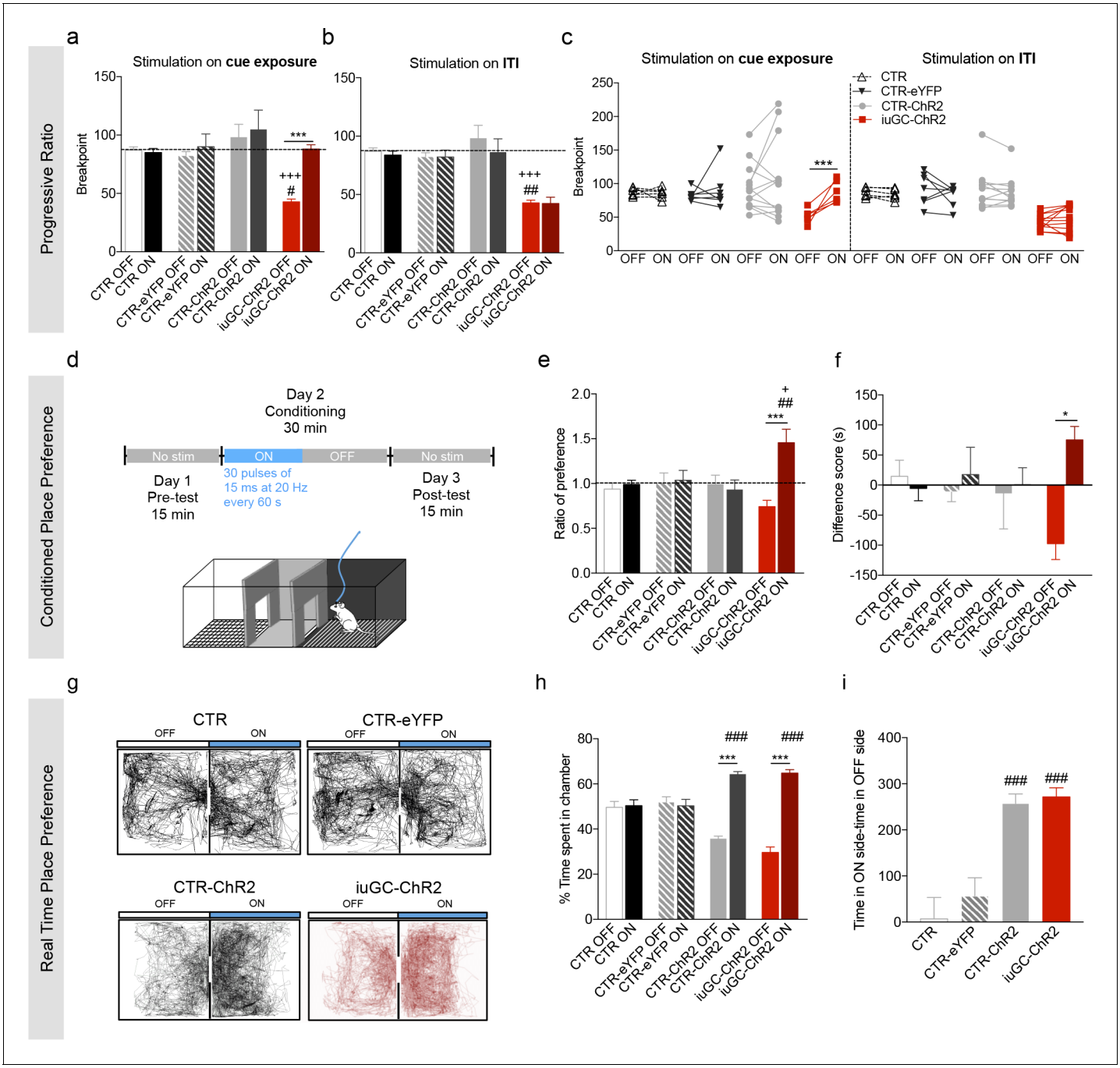

Figure 4. Optogenetic activation of LDT-VTA rescues motivational deficits of iuGC-ChR2 animals and induces conditioning. (**a**) Optogenetic stimulation of LDT terminals in the VTA during cue exposure (30 pulses of 15 ms at 20 Hz) rescues the breakpoint deficits in the PR test of iuGC-ChR2 animals, with no effect in other groups (n_{CTR} = 6; $n_{CTR\text{-}eYFP}$ = 7; $n_{CTR\text{-}ChR2}$ = 13; $n_{iuGC\text{-}ChR2}$ = 12). (**b**) Activation of LDT terminals in the VTA in an *irrelevant* period, such as for example during inter-trial interval (ITI) does not change breakpoint of iuGC-ChR2 animals. (**c**) Individual performance in the PR test. All iuGC-ChR2 animals increase their breakpoint when stimulation is associated with the cue but not during the ITI. (**d**) Schematic representation of the CPP protocol. Laser stimulation (30 pulses of 15 ms at 20 Hz, every 60 s) is associated to one chamber. (**e**) Optogenetic stimulation of LDT terminals in the VTA increases preference for the stimulation-paired box (ON) in iuGC-ChR2 but not in CTR-eYFP nor CTR-ChR2 animals (n_{CTR} = 6; $n_{CTR\text{-}eYFP}$ = 7; $n_{CTR\text{-}ChR2}$ = 5; $n_{iuGC\text{-}ChR2}$ = 6). (**f**) Difference score of CPP protocol shown as the difference in time spent in pre- and post-test. iuGC-ChR2 animals present a shift in preference for the ON chamber. (**g**) Real Time Place Preference (RTPP) protocol: animals were placed in a box with two identical chambers for 15 min and allowed to freely explore. When animals crossed to the ON side, optical stimulation was given until exiting the chamber. Shown are representative tracks from a CTR, CTR-eYFP, CTR-ChR2 and an iuGC-ChR2 animal. (**h**) CTR-ChR2 and iuGC-ChR2 rats spend a significantly higher percentage of time in the stimulation-associated box (ON side) (n_{CTR} = 6; $n_{CTR\text{-}eYFP}$ = 7; $n_{CTR\text{-}ChR2}$ = 8; $n_{iuGC\text{-}ChR2}$ = 6). (**i**) Difference between time spent

Figure 4 continued on next page

Figure 4 continued

in the ON versus OFF side. Data represented as mean ±s.e.m. *p<0.05, **p<0.001, ***p<0.0001. #: comparison with CTR-eYFP; +: comparison with CTR-ChR2. Additional data is depicted in ***Figure 4—figure supplements 1–3***.

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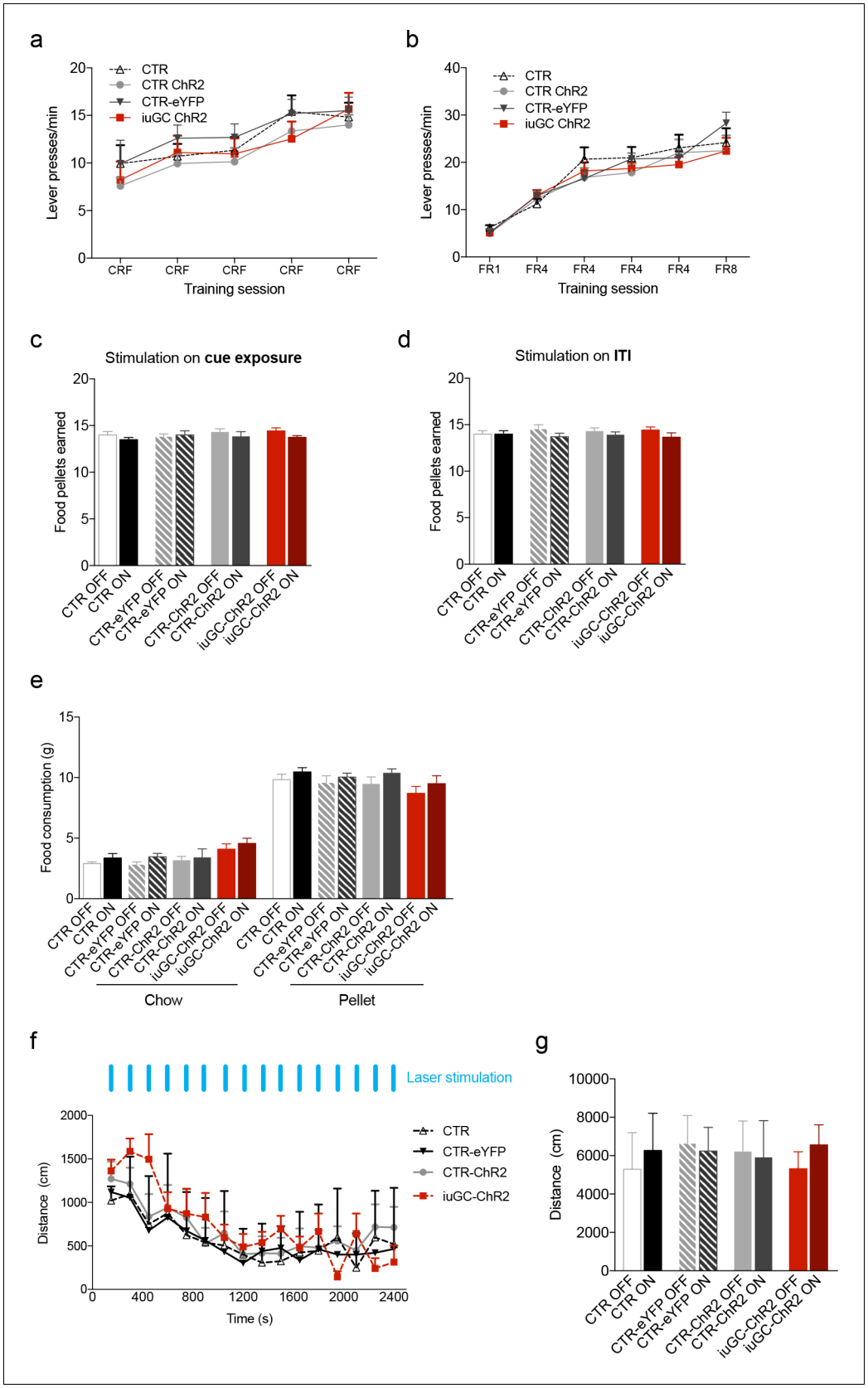

Figure 4—figure supplement 1. Effects of iuGC treatment or optogenetic activation of the LDT-VTA circuit in operant learning, food consumption and locomotion. Performance in the (**a**) Conditioning Reinforcement (CRF)

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Figure 4—figure supplement 1 continued

and (**b**) Fixed Ratio (FR) sessions was similar between groups (n_{CTR} = 6; $n_{CTR\text{-}eYFP}$ = 7; $n_{CTR\text{-}ChR2}$ = 13; $n_{iuGC\text{-}ChR2}$ = 12). (**c**) Total number of food pellets earned in the PR session, in which optical stimulation was given during cue exposure and (**d**) during the ITI. (**e**) No effects in free feeding behavior – regular chow or pellets in stimulated animals (n_{CTR} = 6; $n_{CTR\text{-}eYFP}$ = 7; $n_{CTR\text{-}ChR2}$ = 8; $n_{iuGC\text{-}ChR2}$ = 6). (**f**) No differences were observed in the locomotor activity of CTR, CTR-eYFP, CTR-ChR2 and iuGC upon stimulation (30 pulses of 15 ms at 20 Hz; given every minute). (**g**) Total distance travelled revealed no differences between groups (n_{CTR} = 6; $n_{CTR\text{-}eYFP}$ = 7; $n_{CTR\text{-}ChR2}$ = 8; $n_{iuGC\text{-}ChR2}$ = 6). Data represented as mean ±s.e.m.

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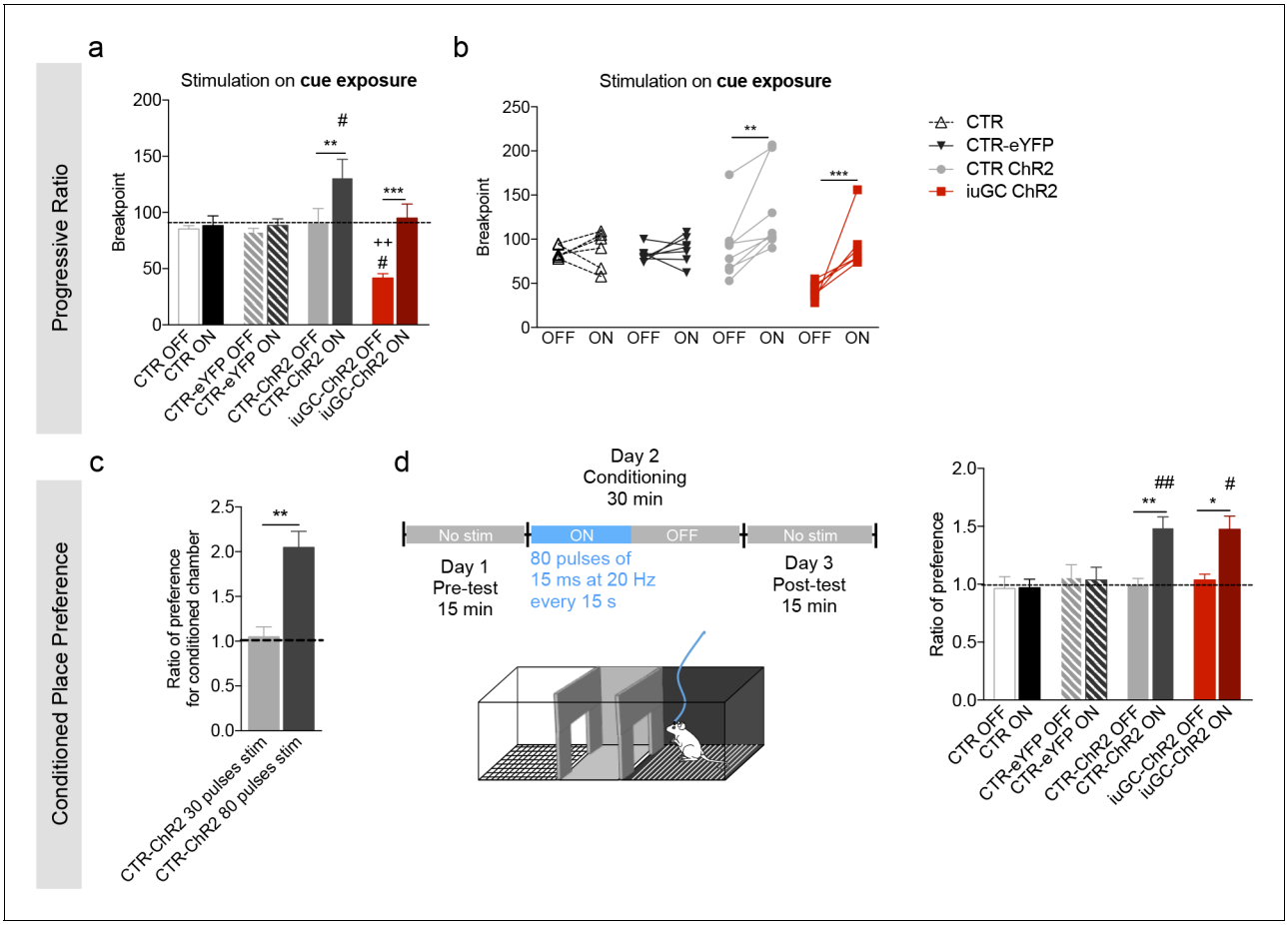

Figure 4—figure supplement 2. Behavioral effects of higher stimulation of LDT-VTA terminals. (**a**) To verify the reinforcing properties of LDT-VTA stimulation, we performed a different protocol of optogenetic stimulation of LDT terminals that consists in higher number of pulses - 80 pulses of 15 ms at 20 Hz. (**a**) This stimulation is able to increase the motivational levels of both CTR-ChR2 and iuGC-ChR2 animals as seen by their increased breakpoint. No effect in CTR and CTR-eYFP groups. (**b**) Individual performance in the progressive ration task during laser OFF and laser ON sessions (n_{CTR-}=6; $n_{CTR-ChR2}$ = 8; $n_{iuGC-ChR2}$ = 6). (**c**) Ratio of preference of CTR-ChR2 and iuGC-ChR2 rats increases in the CPP in response to higher number of stimuli (80 pulses of 15 ms at 20 Hz) ($n_{CTR-ChR2-stim}$=5, $n_{CTR-ChR2-high\ stim}$=8). (**d**) Higher number of stimuli (80 pulses of 15 ms at 20 Hz, every 15 s) induces preference in CTR-ChR2 and iuGC-ChR2 animals, but not in control groups (n_{CTR} = 6; $n_{CTR-eYFP}$ = 7; $n_{CTR-ChR2}$ = 8; $n_{iuGC-ChR2}$ = 6). Data represented as mean ±s.e. m.*p<0.05; **p<0.01; ***p<0.001. #: Comparison with CTR-eYFP.

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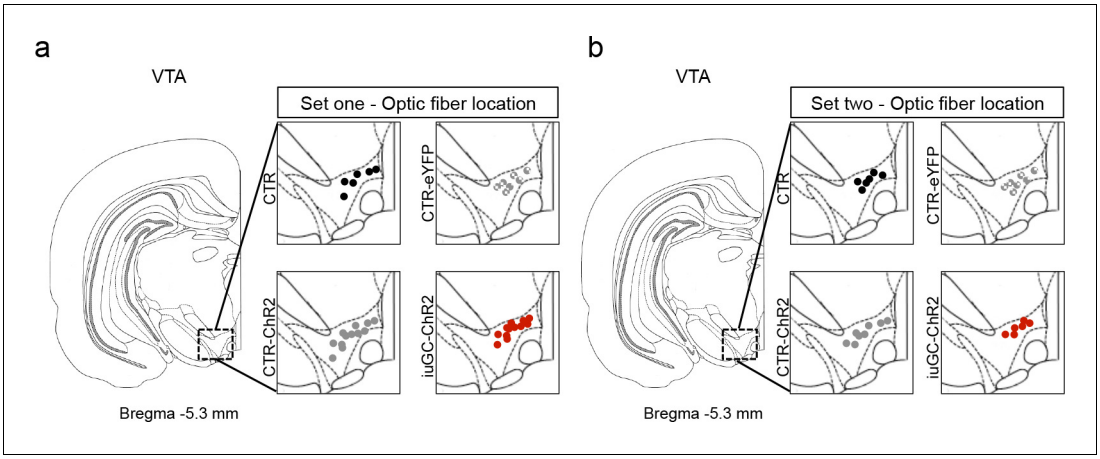

Figure 4—figure supplement 3. Optic fiber placement of animals used for behavioral experiments. Schematic representation of optic fiber placement in LDT terminals in the VTA of the first (**a**) and second (**b**) set of rats that performed behavioral experiments.

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Chapter 2.2

Role of laterodorsal tegmentum projections to VTA in multiple phases of reward behaviour

Manuscript in preparation

Role of laterodorsal tegmentum projections to VTA in multiple phases of reward behaviour

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Abstract

The laterodorsal tegmentum (LDT) has been classically involved in rapid eye movement (REM) sleep and attention. Lately, due to the fact that LDT tightly controls the activity of ventral tegmental area (VTA) dopaminergic neurons, modulating dopamine release in the nucleus accumbens, this region has also been associated with reward-related behaviours.

To further understand the role of LDT-VTA inputs in reward-related behaviours, we used optogenetics to specifically manipulate these inputs during different behavioural paradigms. We found that phasic activation of LDT-VTA terminals shifts and amplifies preference to a laser-paired reward in comparison to an otherwise equal reward. In addition, in the progressive ratio task, LDT-VTA stimulation boosts motivation, since animals present increased breakpoint, i.e, an enhancement in the willingness to work to get the reward associated with the laser-paired lever. Interestingly, animals still manifest preference for the laser-associated reward in laser extinction conditions, but this preference was abolished if the reward was omitted.

In addition, we show that LDT-VTA optogenetic activation conveys positive reinforcement signals since animals will self-stimulate, and present a prominent preference for the laser-associated chambers in the conditioned place preference and in the real-time place preference tests.

Collectively, these data suggest that the LDT-VTA inputs encode positive reinforcement signals and are important in different dimensions of reward-related behaviours.

Introduction

Integrating endogenous and environmental stimuli to motivate behaviour is crucial for survival. The neuroanatomical substrates of motivation are complex, and include the mesolimbic pathway, comprising dopaminergic projections from the ventral tegmental area (VTA) to the nucleus accumbens (NAc) (Koob and Le Moal, 2008; Mogenson et al., 1980; Russo and Nestler, 2013). VTA dopaminergic neurons change their discharge mode from tonic to phasic in response to sensory events that predict a reward outcome (Montague et al., 2004), and encode a prediction error signal that is crucial for reinforcement learning (Schultz, 2013). Thus, this switch in the firing mode of dopaminergic neurons, which is triggered by excitatory drive, is critical for the expression of reward-oriented behaviours (Tsai et al., 2009).

The laterodorsal tegmentum (LDT) is comprised by populations of acetylcholine, glutamate and GABA neurons (Wang and Morales, 2009), and provides a regulatory input to the VTA (Lammel et al., 2012; Oakman et al., 1995; Watabe-Uchida et al., 2012; Woolf and Butcher, 1986). Specifically, the LDT provides asymmetric (excitatory-type) inputs to VTA dopaminergic neurons that preferentially innervate the NAc (Omelchenko and Sesack, 2005). Additionally, LDT-VTA cholinergic terminals synapse on VTA-GABA neurons that innervate the NAc and the prefrontal cortex (Omelchenko and Sesack, 2006). It has been proposed a divergent excitation/inhibition LDT influence on mesoaccumbens neurons that is likely to excite dopaminergic cells and inhibit GABA neurons of the VTA (Omelchenko and Sesack, 2005, 2006). Indeed, previous reports have shown that electrical stimulation of the LDT increases NAc dopamine levels through both VTA glutamatergic and cholinergic receptors (Forster and Blaha, 2000; Forster et al., 2002). In addition, LDT activity is essential for VTA dopaminergic burst firing (Lodge and Grace, 2006), which is considered to be the functionally relevant signal that encodes reward prediction or incentive salience (Berridge and Robinson, 1998; Cooper, 2002; Grace and Bunney, 1984; Schultz, 1998).

Considering that dopaminergic neurons encode the association between sensory and environmental modalities that signal reward (Berridge, 2007; Schultz, 2007; Wise, 2004), one could hypothesize that LDT neurons are involved in tuning that activity and update the predictive value of stimuli during associative learning for future reinforcement. In line with this, optogenetic excitation of LDT-VTA cells results in the acquisition of conditioned place preference in mice (Lammel et al., 2012) and reinforces lever pressing in rats (Steidl and Veverka, 2015).

To further investigate the role of LDT signalling in reinforcement and motivation, we combined *in vivo* optogenetic stimulation of LDT-VTA terminals during distinct reward-related behavioural tests.

Material and Methods

Animals and Treatments

Wistar rats were individually housed under standard laboratory conditions (light/dark cycle of 12/12h; 22°C); food and water ad libitum. At the start of the experiments, two-three months old male progeny were used for electrophysiological and behavioural experiments. Two different experiments were performed: in a group of animals we assessed the basal activity of LDT and VTA and also the LDT-VTA activity. All manipulations were conducted in accordance with European Regulations (European Union Directive 2010/63/EU) and FELASA guidelines (Nicklas et al., 2002). Animal facilities and the people involved in animal experiment and design were certified by the Portuguese regulatory entity — Direção Geral de Alimentação e Veterinária (DGAV). All protocols were approved by the Animal Ethics Committee of University of Minho (SECVS protocol #107/2015) and DGAV (#19074).

Constructs and virus preparation

AAV5–EF1a–WGA–Cre–mCherry and AAV5–EF1a–DIO–hChR2–YFP were obtained directly from the Gene Therapy Centre Vector Core (UNC) centre. AAV5 vector titres were 2.1-6.6x10^{12} virus molecules ml^{-1} as determined by dot blot.

Surgery and cannula implantation

Rats designated for behavioural experiments were anesthetized with 75mg kg^{-1} ketamine (Imalgene, Merial) plus 0.5mg kg^{-1} medetomidine (Dorbene, Cymedica). One μL of AAV5–EF1a–WGA–Cre–mCherry and AAV5–EF1a–DIO–hChR2–YFP were unilaterally injected into the VTA (coordinates from bregma, according to Paxinos and Watson: -5.4mm anteroposterior, +0.6mm mediolateral, and -7.8mm dorsoventral) and LDT (coordinates from bregma: -8.5mm anteroposterior, +0.9mm mediolateral, and -6.5mm dorsoventral), respectively (ChR2 group). Another group of animals was injected in the LDT only with 1μL AAV5–EF1a–DIO–hChR2–YFP. Rats were then implanted with an optic fibre (200μm core fibre optic; Thorlabs) with 2.5mm stainless steel ferrule (Thorlabs) using the injection coordinates for the VTA (with the exception of dorsoventral: -7.7mm) that were secured to the skull using 2.4mm screws (Bilaney, Germany) and dental cement (C&B kit, Sun Medical). Rats were removed from the stereotaxic frame and sutured. Anaesthesia was reverted by administration of atipamezole (1mg kg^{-1}). After surgery animals were

given anti-inflammatory (Carprofeno, 5mg kg^{-1}) for one day, analgesic (butorphanol, 5mg kg^{-1}) for 3 days, and were let to fully recover before initiation of behaviour. Optic fibre placement was confirmed for all animals after behavioural experiments (Fig. 2a). Animals that were assigned for electrophysiological experiments were not implanted with an optic fibre.

Behaviour

Subjects and apparatus

Rats were placed and maintained on food restriction (≈7g per day of standard lab chow) to maintain 90% free-feeding weight. Behavioural sessions were performed in operant chambers (Med Associates) containing a central magazine that provided access to 45mg food pellets (Bio-Serve), two retractable levers located on each side of the magazine with cue lights above them. A 2.8W, 100mA house light positioned at the top-centre of the wall opposite to the magazine provided illumination. A computer equipped with Med-PC software (Med Associates) controlled the equipment and recorded the data.

Two-choice schedule of reinforcement

During instrumental training, rats are presented two illuminated levers, one on either side of the magazine. Presses on one lever (Laser + pellet lever (stim+)) leads to instrumental delivery of a pellet plus 4s blue (473nm) laser stimulation at 10mW, accompanied by a 4s auditory cue (white noise or tone; always the same paired for a particular rat, but counterbalanced assignments across rats). In contrast, pressing the other lever (stim- lever) delivered a single pellet accompanied by another 4s auditory cue (tone or white noise), but with no laser illumination. For both levers, presses during the 4s after pellet delivery have no further consequence. After 2 days of habituation, each daily session begins with a single lever presented alone to allow opportunity to earn its associated reward (either stim+ or pellet Alone), after which the lever is retracted. Then, the alternative lever is presented by itself to allow opportunity to earn the other reward, to ensure that the rat sampled both reward outcomes. Finally, both levers together are extended for the remainder of the session (30min total), allowing the rat to freely choose between the two levers and to earn respective rewards in any ratio. Whenever the schedule of reinforcements is completed on either lever (FR1, FR4, RR4, RR6), a pellet is immediately delivered, accompanied by 4s of the appropriate auditory cue. For the stim+ lever, delivery of the pellet is also accompanied by

additional simultaneous laser stimulation. During those 4s, lever pressing is recorded but no additional stimulation or reward is delivered.

Progressive ratio

The progressive ratio test was performed with either the stim+ lever or with the pellet alone lever without any laser (order of test conditions is balanced across animals) and repeated for each animal with the other lever. The number of presses required to produce the next reward delivery increases after each reward, according to an exponential progression (progressive ratio schedule: 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, 145, 178, 219, 268, . . .) derived from the formula PR = [5e(reward number x 0.2)] - 5 and rounded to the nearest integer. To determine whether any preference in responding is the result of increased workload, animals are given a FR1 session after PR, identical to the initial day of training.

Extinction of food (laser self-stimulation)

To conversely assess whether laser stimulation alone can maintain responding on a pellet-laser-associated lever when the reward is discontinued, rats are given the opportunity to earn the same levers but without pellet (pellet extinction). Each completed trial (RR4) on the stim+ lever results in the delivery of laser stimulation and the previously paired auditory cue but no pellet delivery. Each completed trial on the other lever (previously pellet alone) resulted in the delivery of its auditory cue.

Persistence of choice preference in the absence of laser stimulation

To test the persistence of laser-induced preference, rats that have received 2 days refresher training with stim+ versus reward alone, underwent 4 consecutive days of laser-extinction testing, where outcomes for both levers consisted in the delivery of a pellet and the associated auditory cue, with no administration of laser stimulation.

Reversal procedure

After a reminder session on a RR4, rats performed a one-day (30min) reversal procedure during which the stim+ and reward alone levers were switched. In this paradigm, a single response on the previously inactive lever (reward alone lever) was paired to the delivery of phasic optical stimulations.

Intra-cranial self-stimulation (ICSS)

Operant conditioning chambers (MedAssociates Inc.) were equipped with two retractable levers located on one wall of the chamber, a house light, and a cue light located above the two retractable levers. Rats were connected to optical tethers immediately prior to each behavioural testing session. All animals underwent 60min sessions during which pressing of one retractable lever (the reinforced lever – stim+) resulted in 80 10ms pulses of 473nM light at 20Hz and illumination of the cue light for 4s. No priming stimulation was given at any time and presses on the reinforced lever made within 4s of the previous lever press were recorded but did not result in laser activation. Presses on the alternate retractable lever (the non-reinforced lever) were recorded but had no effect.

Conditioned Place Preference – CPP

The CPP apparatus consisted of two compartments with different patterns, separated by a neutral area (Med Associates): a left chamber measuring 27.5 cm x 21cm with black walls and a grid metal floor; a centre chamber measuring 15.5cm x 21cm with gray walls and gray plastic floor; and a right chamber measuring 27.5cm x 21cm with white walls and a mesh metal floor. Rat location within the apparatus during each preference test was monitored using a computerized photo-beam system (Med Associates). Briefly, on day 1, individual rats were placed in the centre chamber and allowed to freely explore the entire apparatus for 15min (pre-test). On day 2, rats were confined to one of the side chambers for 30min and paired with optical stimulation, ON side; in the second session, rats were confined to the other side chamber for 30 min with no stimulation, OFF side. Conditioning sessions were counterbalanced. On day 3 rats were allowed to freely explore the entire apparatus for 15min (test). Optical stimulation consisted of 80 pulses of 10ms at 20Hz, every 15s. Results are expressed as the ratio of preference in the conditioned chamber and total time spent on each side.

Real-Time Place Preference – RTPP

RTPP test was performed in a custom-made black plastic arena (60 x 60 x 40cm) comprised by two indistinguishable chambers, for 15min. One chamber was paired with light stimulation of 10ms pulses at 20Hz during the entire period that the animal stayed in the stimulus-

paired side. The choice of paired chamber was counterbalanced across rats. Animals were placed in the no-stimulation chamber at the start of the session and light stimulation started at every entry into the paired chamber. Animal activity was recorded using a video camera and time spent in each chamber was manually assessed. Results are presented as percentage of time spent in each chamber.

Free food consumption monitoring

Food intake tests were conducted in a familiar chamber containing bedding and rats had serial access to pre-weighed quantities of regular chow pellets (20-22g; 4RF21, Mucedola SRL) and palatable food pellets (20-22 g; F0021, BioServ) while also having constant access to water. Each food intake session consisted of 20 min access to 20g of regular chow followed by 20min of access to 20-22g of palatable food pellets and chow. Laser stimulation was given at every minute and consisted of 80 pulses of 10ms at 20 Hz. Food consumption was repeated on 3 consecutive days. Laser stimulation was administered only on 1 day, which occurred on either day 2 or 3 (counterbalanced across rats). Control intake was measured in the absence of any laser stimulation on the 2 remaining days (day 1 and either day 2 or 3, averaged together). Chow and palatable food pellets were reweighed at the end of the test to calculate total consumption.

Immunofluorescence (IF)

Animals were anaesthetized with pentobarbital (Eutasil, Lisbon, Portugal) 90 min after initiation of the PR test, and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde. Brains were removed and sectioned coronally at a thickness of 50μm, on a vibrating microtome (VT1000S, Leica, Germany). Sections were incubated with the primary antibody mouse anti-TH (1:1000, Abcam); rabbit anti-c-fos (1:1000, Ab-5, Merck Millipore) and goat anti-GFP (1:500, Abcam) and goat anti-ChAT (1:750, AB144P, Millipore) followed by appropriate secondary fluorescent antibodies (1:1000, Invitrogen, MA, USA). All sections were stained with 4',6-diamidino-2-phenylindole (DAPI; 1mg ml-1) and mounted using mounting media (Permafluor, Invitrogen, MA, USA). Positive cells within the brain regions of interest were analysed and cell counts were performed by confocal microscopy (Olympus FluoViewTMFV1000). Estimation of cell density was obtained by dividing cell number values with the corresponding areas, determined using an Olympus BX51 optical microscope and the StereoInvestigator software (Microbrightfield).

Statistical Analysis

Statistical analysis was performed in GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA) and SPSS Statistics v19.0 (IBM corp, USA). Normality tests were performed for all data analysed. Two-way analysis of variance (ANOVA) was used when appropriate. Bonferroni's post hoc multiple comparison test was used for group differences determination. Statistical analysis between two groups was made using Student's t-test or Mann-Whitney tests. Non-parametric analysis (Mann-Whitney test) was used when normality of data was not assumed. Pearson correlations were calculated to obtain the strength of the relation between the test score and cell activation. Results are presented as mean ± SEM. Statistical significance was accepted for $p \leq 0.05$.

Results

Optogenetic stimulation of LDT terminals increases VTA firing rate

We first used a combined viral approach to specifically target LDT direct inputs to the VTA. We injected an adeno-associated virus (AAV5) containing a WGA–Cre fusion construct in the VTA (AAV–EF1a–DIO–WGA–Cre–mCherry), and a vector encoding a Cre-dependent ChR2 in the LDT (AAV-EF1a-DIO-hChR2-eYFP) (ChR2 group). Control animals were only injected with AAV-EF1a-DIO-hChR2-eYFP in the LDT (CTR group). The WGA–Cre fusion protein is retrogradely transported (Gradinaru et al., 2010; Xu and Südhof, 2013), inducing the expression of Cre-dependent ChR2-YFP only in LDT neurons that directly project to the VTA (Fig. 1a). We were able to observe YFP staining throughout soma and dendrites of LDT neurons and in terminals located in the VTA (Fig. 1b-c).

We next performed single cell electrophysiological recordings in anesthetized rats (Fig. 1a,d), while optically stimulating LDT terminals in the VTA (Coimbra et al., 2017). Optical stimulation of LDT terminals evoked excitatory responses in 53% of VTA neurons and inhibitory responses in 20% of recorded neurons (Fig. 1f; n = 70 neurons; n = 6 rats); most of the cells (62%) returned to baseline levels after stimulation. We found that 80 10ms light-pulse trains delivered at 20 Hz were able to induce a significant increase in the net firing rate of VTA (Fig. 1g-h; $F(1.59, 109.7) = 10.3$, $p = 0.0003$), as previously described (Coimbra et al., 2017).

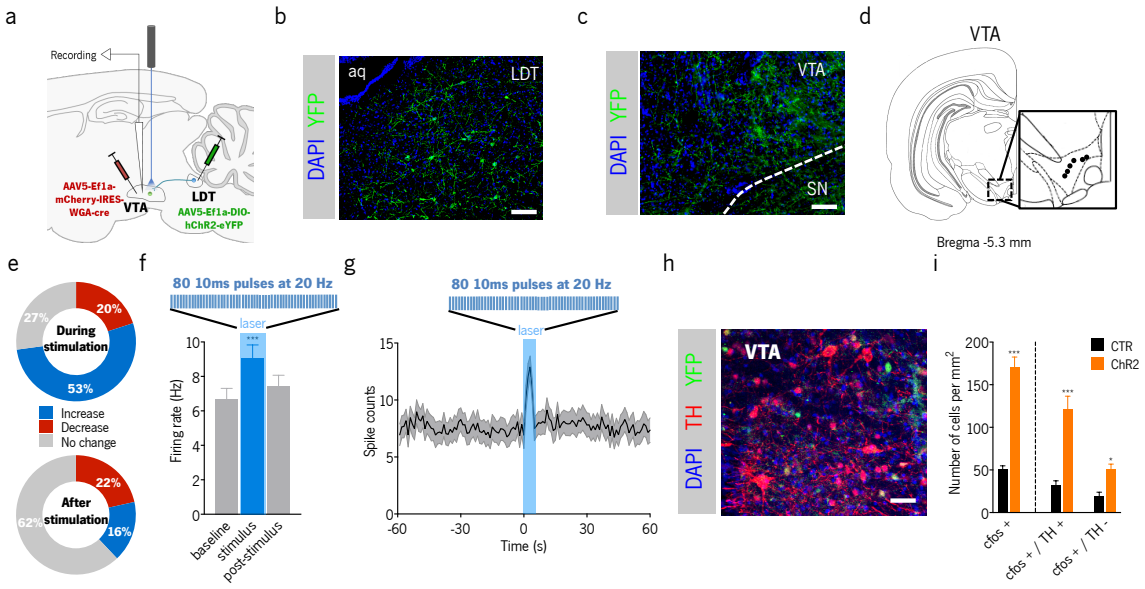

Figure 1. Optogenetic activation of LDT terminals increases VTA firing rate. (a) Strategy used for optogenetic manipulation and *in vivo* electrophysiological recordings. An AAV-WGA-Cre fusion vector was injected unilaterally in the VTA and Cre-dependent ChR2 in the LDT. (b) Representative immunofluorescence showing eYFP staining in the LDT and (c) the VTA (scale bar=200µm). (d) Representative scheme showing electrode placement in the VTA. (e) Around half of VTA recorded cells increased their firing rate upon stimulation (stimulation: 80 10 ms pulses at 20 Hz) and returned to baseline levels after stimulation. (f) VTA neurons increase firing rate upon optical activation of LDT terminals. (g) Representative recording of temporal variation that shows an increase during LDT terminal stimulation. (h) Representative immunofluorescence for c-fos, eYFP and TH scale bar=25µm) and (i) quantification of double positive cells, after optical stimulation of LDT terminals in the VTA. Values are shown as mean ± SEM. *p<0.05; **p<0.01; ***p<0.001.

LDT: laterodorsal tegmentum; VTA: ventral tegmental area.

We also analysed the number of c-fos$^+$ cells in the VTA after performing optical stimulation (Fig. 1i-j). ChR2 animals showed a significant increase in c-fos$^+$ cells when compared with CTR animals (Bonferroni *post-hoc* t test, $t(24) = 8.852$, $p < 0.001$); this was due to an increase in the recruitment of both TH$^+$ and TH$^-$ cells (Bonferroni *post-hoc* t test, cfos$^+$/TH$^+$: $t(24) = 7.315$, $p < 0.001$; cfos$^+$/TH$^-$: $t(24) = 2.639$, $p = 0.0432$).

Optogenetic activation of LDT-VTA inputs enhances preference for a laser-paired reward

To test the impact of LDT-VTA stimulation in behaviour, we unilaterally activated these inputs in freely moving rats during a two choice instrumental task (Fig. 2a). Animals were trained to press two levers to get food pellets, one of the levers was arbitrarily selected to deliver the pellet with simultaneous LDT-VTA optogenetic stimulation (stim+; blue laser stimulation: 80 10ms pulses at 20Hz) wherease pressing the other lever delivered only the pellet (Fig. 2b); the effort to get the pellet was increased until a random ratio of 6 lever presses per reward.

ChR2 rats progressively discriminated and preferred the stim+ lever from the FR4 session until the end of acquisition (Fig. 2b; 2way ANOVA; $F(21, 462) = 12.8$, $p < 0.0001$). At the end of the acquisition, ChR2 animals exhibited a 4.6:1 ratio of preference for the stim+ lever in comparison to stim- lever (Bonferroni *post-hoc* t test, $t(528) = 9.113$, $p < 0.001$). To further evaluate motivation to work for the laser-associated reward, animals were subjected to the progressive ratio task, in which effort increases throughout the session. ChR2 animals presented increased cumulative presses in the stim+ lever (Fig. 2c; Bonferroni's *post hoc* t test, $t(396) = 7.089$, ***$p<0.001$); and when compared to stim+ lever in CTR animals (Bonferroni's *post hoc* t test, $t(396) = 425$, +++$p<0.001$). In accordance, ChR2 animals also presented a higher breakpoint in the laser-associated lever than the stim- lever (Fig. 2d; 2way ANOVA; Bonferroni's *post hoc* t test, $t(33) = 9.324$, ***$p < 0.001$), that was also significantly higher when compared to stim+ in CTR animals (Bonferroni's *post hoc* t test, $t(66) = 5.739$, +++$p < 0.0001$).

These results suggest that LDT-VTA optogenetic stimulation enhances the value of a laser-paired reward and enhances motivational drive to obtain that reward, considering that no changes in a free food consumption paradigm were noted (Supplementary Fig. 1). Next, we evaluated neuronal activation by c-fos staining in animals that performed the PR test (Fig. 2h-j). ChR2 animals that performed the test with the stim+ lever showed a significant increase in c-fos$^+$ cells in the VTA when compared to ChR2 animals stim- (Fig. 2j; 2way ANOVA; Bonferroni *post-hoc* t test, $t(48) = 6.622$, ***$p < 0.001$) or when compared to CTR animals stim+ ($t(48) = 7.902$, ^^^$p < 0.001$); this increase was more prominent in TH$^+$ cells (ChR2 stim+ vs. stim- lever: $t(48) = 4.931$, ***$p < 0.001$; ChR2 vs. CTR stim+ lever: $t(48) = 5.883$, ^^^$p < 0.001$). In addition, the number of c-fos$^+$/TH$^+$ cells was positively correlated with individual breakpoint (Fig 2k; Pearson's correlation: $r = 0.7385$, $p < 0.0001$, $n = 20$).

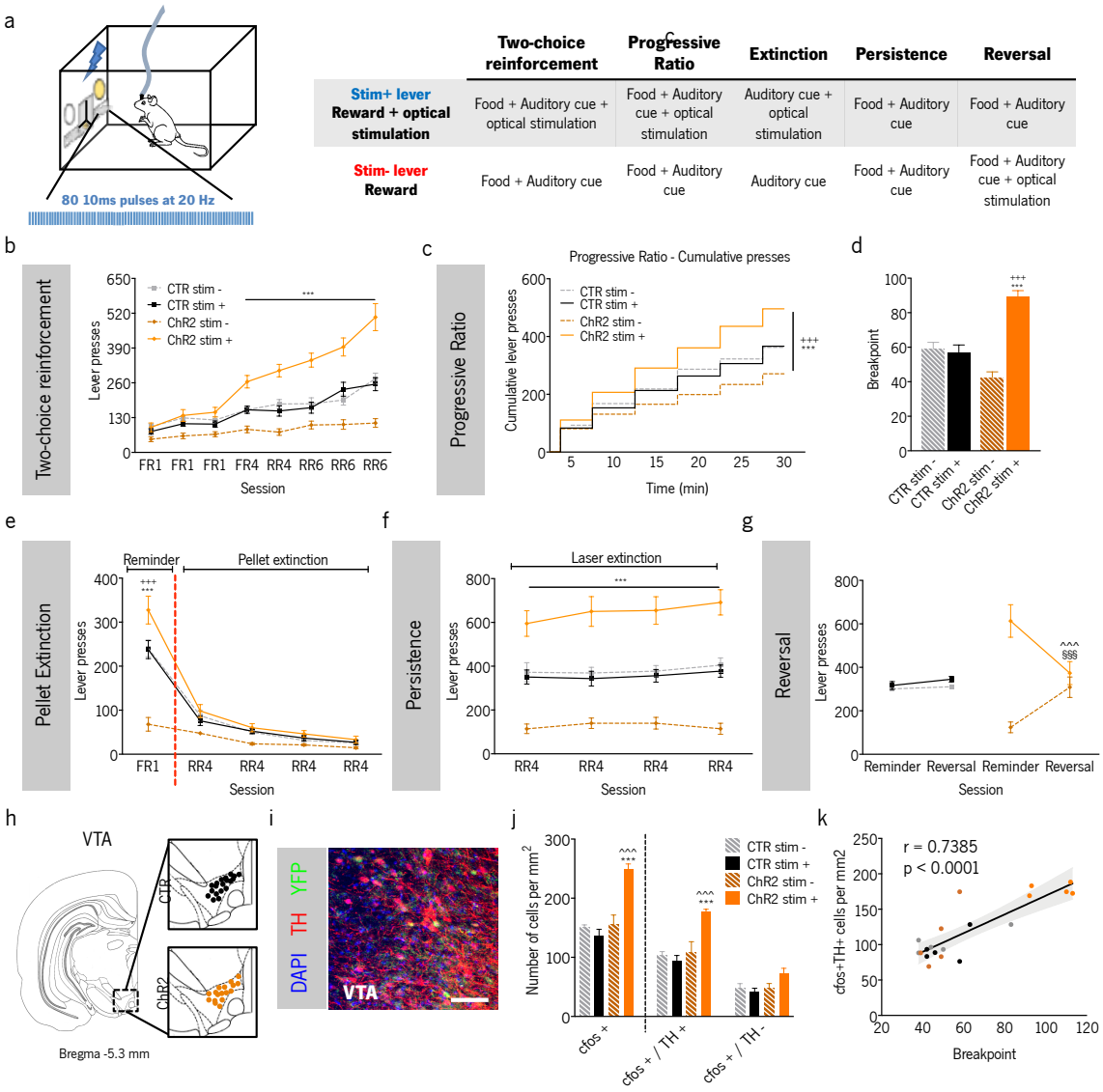

Figure 2. Optogenetic activation of LDT terminals in the VTA facilitates positive reinforcement during food-seeking behaviour. (a) Procedure for enhancement of laser-paired pellet reward in the two–choice task. Schematic shows apparatus for simultaneous choice tests. Two levers protrude on neither side and pressing either lever earned an equivalent pellet and a distinctive 4 s sound, where the outcome of only one lever was also paired simultaneously with onset of 4 s laser pulse (stim+). The opposing lever only delivered a food reward and associated sound (stim-). Timeline of the experimental strategy and instrumental conditions during the acquisition, extinction, persistence and reversal phases of the operant behavioural task. (b) Time-course representation of the behavioural responses during acquisition of food-seeking behaviour in ChR2 (orange; n = 15) and CTR (black; n = 19) rats. Optogenetic activation of LDT terminals in the VTA increases choice for laser-paired stim+ lever. (c) Cumulative presses performed during the progressive ratio task show ChR2 animals pressing increasingly more on the stim+ lever. (d) Performance during the progressive ratio task. (e) In the absence of food, responses on either lever does not deliver a pellet reward, but pressing on the stim+ results on laser stimulation. All animals decrease lever-

pressing responses during pellet extinction conditions. (f) Number of responses in the absence of laser stimulation showing persistent preference of ChR2 animals. (g) Reversal session with position alteration of stim+ and stim– levers. Apart from CTR animals, ChR2 group shifted response on both levers. (h) Schematic representation of cannula implantation. (i) Representative immunofluorescence for c-fos and TH scale bar=50μm) and (j) respective quantification of double positive cells of ChR2 and CTR rats after PR performance on the stim+ or the stim- lever. (k) Positive Pearson's correlation between individual breakpoint and number of c-fos$^+$/TH$^+$. Values are shown as mean ± SEM. * Significant difference between ChR2 stim+ and stim- levers; + Significant difference between ChR2 stim+ and CTR stim+ levers; ^ Significant difference between ChR2 stim- and ChR2 stim- levers in the reversal session ($p<0.05$); § Significant difference between ChR2 stim+ and ChR2 stim+ levers in the reversal session ($p<0.05$).

VTA: ventral tegmental area.

Stimulation of LDT-VTA inputs adds value to a food reward

Next, animals underwent a food extinction procedure during which there was no delivery of food pellets in any of the levers, but the stim+ lever still originated optical stimulation if pressed (Fig. 2e). Both groups decreased lever pressing throughout the sessions and, at the end of the extinction phase, ChR2 animals exhibited similar lever presses on stim+ and stim- levers. These results suggested that the pairing of the stimulation with the reward is crucial for the positive reinforcing properties of LDT-VTA stimulation in operant behaviour.

In order to further understand the reinforcing properties of LDT-VTA activation on the value given to a reward, animals were subjected to the two-choice instrumental task but now in laser extinction conditions, i.e., the outcome of both stim- and stim+ lever is merely a pellet (equal choice); this allows to evaluate persistence (Fig. 2f). Surprisingly, ChR2 animals still manifested preference for the laser-associated lever in four consecutive trials, despite the outcome being similar between levers (2way ANOVA; $F(3, 66) = 28.43$, $p < 0.0001$), suggesting that the value of the laser-paired reward was increased.

To rule out possible confounding factors such as habit towards the stim+ lever, we also evaluated the behavioural flexibility using a single-session reversal paradigm during which the stim+ and stim- levers were switched (Fig. 2g). We found that ChR2 animals switched their responses towards the new stim+ lever, indicative of goal-direct behaviour (Bonferroni *post-hoc* t test, Reminder vs. Reversal session ChR2 stim+: $t(66) = 7.767$, ***$p < 0.0001$; ChR2 stim-: $t(66) = 5.996$, ^^^$p < 0.0001$).

Optogenetic activation of LDT terminals is sufficient for ICSS and place preference

In a separate experiment, we tested whether optogenetic activation of LDT-VTA inputs was able to induce optical intracranial self-stimulation (ICSS). To assess this, stim+ lever was paired with phasic optical stimulation (80 10ms pulses at 20 Hz) while the other lever had no outcome (Fig. 3a). Two way ANOVA analysis showed a significant effect of group (Fig. 3b; 2way ANOVA; $F(3, 20) = 53.41$, $^{***}p < 0.0001$). As depicted in Figure 3a, ChR2 preferred the stim+ lever in comparison to stim- lever (Bonferroni *post-hoc* t test, $t(60) = 6.9$, $^{***}p < 0.001$), whereas CTR animals did not discriminate between levers and presented fewer lever presses.

In a laser extinction session, ChR2 animals decreased lever pressing in the stim+ lever (Bonferroni *post-hoc* t test, $t(20) = 11.02$, $p < 0.001$) while slightly increasing the number of presses in the inactive lever ($t(20) = 4.018$, $p = 0.0027$). During a reversal session where the stim+ and stim- levers were switched, ChR2 animals increased lever pressing in the new stim+ lever (Bonferroni *post-hoc* t test, $t(20) = 3.982$, $p = 0.0029$).

To have additional measures of the reinforcing properties of LDT-VTA terminal stimulation, we performed the CPP (non-contingent) and RTPP (contingent) tests, pairing one chamber of each apparatus with laser stimulation (Fig. 3e,h). Activating LDT-VTA terminals elicited place preference in both paradigms, as shown by the increase of the ratio of preference of ChR2 animals for the conditioned chamber in the CPP (Fig. 3f; t test, $t(16) = 4.001$, $p = 0.0010$) or the total time spent on the ON versus the OFF side of the CPP chamber (Fig. 3g; Bonferroni's *post hoc* t test, $t(17) = 5.604$, $p < 0.001$). In the RTPP test, ChR2 animals spent more time in the ON side of the RTPP chamber than CTR animals ($t(40) = 5.19$, $p < 0.001$). Collectively, these results suggest that activation of LDT-VTA neurons conveys positive reinforcing properties.

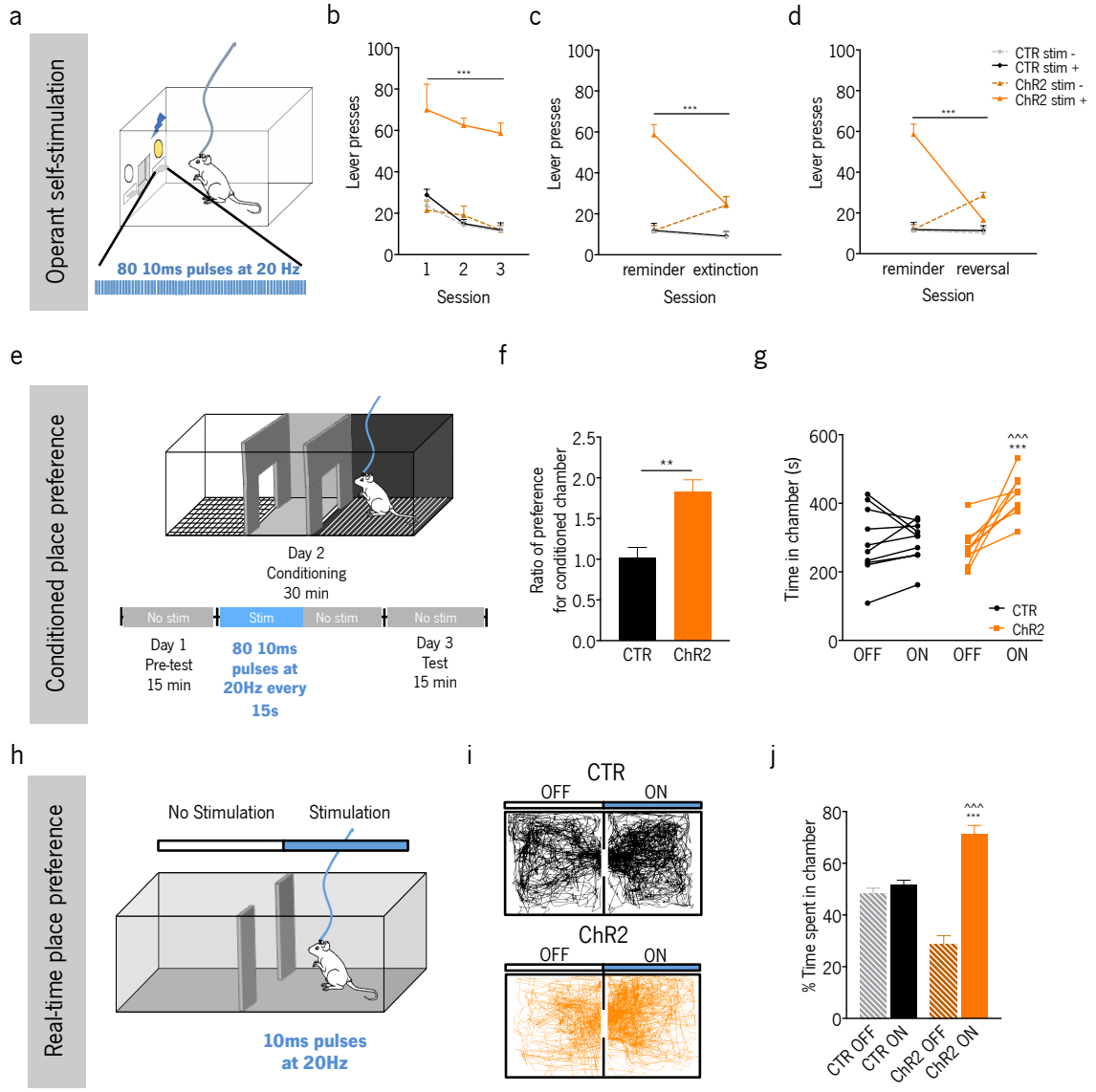

Figure 3. Optical activation of LDT-VTA induces ICSS and place preference recruitment of NAc D1-MSNs. (a) Schematic representation of intracranial self-stimulation paradigm: pressing on the stim + lever results on the delivery of optical stimulation. (b) Performance during 3 sessions of ICSS, (c) extinction and (d) reversal sessions. (e) Procedure for conditioned place preference and (h) real time place preference where one chamber is associated with laser stimulation (ON side). (f) Ratio of preference after CPP conditioning and (g) total time spent on each chamber, showing an increase in preference of ChR2 animals. (i) Representative tracks in the RTPP for CTR and ChR2 groups. (j) Percentage of time spent on the ON and OFF sides. * Significant difference between ChR2 stim + and stim - levers; ^ Significant difference between ChR2 stim + and CTR stim + levers. Values are shown as mean ± SEM. *p<0.05; **p<0.01; ***p<0.001.

Discussion

Assigning valence to environmental stimuli by determining whether they are rewarding and should be approached, or are aversive and should be avoided is crucial for survival. VTA dopaminergic neurons are proposed to encode value-related signals and the VTA is a key region in both reward and aversion (Lammel et al., 2014; Matsumoto and Hikosaka, 2009; Morales and Margolis, 2017; Schultz, 2013, 2015). Due to its privileged role in controlling VTA dopaminergic activity and subsequent dopamine release in the NAc, the LDT has been proposed to modulate reward-related behaviours. Indeed, recent studies have shown that optogenetic excitation of LDT-VTA inputs is reinforcing, since it induces place preference and enhances instrumental behaviours (Dautan et al., 2016; Lammel et al., 2012; Steidl and Veverka, 2015; Steidl et al., 2017a; Xiao et al., 2016). Despite these important contributions, the role of LDT-VTA inputs in reward-related behaviours needs to be characterized in more detail.

Here, we optogenetically activated LDT-VTA terminals in freely moving rats during multiple phases of reward behaviour. First, animals were exposed to a two-choice instrumental paradigm, in which pressing either lever delivered one pellet, but one of the rewards was paired with simultaneous optogenetic stimulation (stim+ lever). ChR2 animals progressively press more in the laser-associated lever, i.e., LDT-VTA stimulation enhanced preference of an otherwise equal choice (one pellet). These effects are unlikely to reflect changes in "liking" the pellet since we observed no differences in free food consumption of chow or palatable pellets (Supplementary Fig. 1). In addition, animals presented a substantial increase in motivation to work for the pellet reward associated with the laser stimulation, suggesting that excitation of these inputs amplified the motivational attractiveness of its associatively paired reward representation, raising its incentive value. In agreement, laser extinction had no effect whatsoever in lever pressing, as long as the reward was delivered. Conversely, removing the reward (pellet) dramatically decreased lever pressing in ChR2 animals, hinting that LDT-VTA excitation did not have any residual reward value on its own in these animals that had *imprinted* the lever-laser-reward association. These findings resemble one study in which optogenetic stimulation of central amygdala (CeA) specifically added value to a laser-paired external reward, but when that reward was absent, preference was no longer observed (Robinson et al., 2014).

However, these results are puzzling to conciliate with the fact that LDT-VTA terminal stimulation appears to be rewarding/reinforcing *per se*, since it induced place preference in both

the CPP and RTPP tests (this study and (Lammel et al., 2014; Steidl and Veverka, 2015; Steidl et al., 2017a; Xiao et al., 2016), and considering the fact that ChR2 animals would readily self-stimulate. This suggests that excitation of these inputs can add value to external rewards, but also act as an independent reward/reinforcer. Considering the two-choice task, animals are food deprived and their main goal is to press the lever to obtain a food pellet. Once the pellet is removed, the instrumental action is no longer reinforced, so animals quit responding because the outcome is absent. In the self-stimulation paradigm, the instrumental action occurs initially by chance, and the persistence of instrumental behaviour is a decision solely based on the rewarding properties of the laser stimulus, not on a physiological need of the animal. Our findings highlight the complexity of reward behaviours, and the importance of the context and associative learning in the process. Other studies have also originated puzzling effects in different tests that evaluate the rewarding properties of a stimulus. For example, optogenetic stimulation of GABAergic projections from the CeA to ventromedial PFC increases motivation to get a food reward and induces place preference in the RTPP, but is not able to induce self-stimulation behaviour (Seo et al., 2016), in line with the notion that a rewarding stimulus can act as reinforcer in one context but not in others.

Stimulation of LDT axons produced an overall focused effect and increase in salience/motivation that is likely the result of enhancing dopamine release from mesolimbic neurons into the NAc (Forster and Blaha, 2000; Steidl et al., 2017b). Indeed, an elegant study has shown that LDT cholinergic axonal stimulation increases the number of spikes in bursts in the VTA, i.e., is equivalent to increasing the value (via dopamine) associated with reward prediction (Dautan et al., 2016). However, a remaining question is how LDT inputs of different nature (glutamatergic and GABAergic) are integrated in the VTA to control striatal activity and shape behaviour.

In summary, we show that LDT-VTA optogenetic activation elicits a predominantly excitatory response in the VTA in both dopaminergic and non-dopaminergic neurons, adding value to an associated external reward but it is also inherently rewarding/reinforcing *per se*.

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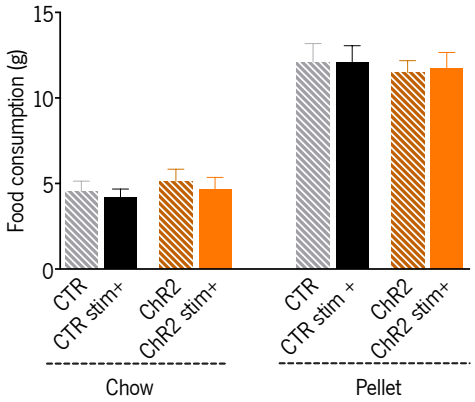
Supplementary Information

Role of laterodorsal tegmentum projections to VTA in multiple phases of reward behaviour

Manuscript in preparation



Supplemental Figure 1. Effect of LDT terminals stimulation on food consumption. No effect of optical stimulation in free-feeding behaviour for either normal chow or palatable food pellets. Values are shown as mean ± SEM.

Chapter 3

Role of laterodorsal tegmentum projections to nucleus accumbens in reward-related behaviours

Manuscript in preparation

Role of laterodorsal tegmentum projections to nucleus accumbens in reward-related behaviours

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Abstract

The laterodorsal tegmentum (LDT) has been associated with reward and positive reinforcement due to its role in regulating firing activity of ventral tegmental area (VTA), indirectly controlling dopamine release in the nucleus accumbens (NAc). Interestingly, recent anatomical evidence shows that the LDT also directly projects to the NAc, but the biological role of these connections remains unknown.

Here, we show that optogenetic activation of LDT terminals in the NAc induces a net increase in accumbal firing rate. Stimulation of LDT-NAc projections enhances motivational drive and induces preference for the laser-associated lever, but only when it was paired with a reward (pellet). We further demonstrate that specific activation of LDT-NAc cholinergic projections is sufficient for this shift in preference, suggesting that these inputs enhance and narrow incentive motivation for a reward above another.

These results provide the first evidence that LDT-NAc projections are functional and play an important role in motivated behaviours and positive reinforcement.

Introduction

Pharmacological, lesion and optogenetic studies have involved the laterodorsal tegmentum (LDT) in reward processing and reinforcement. These processes are thought to be mediated by cholinergic, glutamatergic, and GABAergic LDT inputs to the ventral tegmental area (VTA) (Wang et al., 2010), that either target the dopaminergic population (H.-L. Wang & Morales, 2009; Watabe-Uchida, Zhu, Ogawa, Vamanrao, & Uchida, 2012) and/or the GABAergic neurons of this region (Clements et al., 1991; Cornwall et al., 1990; Forster and Blaha, 2000; Forster et al., 2002). LDT projections can regulate the firing activity of midbrain dopamine neurons and indirectly nucleus accumbens (NAc) dopamine levels (Forster and Blaha, 2000; Forster et al., 2002; Lodge and Grace, 2006). In fact, LDT electrical stimulation increases dopamine cell burst firing and boosts dopamine release in the NAc, whereas LDT lesions reduce dopamine burst firing (Blaha et al., 1996; Grace et al., 2007; Hernández-López et al., 1992; Lodge and Grace, 2006).

Recent behavioural studies have shown that optogenetic stimulation of LDT-VTA neurons enhance place preference (Lammel et al., 2012; Steidl et al., 2017a) and reinforce intra-cranial self-stimulation responding in rats (Steidl and Veverka, 2015; Steidl et al., 2017b). However, LDT effects in reward-related behaviours may go beyond this direct control of VTA since a neuroanatomical study has shown that LDT also directly innervates the NAc (Dautan et al., 2014). It is proposed that LDT neurons preferentially innervate medial striatum and NAc core, forming asymmetric synapses, thought to be excitatory (Dautan et al., 2014). Interestingly, collaterals of some of these neurons have been found to innervate dopaminergic midbrain neurons (and thalamus) as well. Around 60% of these projections are cholinergic, with an additional contribution of GABAergic and glutamatergic projections (Dautan et al., 2014). Remarkably, to date, the functional role of LDT-NAc direct projections and how they contribute for reward and reinforcement has not been evaluated.

Considering this, in this work we optogenetically modulated all LDT-NAc projections (and also only cholinergic inputs) and evaluated different dimensions of reward-related behaviours.

Material and Methods

Animals and Treatments

Rats

Wistar Han rats, aged 2 months at the beginning of experiments, were housed under standard laboratory conditions (light/dark cycle of 12/12 h; 22°C); food and water ad libitum. Health monitoring was performed according to FELASA guidelines, confirming the Specified Pathogen Free health status of sentinel animals maintained in the same animal room. All manipulations were conducted in strict 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 – DGAV. All the experiments were approved by the Ethics Committee of the University of Minho (SECVS protocol #107/2015). The experiments were also authorized by the national competent entity DGAV (#19074).

Mice

Male and female C57/Bl6 transgenic mice (age of 2 months at the beginning of the experiments) were housed at weaning in groups of 3-5 animals per cage, under standard laboratory conditions (light/dark cycle of 12/12 h; 22°C); food and water ad libitum. The progeny produced by mating ChAT-cre (B6;129S6-*Chat*$^{tm2(cre)Lowl}$/J/ChAT-IRES-Cre, #006410, The Jackson Laboratory) heterozygous transgenic male mice with wild-type C57/Bl6 females were genotyped at weaning by PCR fragment analysis.

All behavioural experiments were performed during the light period of the light/dark cycle. Health monitoring was performed according to FELASA guidelines, 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 the experiments were approved by the Ethics Committee of the University of Minho (SECVS protocol #107/2015). The experiments were also authorized by the national competent entity DGAV (#19074).

Genotyping

DNA was isolated from tail biopsy using the Citogene DNA isolation kit (Citomed). In a single PCR genotyping tube, the primers CRE F (5'-AGCCTGTTTTGCACGTTCACC-3') and Cre R (5'-GGTTTCCCGCAGAACCTGAA-3') were used to amplify the ChAT-cre transgene. An internal control gene (metallothionein 3) was used in the PCR (MT3_F (5'-GGTCCTCACTGGCAGCAGCTGCA-3') and MT3_2 (5'-CCTAGCACCCACCCAAAGAGCTG-3'). Heterozygous mice were discriminated from the wild-type mice by the presence of two amplified DNA products corresponding to the transgene and the internal control gene. Gels were visualized with GEL DOC EZ imager (Bio-Rad, Hercules, CA, USA) and analysed with the Image Lab 4.1 (Bio-Rad).

Optogenetics - Constructs

AAV5–EF1a–WGA–Cre–mCherry, AAV5–EF1a–DIO–hChR2–YFP and AAV5–EF1a–DIO-eNpHR 3.0–YFP were obtained directly from the Gene Therapy Centre Vector Core (UNC) centre. AAV5 vector titres were 2.1-6.6X10^{12} virus molecules/ml.

Surgery and cannula implantation

Rats designated for behavioural experiments were anesthetized with 75mg kg-1 ketamine (Imalgene, Merial) plus 0.5mg kg-1 medetomidine (Dorbene, Cymedica). One μl of AAV5–EF1a–WGA–Cre–mCherry was unilaterally injected into the NAc (coordinates from bregma, according to Paxinos and Watson (Paxinos and Watson, 2007): +1.5mm anteroposterior, +0.9mm mediolateral, and -6.5mm dorsoventral) and 1μl of AAV5–EF1a–DIO–hChR2–YFP was injected in the LDT (coordinates from bregma: -8.5mm anteroposterior, +0.9mm mediolateral, and -6.5mm dorsoventral) (ChR2). Another group of animals was injected in the LDT with 1μl of AAV5–EF1a–DIO–eNpHR 3.0–YFP (eNpHR) We had an additional group: a control group (CTR) that was injected only with 1μl AAV5–EF1a–DIO–hChR2–YFP in the LDT. Rats were then implanted with an optic fiber (200μm core fiber optic; Thorlabs, NJ, USA) with 2.5mm stainless steel ferrule (Thorlabs, NJ, USA) 0.1mm above the injection coordinates for the NAc. Ferrules were secured to the skull using 2.4mm screws (Bilaney, Germany) and dental cement (C&B kit, Sun Medical). Rats were removed from the stereotaxic frame and sutured. Anaesthesia was reverted by administration of atipamezole (1mg kg^{-1}). After surgery animals were given anti-inflammatory (Carprofeno, 5mg kg^{-1}) for one day, analgesic (butorphanol, 5mg/kg) for 3 days, and were let to fully recover before initiation of behaviour. Optic fibre placement was confirmed for all animals after behavioural experiments.

Animals that were assigned for electrophysiological experiments were not implanted with an optic fibre.

Mice designated behavioural experiments were anaesthetized with 75 mg kg^{-1} ketamine (Imalgene, Merial) plus 1mg kg^{-1} medetomidine (Dorbene, Cymedica). Virus (500nl) was unilaterally injected into the LDT (coordinates from bregma, according to Paxinos and Franklin (Paxinos and Franklin, 2004): -5mm anteroposterior (AP), +0.5mm mediolateral (ML), and -3.0mm dorsoventral (DV), using an 30-gauge needle Hamilton syringe (Hamilton Company, Switzerland), at a rate of 100nl min^{-1} and the syringe was left in place for 5 minutes to allow diffusion. Mice were implanted with an optic fiber (200 μm core fiber optic; Thorlabs) with 2.5mm stainless steel ferrule (Thorlabs) in the NAc (coordinates from bregma, according to Paxinos and Franklin (Paxinos and Franklin, 2004): +1.2mm anteroposterior (AP), +0.8mm mediolateral (ML), and -3.8mm dorsoventral (DV) and secured to the skull using dental cement (C&B kit, Sun Medical). Mice were removed from the stereotaxic frame, sutured and left to recover for two weeks before initiation of the behavioural protocols. All animals were treated 30 minutes before surgery and 6 hours after surgery with an analgesic – buprenorphine at 0.05 mg kg^{-1} (Bupaq, Richterpharma).

In vivo electrophysiology recordings

4 weeks after injection of the virus, animals were submitted to a stereotaxic surgery for the placement of the optic fiber and recording electrodes, following anatomical coordinates. Animals were anesthetized with urethane (1.75 g Kg-1, Sigma). The total dose was administered in 3 separate intra peritoneal injections, 30 min apart. Body temperature was maintained at approximately 37°C with a homoeothermic heat pad system (DC temperature controller, FHC, ME, USA). Adequate anaesthesia was confirmed by observation of general muscle tone, by assessing withdrawal responses to noxious pinching and by whiskers movement.

Stimulating and recording electrodes were placed in the following coordinates: for rats - LDT: -8.5 from bregma, 0.8 lateral from midline, -5.5 to -7.9 ventral to brain surface; NAc: 1.5 from bregma, 0.9 lateral from midline, -6.0 to -7.0 ventral to brain surface; for mice – LDT: -5mm from bregma, 0.5mm from midline and -2.5 to -3.5 ventral to brain surface; NAc: 1.2 from bregma, 0.8 lateral from midline and -3.2 to -4.2 ventral to brain surface. A reference electrode was fixed in the skull, in contact with the dura.

Extracellular neural activity from the LDT and the NAc was recorded using a recording electrode (3 – 7 MΩ at 1 kHz). Recordings were amplified and filtered by the Neurolog amplifier

(NL900D, Digitimer Ltd, UK) (low-pass filter at 500 Hz and high-pass filter at 5kHz). A recording electrode coupled with a fibre optic patch cable (Thorlabs) was placed in the NAc or LDT. Spontaneous activity of single neurons was recorded to establish baseline for at least 60s. The DPSS 473nm laser system (CNI), controlled by a stimulator (Master-8, AMPI), was used for intracranial light delivery and fibre optic output was pre-calibrated to 10-1 mW. Optical stimulation consisted of 80 pulses of 10 ms at 20 Hz. Firing rate was calculated for the baseline, stimulation period and post stimulation period (60 s after the end of stimulation). Neurons showing a firing rate increase or decrease by more than 20% from the mean frequency of the baseline period were considered as responsive, as previously reported by Benazzouz and colleagues (Benazzouz et al., 2000).

We classified single units in the NAc into three separated groups of putative neurons: putative medium spiny neurons (MSNs), cholinergic interneurons (CINs), and fast-spiking (FS) interneurons, according to previous descriptions: FS interneurons were identified has having a waveform half-width of less that 100μs and a baseline firing rate higher that 10Hz; tonically active putative CINs (pCINs) were identified as those with a waveform half-width bigger that 300μs. Putative MSNs (pMSNs) were identified as those with baseline firing rate lower that 5Hz. Cells that did not fit in any classifications were not considered for the analysis. At the end of each electrophysiological experiment, all brains were collected and processed to identify recording region.

Behaviour

Two-choice schedule of reinforcement

Rats

During instrumental training, rats are presented two illuminated levers, one on either side of the magazine (Robinson et al., 2014). Presses on one lever (Laser + pellet lever (stim+)) leads to instrumental delivery of a pellet plus 4s blue (473nm) laser stimulation at 10mW, accompanied by a 4s auditory cue (white noise or tone; always the same paired for a particular rat, but counterbalanced assignments across rats). In contrast, pressing the other lever (pellet alone lever) delivered a single pellet accompanied by another 4s auditory cue (tone or white noise), but with no laser illumination. For both levers, presses during the 4s after pellet delivery have no further consequence. After 2 days of habituation, each daily session begins with a single lever presented alone to allow opportunity to earn its associated reward (either stim+ or stim-), after which the lever

is retracted. Then, the alternative lever is presented by itself to allow opportunity to earn the other reward, to ensure that the rat sampled both reward outcomes. Finally, both levers together are extended for the remainder of the session (30min total), allowing the rat to freely choose between the two levers and to earn respective rewards in any ratio. Whenever the schedule of reinforcements is completed on either lever (FR1, FR4, RR4, RR6), a pellet is immediately delivered, accompanied by 4s of the appropriate auditory cue. For the stim+ lever, delivery of the pellet is also accompanied by additional simultaneous laser stimulation. During those 4s, lever pressing is recorded but no additional stimulation or reward is delivered.

Mice

Mice were trained for food-seeking operant task and optogenetic stimulation during 20 minutes daily sessions (Adamantidis et al., 2011). Single press on the stim+ lever was paired with the delivery of one 20mg food pellet (BioServ, USA) and optical stimulations (80 light 10ms pulses delivered at 20Hz over 4s) under a FR1. Both ChR2 and eYFP mice received this optical stimulation when food rewards were earned. Single press on the reward alone lever was only paired with the delivery of one 20mg food pellet (i.e., without optical stimulation) under the same schedule of reinforcement (FR1). During the acquisition session, cue lights above each lever were on. Responses on the stim+ and the reward alone lever during those 4s periods were recorded but had no additional stimulation or reward. Animals progressed in the behavioural protocol if the acquisition of stable lever-pressing behaviour (i.e., < 30 % variation in lever press activity over three consecutive sessions) was met.

Progressive ratio

For both rats and mice, the progressive ratio (PR) test was performed with either the stim+ lever or with the pellet alone lever without any laser (order of test conditions is balanced across animals) and repeated for each animal with the other lever. The number of presses required to produce the next reward delivery increases after each reward, according to an exponential progression (progressive ratio schedule: 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, 145, 178, 219, 268, . . .) derived from the formula PR = [5e(reward number*0.2)] - 5 and rounded to the nearest integer. To determine whether any preference in responding is the result of increased workload, animals are given a FR1 session after PR, identical to the initial day of training.

Extinction of food (laser self-stimulation)

Rats

To conversely assess whether laser stimulation alone can maintain responding on a pellet-laser-associated lever when the reward is discontinued, rats are given the opportunity to earn the same levers but without pellet (pellet extinction). Each completed trial (RR4) on the stim+ lever results in the delivery of laser stimulation and the previously paired auditory cue but no pellet delivery. Each completed trial on the other lever (previously pellet alone) resulted in the delivery of its auditory cue.

Mice

Mice underwent 30 minutes daily extinction sessions, during which food reward was absent in a FR1 schedule. Each press on the stim+ lever results in the delivery of laser stimulation with no pellet delivery. Presses on the opposite lever (previously pellet alone) had no consequence. Animals were maintained in this condition until behavioural responses were extinguished (<30% variation in lever press activity over three consecutive sessions).

Persistence of choice preference in the absence of laser stimulation

Rats

To test the persistence of laser-induced preference, rats that have received 2 days refresher training with stim+ versus reward alone, underwent 4 consecutive days of laser-extinction testing, where outcomes for both levers consisted in the delivery of a pellet and the associated auditory cue, with no administration of laser stimulation.

Mice

Following reactivation procedure, animals were tested for 4 days in a persistence procedure (similar as rats), where one lever press on either lever resulted in the delivery of a pellet but with no laser stimulation on the previously associated lever.

Reversal procedure

Both rats and mice, after a reminder session on a RR4 (rats) or FR1 schedule (mice), performed a one-day (30min) reversal procedure during which the stim+ and reward alone levers

were switched. In this paradigm, a single response on the previously inactive lever (reward alone lever) was paired to the delivery of phasic optical stimulations.

Conditioned Place Preference – CPP

The CPP protocol was adapted from a previously published report (Lammel et al., 2012). The CPP apparatus consisted of two compartments with different patterns, separated by a neutral area (Med Associates): a left chamber measuring 27.5 cm x 21 cm with black walls and a grid metal floor; a centre chamber measuring 15.5 cm x 21 cm with gray walls and gray plastic floor; and a right chamber measuring 27.5 cm x 21 cm with white walls and a mesh metal floor. Rat location within the apparatus during each preference test was monitored using a computerized photo-beam system (Med Associates). Briefly, on day 1, individual rats were placed in the centre chamber and allowed to freely explore the entire apparatus for 15min (pre-test). On day 2, rats were confined to one of the side chambers for 30min and paired with optical stimulation, ON side; in the second session, rats were confined to the other side chamber for 30min with no stimulation, OFF side. Conditioning sessions were counterbalanced. On day 3 rats were allowed to freely explore the entire apparatus for 15min (post-test). Optical stimulation consisted of 80 pulses of 10ms at 20Hz, every 15s. Results are expressed as the ratio of preference in each side.

Real-Time Place Preference – RTPP

RTPP test was performed in a custom-made black plastic arena (60 x 60 x 40cm) comprised by two indistinguishable chambers, for 15min. One chamber was paired with light stimulation of 10ms pulses at 20Hz during the entire period that the animal stayed in the stimulus-paired side. The choice of paired chamber was counterbalanced across rats. Animals were placed in the no-stimulation chamber at the start of the session and light stimulation started at every entry into the paired chamber. Animal activity was recorded using a video camera and time spent in each chamber was manually assessed. Results are presented as percentage of time spent in each chamber.

Intra-cranial self-stimulation (ICSS)

Operant conditioning chambers (MedAssociates Inc.) were equipped with two retractable levers located on one wall of the chamber, a house light, and a cue light located above the two retractable levers. Rats or mice were connected to optical tethers immediately prior to each

behavioural testing session. All animals underwent 60min sessions during which pressing of one retractable lever (the reinforced lever – stim+) resulted in 80 10ms pulses of 473nM light at 20Hz and illumination of the cue light for 4s. No priming stimulation was given at any time and presses on the reinforced lever made within 4s of the previous lever press were recorded but did not result in laser activation. Presses on the alternate retractable lever (stim- lever) were recorded but had no effect.

Locomotor Activity and Food consumption monitoring

Locomotor behaviour was monitored using the open field test. Briefly, rats were attached to an optical fibre connected to a laser (475nm) and immediately placed in the centre of an arena (Med Associates). Locomotion was monitored online over a period of 40min. Stimulation consisted of 80 light pulses of 10ms (473nm; 10mW at the tip of the implanted fibre) delivered at 20Hz every 150s, after the animals were placed in the arena. Total distance travelled was used as indicator of locomotor activity.

Food intake tests were conducted in a familiar chamber containing bedding and rats had serial access to pre-weighed quantities of regular chow pellets (20-22g; 4RF21, Mucedola SRL) and palatable food pellets (20-22g; F0021, BioServ) while also having constant access to water. Each food intake session consisted of 20min access to 20g of regular chow followed by 20min of access to 20-22g of palatable food pellets and chow. Food consumption was repeated for 3 consecutive days. Laser stimulation was administered only on 1 day (80 pulses of 10ms at 20Hz at every 60s), which occurred on either day 2 or 3 (counterbalanced across rats). Control intake was measured in the absence of any laser stimulation on the 2 remaining days (day 1 and either day 2 or 3, averaged together). Chow and palatable food pellets were reweighed at the end of the test to calculate total consumption.

Immunofluorescence (IF)

Animals were anaesthetized with pentobarbital (Eutasil, Lisbon, Portugal) 90min after initiation of the PR test, and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde. Brains were removed and sectioned coronally at a thickness of 50μm, on a vibrating microtome (VT1000S, Leica, Germany). Sections were incubated with the primary antibody mouse anti-D2 receptor (1:500, B-10, Santa Cruz Biotechnology); rabbit anti-c-fos (1:1,000, Ab-5, Merck Millipore) and goat anti-GFP (1:500, ab6673, Abcam), mouse anti-D1

receptor (1:100, NB110-60017, Novus), goat anti-ChAT (1:750, AB144P, Millipore) and sheep anti-ChAT (1:500, Abcam) followed by appropriate secondary fluorescent antibodies (1:1000, Invitrogen, MA, USA). All sections were stained with 4',6-diamidino-2-phenylindole (DAPI; 1 mg ml^{-1}) and mounted using mounting media (Permafluor, Invitrogen, MA, USA). Positive cells within the brain regions of interest were analysed and cell counts were performed by confocal microscopy (Olympus FluoViewTMFV1000). Estimation of cell density was obtained by dividing cell number values with the corresponding areas, determined using an Olympus BX51 optical microscope and the StereoInvestigator software (Microbrightfield).

Statistical Analysis

Statistical analysis was performed in GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA) and SPSS Statistics v19.0 (IBM corp., USA). Parametric tests were used whenever Shapiro-Wilk normality test SW>0.05. One or two-way analysis of variance (ANOVA) was used when appropriate. Bonferroni's post hoc multiple comparison tests were used for group differences determination. Statistical analysis between two groups was made using Student's t-test. Results are presented as mean ± SEM. Statistical significance was accepted for $p<0.05$.

Results

Anatomical and electrophysiological confirmation of LDT-NAc direct projections

To confirm previous findings showing that the LDT sends direct projections to the NAc (Dautan et al., 2014), we injected in the NAc of rats an adeno-associated virus (AAV5) containing a vector encoding for wheat germ agglutinin (WGA)-Cre fusion protein (AAV5–EF1a–WGA–Cre–mCherry) in combination with an injection of a Cre-dependent channelrhodopsin or hallorhodopsin in the LDT (AAV5-Ef1a-DIO-hChR2(H134R)-eYFP or AAV5-Ef1a-DIO-eNpHR3.0-eYFP) (Fig 1a). WGA-Cre protein is able to migrate trans-synaptically and induce ChR2 expression in regions directly connected to the NAc. We found abundant YFP expression in the LDT, indicative of direct LDT-NAc projections. YFP expression was distributed throughout their soma, dendrites and axons (Fig. 1b). As expected, YPF was also observed in LDT terminals in the NAc (Fig. 1c). Nearly 50% of YFP transfected LDT neurons were cholinergic, 29% glutamatergic and 23% GABAergic (Fig. 1d-e).

In order to ensure minimal second order transynaptic migration of WGA-Cre (Dautan et al., 2014; Witten et al., 2011), we performed single-cell *in vivo* electrophysiological recordings 4 weeks post-injection (Fig. 1f). Optical activation (stimulation: 20Hz, 80 10ms light pulses) of LDT cells induced an increase in the firing rate of 48% of recorded neurons, 12% presented decreased activity and 40% did not respond (Fig. 1g), with a spike latency below 2ms (Supplementary Fig. 1a). The overall net firing rate was increased in this region (Fig. 1h; RM ANOVA, $F(1.182, 28.36)=11.66$, $p=0.0012$, n=25 cells).

To avoid indirect activation of other brain regions due to LDT collaterals (Dautan et al., 2014), we stimulated LDT terminals in the NAc. This stimulation evoked an excitatory response in 53% of NAc cells, and an inhibitory response in 13% (Fig. 1j). LDT terminal stimulation elicited an increase in NAc net firing rate (Fig. 1k; RM ANOVA, $F(1.482, 93.39)=159.7$, $p<0.0001$, n=65 cells); with a short latency to fire to stimulation (Supplementary Fig. 1b), indicative of monosynaptic transmission. We divided NAc cells into putative medium spiny neurons (pMSNs), cholinergic interneurons (pCIN) and fast spiking GABAergic interneurons (pFS) based on their waveform characteristics. 51.8% of recorded pMSNs increased their firing rate upon LDT terminal activation (Fig. 1m), whereas only a few decreased firing rate. Regarding pCINS and pFS interneurons, 67% and 50% showed an increase in firing rate upon LDT axon terminal stimulation, respectively.

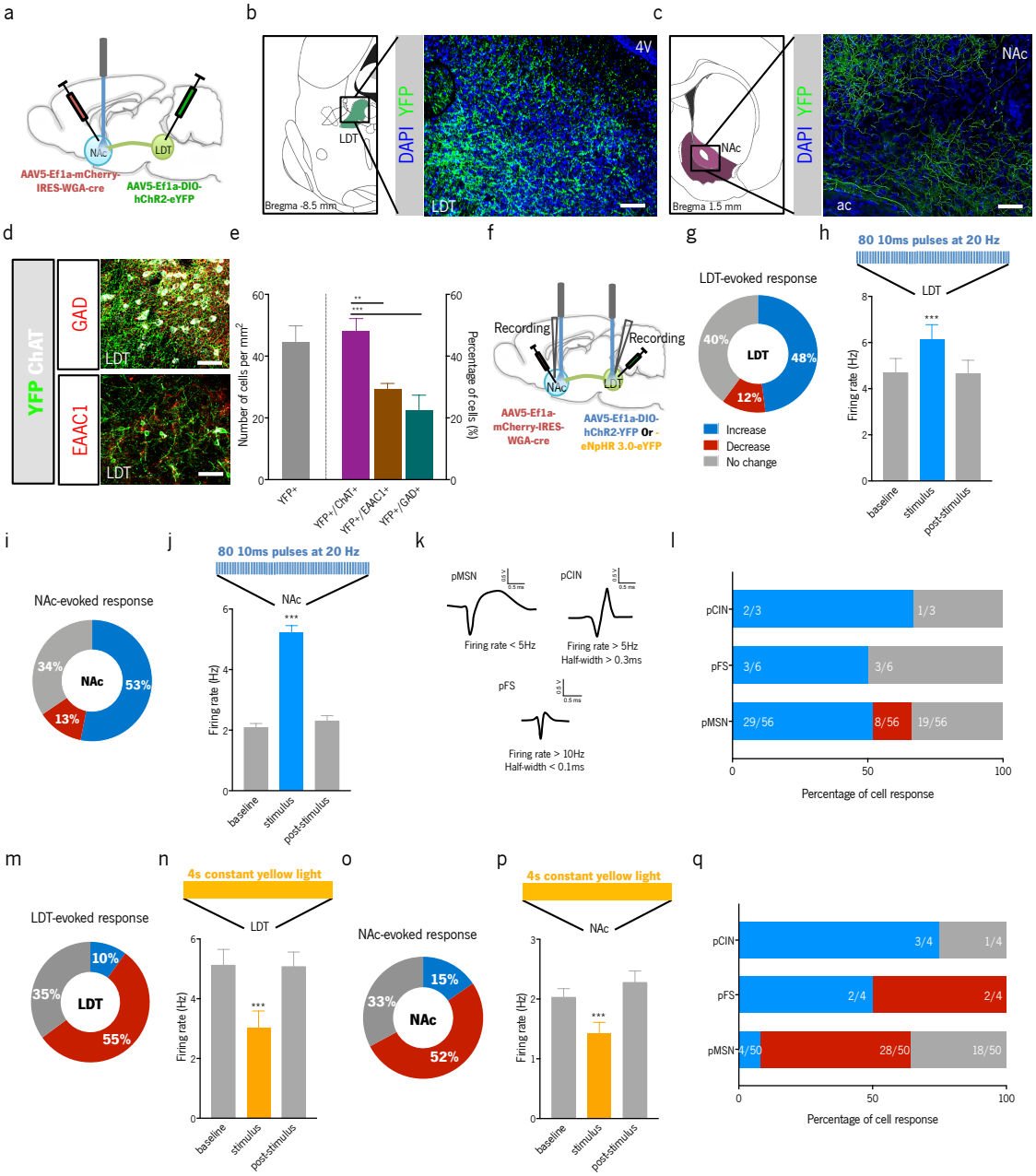

Figure 1. LDT stimulation directly excites NAc. (a) Strategy used for optogenetic manipulation. An AAV-WGA-Cre fusion vector was injected unilaterally in the NAc and a Cre-dependent ChR2 was injected in the LDT. (b) Representative immunofluorescence showing eYFP staining in the LDT and (c) LDT terminals in the NAc (scale bar=200µm). (d) Representative immunofluorescence for eYFP and ChAT, EAAC1 or GAD65/67 (scale bar=50µm) and (e) respective quantification of double positive cells (n=10 animals). ~50% of transfected LDT neurons were cholinergic, 29% glutamatergic and 23% GABAergic. (f) Schematic representation of the *in vivo* electrophysiological recordings. (g) The majority of LDT neurons (48%) increase firing rate in response to optical activation (80 10 ms pulses at 20 Hz) of LDT cell bodies. (h) Net firing rate

increase in the LDT during optical stimulation. (i) LDT terminal stimulation evokes a predominantly excitatory response in NAc cells (53% of cells). (j) Net firing rate increase in NAc during LDT terminal stimulation. (k) NAc neurons were separated into 3 categories according to firing rate and waveform characteristics: putative medium spiny neurons (pMSNs), cholinergic interneurons (pCINs) and fast spiking GABAergic interneurons (pFS). (l) LDT terminal stimulation evoked a predominantly excitatory response in either of the cell types. (m) The majority of LDT neurons (55%) decrease firing rate in response to optical inhibition (4s of constant yellow light at 10mW) of LDT cell bodies. (n) Net firing rate decrease in the LDT during optical inhibition. (o) LDT terminal inhibition evokes a predominantly inhibitory response in NAc cells (52% of cells). (p) Firing rate in the NAc upon inhibition of LDT terminals. (q) LDT terminal inhibition evoked both excitatory and inhibitory responses in either of the NAc cell types. *Significant difference between baseline and stimulus ($p<0.001$). Values are shown as mean ± SEM.
NAc: nucleus accumbens; LDT: laterodorsal tegmentum.

Next we performed LDT optogenetic inhibition experiments using hallorhodopsin (eNpHR). Optical inhibition (4s constant yellow light) of LDT cell bodies induced a decrease in the firing rate of 55% of recorded cells, whereas 35% did not show any changes in activity upon inhibition; 10% of recorded cells increased firing rate (Fig. 1n-o; RM ANOVA, $F(1.198, 46.74)=25.34$, $p<0.0001$, n=40 cells). Spike latency was below 2ms in LDT cells (Supplementary Fig. 1c). The same inhibition protocol in LDT terminals in the NAc significantly decreased the firing rate in this region (Fig. 1q-r; RM ANOVA, $F(1.627, 92.72)=10.44$, $p=0.0002$, n=58 cells). 52% of recorded cells decreased activity, 15% increase and 33% did not alter activity in response to optical inhibition with spike latency around 2ms. (Supplementary Fig. 1d). After categorizing NAc cells, 56% of recorded pMSNs decreased activity upon LDT terminal inhibition, 36% did not change activity, and 8% increased activity upon LDT terminal inhibition (Fig. 1t).

Stimulation of LDT-NAc projections shifts preference

To evaluate whether the activation of LDT-NAc projections modulated reward-related behaviours, we used the same viral strategy as above, and added a group injected only with Cre-dependent ChR2 in the LDT (CTR group). We have also evaluated animals solely injected with WGA-Cre in the NAc to ensure that they did not present any behavioural differences (data not shown).

Animals were tested in a two-choice lever operant task, in which both levers give a pellet reward but only one is arbitrarily selected to deliver the pellet with simultaneous optogenetic

manipulation of LDT-NAc terminals (stim+ lever; excitation protocol: 473nm, 80 10ms pulses at 20Hz; inhibition protocol: 589nm, 4 s of constant stimulation at 15mW) (Fig 2a). We observed a significant interaction between sessions and groups (2way ANOVA, $F(35, 308)=9.335$, $p<0.0001$). Specifically, no effect of stimulation was found in CTR animals since they presented a similar number of presses in both levers (Bonferroni's *post hoc* t test, $t(352)=0.1711$, $p>0.999$). However, LDT-NAc stimulation induced a clear preference, since ChR2 animals pressed more in the stim+ lever in comparison to the stim- lever (Fig. 2b, *post hoc* t test, $t(352)=13.1$, $p<0.0001$). Moreover, we also observe a substantial increase in the number of lever presses of ChR2 stimulated in comparison to controls, suggesting increased motivation (*post hoc* t test, $t(352)=7.174$, $p<0.0001$). Optogenetic inhibition of LDT-NAc terminals induced an opposite effect, with eNpHR animals presenting decreased preference for the stim+ lever (*post hoc* t test, $t(352)=10.46$, $p<0.0001$). Importantly, this effect was not due to changes in locomotion nor in appetite/food consumption (Supplementary Fig. 2b-d).

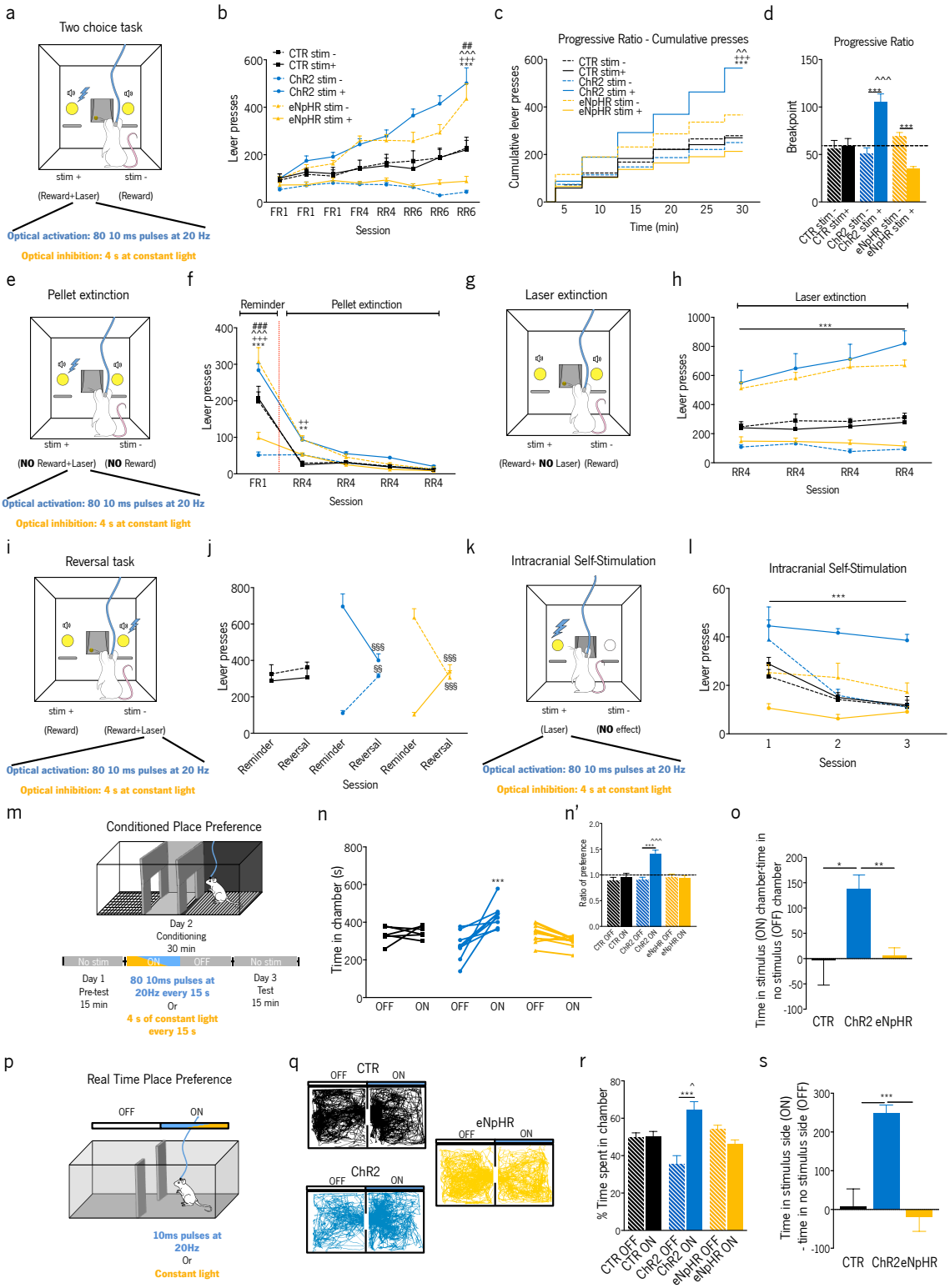

Figure 2. Optogenetic activation of LDT-NAc inputs drives reward. (a) Schematic representation of the two–choice task. One of the levers is randomly associated with the delivery of reward and laser (stim+). ChR2 group refers to optogenetic activation group; eNpHR refers to inhibition group. (b) Time-course representation of the behavioural responses during acquisition of food-seeking behaviour in ChR2 (blue; n=9), eNpHR (yellow; n=10) and CTR (black; n=6) rats. Optogenetic activation of LDT-NAc terminals focuses

choice on stim+ lever, in comparison to an otherwise equivalent food reward (stim-); as opposed to eNpHR that show preference for the stim- lever. (c) Cumulative presses performed during the progressive ratio task show that ChR2 animals press more on the stim+ lever. (d) Breakpoint in the progressive ratio task. ChR2 present increased breakpoint for stim+ lever, whereas eNpHR show a decrease in the breakpoint of the lever associated with optogenetic inhibition. (e) Two-choice task during reward extinction. Responses in either lever does not yield the pellet but laser is still given to stim+ press. (f) All groups decrease lever-pressing responses during pellet extinction conditions. (g) Two-choice task during laser extinction, where no laser stimulation is given on either lever, reward delivery is maintained. (h) Number of responses in laser extinction conditions. ChR2 animals still manifest persistence to press the stim+ lever; eNpHR press more in the stim- lever. (i) Reversal of the two choice task to evaluate behavioural flexibility. (j) CTR animals do not change preference, but ChR2 and eNpHR groups shift response according to the new stim+ lever. (k) Schematic representation of intracranial self-stimulation (ICSS) paradigm: pressing on the stim + lever results on the delivery of optical activation/inhibition. (l) Performance on ICSS, showing that ChR2 readily self-stimulate whereas other groups show no effect. (m) Procedure for conditioned place preference and (p) real time place preference where one chamber is associated with laser stimulation (ON side). (n) Total time spent in the OFF and ON sides. (n') Ratio of preference after CPP conditioning and (o) time difference on the ON and OFF sides, showing preference of ChR2 animals to the ON chamber. No aversion was observed in eNpHR group. (q) Representative tracks during RTPP. (r) Percentage and (s) difference of time spent on the ON and OFF sides, showing that ChR2 animals prefer the ON chamber. No effect was observed in eNpHR animals. * Significant difference between ChR2 stim+ and stim- levers ($p<0.05$); + Significant difference between eNpHR stim+ and stim- levers ($p<0.05$); ^ Significant difference between ChR2 stim+ and CTR stim+ levers ($p<0.05$); # Significant difference between eNpHR stim+ and CTR stim+ levers ($p<0.005$). § Significant difference between reminder and reversal sessions ($p<0.05$) Values are shown as mean ± SEM.

Stimulation of LDT-NAc projections enhances motivation to work for food

Previous data prompt us to evaluate motivation, so we used a progressive ratio schedule of reinforcement, which measures the willingness of an animal to work to get a food pellet. The breakpoint is a direct measure of motivation, and is the maximum effort an animal reaches before giving up when the effort necessary to obtain a reward increases substantially throughout a session. Two-way ANOVA analysis showed that the interaction between the stimulation session and group had a significant effect in the number of cumulative presses (Fig. 2c; 2way ANOVA, $F(25, 220)=11.22$, $p<0.0001$) and the breakpoint value (Fig. 2d; 2way ANOVA, $F(2, 22)=84.64$, $p<0.0001$). ChR2 animals presented increased cumulative presses in the stim+ lever in

comparison to stim- lever (*post hoc* t test, t(44)=4.48, p<0.0001). This was translated into a higher breakpoint in the laser-associated lever in the ChR2 group (*post hoc* t test, t(22)=10.97, p<0.0001), and in comparison to CTR animals (*post hoc* t test, t(44)=5.069, p<0.0001). Inhibition of LDT-NAc terminals decreased motivation since eNpHR animals showed less cumulative presses in the stim+ lever (*post hoc* t test, t(44)=3.186, p<0.05) and a decrease in the breakpoint (t(22)=6.984, p<0.0001). ChR2 animals showed an increase in the number of rewards earned in the stim+ lever session, when compared to the opposite lever or to eNpHR animals (Supplementary Fig. 1a, *post hoc* t test, t(22)=3.799, p=0.0029; t(44)=4.7239, p=0.0003, respectively).

Persistence of preference for the laser-paired lever in laser extinction

In order to further understand the reinforcing properties of LDT-NAc terminals stimulation, animals were subjected to the two-choice task but in laser extinction conditions, which makes the outcome being similar between levers (Fig. 2e). In these conditions, ChR2 animals still manifest preference for the laser-associated stim+ lever in four consecutive trials and, contrariwise, eNpHR group shows preference for the stim- lever (Fig. 2f; 2way ANOVA, F(15, 132)=7.724, p<0.0001). Control group did not present preference for any lever.

Omitting the reward abolishes preference for the laser-paired lever

We next evaluated if LDT-NAc stimulation was reinforcing *per se* or only when paired with a reward. Animals were tested in the same two-choice task but now in pellet extinction conditions (Fig. 2g). All animals decreased instrumental responding for both levers since the first trial (Fig. 2h; 2way ANOVA, F(20, 176)=15.97, p<0.0001). This strongly suggests that the pairing of the stimulation with the reward is crucial for the positive reinforcing properties of LDT-NAc stimulation in operant behaviour. Moreover, it also confirms that the behavioural flexibility is not compromised, considering the immediate decrease in lever pressing once the reward is omitted. Yet, we further tested behavioural flexibility using a single reversal session in which the stim+ lever and the stim- were switched (Fig. 2i). ChR2 animals switch their preference for the novel stim+ lever, whereas eNpHR group decreased preference for the new stim+ lever (Fig. 3j, Reminder vs Reversal session - ChR2 stim+: *post hoc* t test, t(44)=6.089, p<0.0001; ChR2 stim-: t(44)=4.352, p=0.0005; eNpHR stim+: t(44)=5.354, p<0.0001; eNpHR stim-: t(44)=7.462, p<0.0001). Control animals preference remained the same. These results suggest that both ChR2 and eNpHR animals

discriminate the levers based on the *value* that is increased/decreased by laser stimulation, respectively.

Optogenetic activation of LDT terminals is reinforcing

We next decided to evaluate the effects of LDT-NAc optogenetic activation/inhibition in intra-cranial self-stimulation (ICSS) in another set of animals (Fig. 2k). In this test, pressing on the laser-paired lever (stim+) yielded laser stimulation, whereas pressing on the opposite lever did not produce any outcome. Interaction between stimulation and group had a significant effect (Fig. 2l; 2way ANOVA, $F(10, 88)=2.316$, $p=0.0181$). ChR2 animals showed significantly higher lever pressing in the stim+ lever when compared to CTR animals since the first session (Bonferroni's *post hoc* t test, $t(44)=5.435$, $p<0.0001$) while decreasing the presses on stim- lever throughout sessions (Bonferroni's *post hoc* t test, $t(44)=5.188$, $p<0.0001$).). eNpHR animals showed significantly lower lever pressing in the stim+ lever in comparison to the stim- lever (Bonferroni's *post hoc* t test, $t(44)=3.689$, $p=0.0092$), but did not present differences in the number of lever presses in comparison to the control group.

To further understand the reinforcing properties of LDT-NAc activation, we performed the CPP (non-contingent) and RTPP (contingent) tests, pairing one chamber of each apparatus with laser stimulation (ON, Fig. 2m,p,q). The interaction between groups and stimulation produced a significant effect in the CPP (Fig. 2n-n'; 2way ANOVA, $F(2, 22)=16.52$, $p<0.0001$; $F(2, 22)=13.43$, $p=0.0002$, respectively). Activating LDT-NAc terminals elicited place preference in the CPP, shown by the total time spent on the ON side (Fig. 2n; Bonferroni's *post hoc* t test, t 22)=6.152, $p<0.0001$) and by the increase in the ratio of preference between ON and OFF sides (Fig. 2n'; Bonferroni's *post hoc* t test, $t(22)=6.643$, $p<0.0001$) and between ChR2 and CTR groups (Bonferroni's *post hoc* t test, $t(44)=5.097$, $p<0.0001$). The difference of time spent between chambers was increased in the ChR2 group when compared to control (Fig. 2o; Bonferroni's *post hoc* t test, $t(21) = 3.266$, $p < 0.01$) or eNpHR animals (Bonferroni's *post hoc* t test, $t(21)=3.719$, $p<0.05$). eNpHR group presented no preference for any of the chambers (Fig. 2n-o).

Akin, in the RTPP, ChR2 animals also preferred the stimulus-associated ON chamber in comparison to OFF (Bonferroni's *post hoc* t test, $t(22)=4.422$, $p=0.0006$) or to controls (Bonferroni's *post hoc* t test, $t(44)=2.724$, $p=0.0277$). Concordantly, the difference of time spent between chambers was increased in the ChR2 group when compared to control (Fig. 2s;

Bonferroni's *post hoc* t test, t(22)=4.605, p=0.0004). eNpHR group did not manifest preference for any of the chambers in the RTPP.

Optogenetic activation or inhibition of LDT-NAc terminals selectively recruits D1+ or D2+ cells in the NAc

Our next step was to understand what types of cells were being recruited in the NAc during the progressive ratio task with LDT-NAc input excitation/inhibition. So, we quantified the number of c-fos+ cells that expressed either dopamine receptor D1, dopamine receptor D2 or ChAT (cholinergic marker) 90min after the beginning of the PR task (Fig. 3a). A significant effect of group was found in the number of c-fos positive cells (Fig. 3b; 1way ANOVA, $F(5, 21)=11.64$, $p<0.0001$). Stimulated ChR2 stim+ rats showed a significant increase in c-fos staining in the NAc, when compared with non-stimulated ChR2 stim- rats (Bonferroni's *post hoc* t test, $t(21)=6.335$, $p<0.0001$) and with CTR stim+ (Bonferroni's *post hoc* t test, $t(21)=4.601$, $p=0.0023$). The number of double positive c-fos$^+$D1$^+$ cells was increased in ChR2 stim+ animals in comparison with ChR2 stim- rats (Fig. 3b, group effect: 1way ANOVA, $F(5, 21)=11.46$, $p<0.001$, Bonferroni's *post hoc* t test, $t(21)=6.056$, $p<0.001$) as well as with CTR (Bonferroni's *post hoc* t test, $t(21)=4.569$, $p=0.0025$). Interestingly, the number of c-fos$^+$D2$^+$ cells was increased in eNpHR stim+ animals in comparison with non-stimulated eNpHR stim- rats (group effect: 1way ANOVA, $F(5, 21) = 6.459$, $p = 0.0009$, Bonferroni's *post hoc* t test, $t(21)=3.79$, $p=0.0161$). No differences in c-fos$^+$ChAT$^+$ were found.

We then correlated double positive cells with individual breakpoint of all animals (Fig. 3c-e). The number of c-fos$^+$D1$^+$ cells was positively correlated with individual breakpoint (Fig 3c; Pearson's correlation: $r=0.3252$, $p=0.0019$, $n=27$). No significant correlation was found regarding c-fos$^+$/D2$^+$ cells, though there is a trend for inverse correlation with behaviour (Fig 3d; Pearson's correlation: $r=0.0975$, $p=0.1128$, $n=27$). No correlation was found between behavioural performance and the number of c-fos+/ChAT+ cells (Fig. 3e).

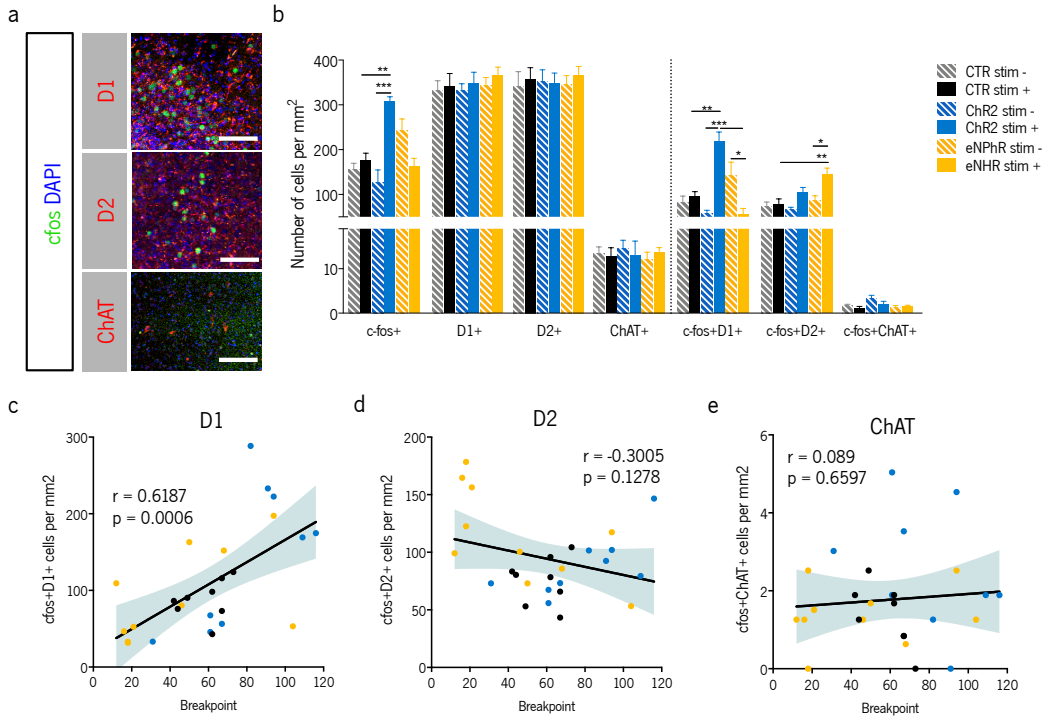

Figure 3. Optical activation of LDT-NAc preferentially recruits NAc D1-MSNs. (a) Representative immunofluorescence for c-fos and dopamine receptor D1, dopamine receptor D2 and ChAT and (b) respective quantification of double positive cells in ChR2 (n_{stim+}=5; n_{stim-}=4), eNpHR (n_{stim+}=5; n_{stim-}=5) and CTR (n_{stim+}=4; n_{stim-}=4) rats after PR performance on the stim+ or the stim- lever. There is an increase in the number of c-fos$^+$D1$^+$ cells in ChR2 stim+ animals and an increase in the number of c-fos$^+$D2$^+$ cells in eNpHR stim+ animals (c) Pearson's correlation between individual breakpoint and number of c-fos$^+$D1$^+$, (d) c-fos$^+$D2$^+$ cells, or (e) c-fos$^+$ChAT$^+$ in the NAc. There is a positive correlation between the number of c-fos$^+$D1$^+$ cells and motivational drive (breakpoint). *p<0.05; **p<0.01; ***p<0.001. Values are shown as mean ± SEM.

Selective optogenetic activation of LDT-NAc cholinergic terminals is sufficient to enhance motivation and induce positive reinforcement

Since the majority of LDT-NAc projections are cholinergic, we decided to selectively manipulate these inputs and evaluate their role in behaviour. For this, we injected in the LDT of ChAT-Cre mice an AAV5 containing a Cre-dependent ChR2 (ChAT-ChR2 group) or eYFP (ChAT-eYFP group) (Fig. 4a). As expected, immunofluorescence against YFP and ChAT revealed a great overlap of both markers (Fig. 4b-c). To test the functionality of this approach, we performed single-cell *in vivo* electrophysiological recordings stimulating either LDT cell bodies, or LDT terminals in the NAc (Fig. 4d).

Optical stimulation of LDT cell bodies increased activity in 77% of local cells (Fig. 4e), 23% did not respond. This was translated into an increase in the net firing rate of this region during

stimulation (Fig. 4f, RM ANOVA, $F(1.315, 27.61)=20.75$, $p<0.0001$, n=22 cells). Optical stimulation of LDT cholinergic terminals evoked an excitatory response in 70% of recorded cells in the NAc, 9% presented inhibitory response, 22% did not respond (Fig. 4g). The net effect was an increase in NAc firing rate (Fig. 4h, RM ANOVA, $F(1.559, 71.71)=63.26$, $p<0.0001$, n=47 cells). By separating cells by their characteristics, we found that LDT terminal stimulation elicited an increase in activity of 83.3% of pMSNs, 16.7% presented no change in activity (Fig. 4i). This stimulation increased firing rate in 50% of recorded pFS; and a decrease in the activity of 60% of pCINs (Fig. 4i).

We then evaluated the role of these projections in behaviour using the two-choice task as in rats. ChAT-ChR2 mice progressively discriminate and prefer the stim+ lever over the sessions (Fig. 4j, 2way ANOVA effect of session: $F(15, 360)=17.1$, $p<0.0001$; effect of group: $F(3, 24)=6.212$, $p=0.0028$). No preference for any lever was observed in ChAT-eYFP control animals. Additionally, no effect of stimulation was observed in general locomotor behaviour (Supplementary Fig. 3a-b).

In the progressive ratio test, ChAT-ChR2 animals presented a stable increase in the cumulative presses in the stim+ lever in comparison to the other lever (Fig. 4k; 2way ANOVA, $F(15, 120)=1.967$, $p=0.0231$). This was translated into a significant increase in the breakpoint of ChAT-ChR2 animals in comparison to ChAT-eYFP animals (Fig. 4l; ANOVA, Bonferroni's *post hoc* t test, $t(24)=4.514$, $p=0.0003$).

In laser extinction conditions in the two choice task, and akin to rats, ChAT-ChR2 mice still display preference for the stim+ lever in comparison to the stim- (Fig. 4m, 2way ANOVA, $F(9, 72)=2.572$, $p=0.0126$, Bonferroni's *post hoc* t test, $t(24)=11.37$, $p<0.0001$); while ChAT-eYFP control animals do not show any preference (Bonferroni's *post hoc* t test, $t(24)=2.987$, $p>0.9999$).

In food extinction conditions in the two choice task, all groups decrease the instrumental responding as soon as the first trial, extinguishing responding (Fig. 4m), similarly to the observations in rats.

To further rule out possible confounding factors such as instrumental habituation, we evaluated the behavioural flexibility of the ChR2 animals using a single-session reversal paradigm in which stim+ was switched to stim- lever. ChAT-ChR2 mice switched their responses towards the new laser-associated lever while decreasing response in the opposite one (Fig. 4o, stim-: Bonferroni's *post hoc* t test, stim-: $t(24)=3.003$, $p=0.0246$; stim+: $t(24)=5.314$, $p<0.0001$).

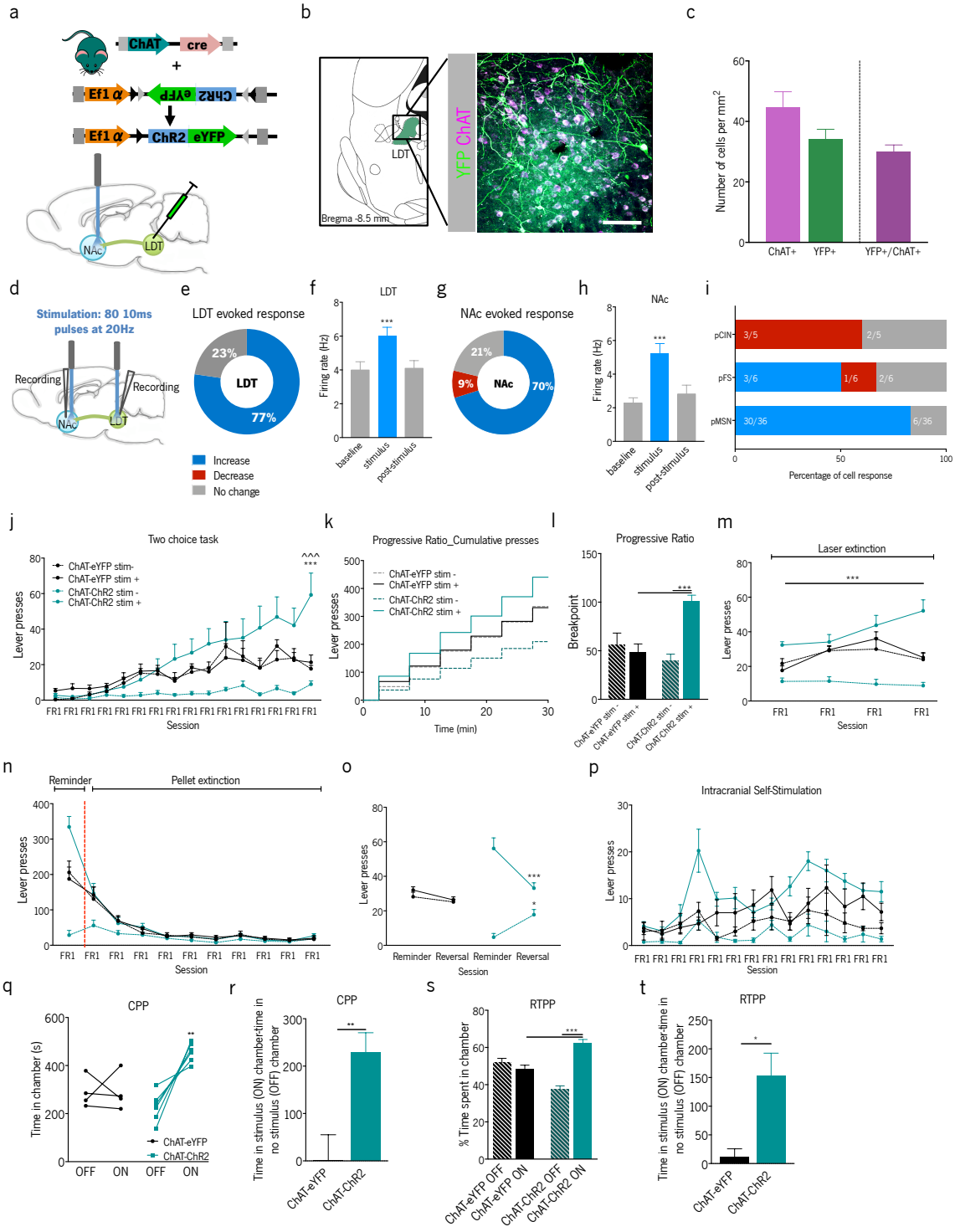

Figure 4. Specific optogenetic activation of LDT-NAc cholinergic terminals drives reward. (a) Strategy used for optogenetic manipulation of cholinergic LDT-NAc terminals. A Cre-dependent ChR2 was injected unilaterally in the LDT of ChAT-Cre mice. (b) Representative immunofluorescence for eYFP and ChAT and (c) respective quantification of double positive cells in the LDT (n=8). (d) Schematic representation of *in vivo* electrophysiological recordings. (e-f) 77% of LDT recorded cells increased firing rate to LDT cell body stimulation (80 10 ms pulses at 20 Hz). (g-h) LDT terminal stimulation evoked an excitatory response in 70% of NAc cells; 9% decreased activity and 21% presented no change in activity. (i)

Percentage of excitatory and inhibitory responses in different NAc cell types in response to LDT cholinergic terminal activation. (j) Time-course representation of the two-choice task of ChAT-ChR2 (green; n=8) and ChAT-eYFP (black; n=6) mice on the stim + (stimulation: 80 10ms pulses at 20Hz) and the stim - lever. ChAT-ChR2 group prefers stim+ lever. (k) Cumulative presses performed during the progressive ratio task, showing that ChAT-ChR2 animals stably press more on the stim+ lever. (l) Breakpoint for stim+ lever is increased in ChAT-ChR2 animals, indicative of increased motivation for the laser-paired reward. (m) Performance in the two choice task in laser extinction conditions, showing persistent preference of ChAT-ChR2 animal for stim+ lever. (n) Similarly to rats, all groups decrease lever-pressing responses during pellet extinction conditions in the two choice task. (o) During a single reversal session, ChAT-ChR2 shift lever-pressing responses towards the new stim+ lever. (p) Time-course representation of the behavioural responses during ICSS behaviour in ChAT-ChR2 and ChAT-eYFP mice. No major differences were observed in the number of lever presses, though there is a trend for preference for the stim+ lever in ChR2 animals in comparison to stim-. (q) Total time spent in the OFF and ON sides in the CPP. (r) Time difference between the ON and OFF sides. (s) Percentage and (t) difference of time in both ON and OFF chambers in the RTPP. Values are shown as mean ± SEM. *p<0.05; **p<0.01; ***p<0.001.

Additionally, we tested whether optogenetic activation of LDT cholinergic terminals facilitates ICSS (Fig. 4p) or place preference as well (Fig. 4q-s). Though there was a trend for increased number of presses in the stim+ lever in comparison to stim-, this was not significantly different from ChAT-eYFP performance, so specific activation of cholinergic LDT-NAc projections was not sufficient to elicit ICSS (at least with these stimulation settings). In the CPP, the interaction between groups and stimulation produced a significant effect (Fig. 4q; 2way ANOVA, $F(1, 8)=11.64$, $p=0.0092$). ChAT-ChR2 animals spent more time in the ON side when compared to OFF side (Bonferroni's *post hoc* t test, $t(8)=5.427$, $p<0.001$) or to the ChAT-eYFP group (Bonferroni's *post hoc* t test, $t(16)=4.292$, $p<0.01$). The difference of time spent between chambers was increased in the ChAT-ChR2 group when compared to ChAT-eYFP (Fig. 4r; t test, $t(8)=3.411$, $p=0.0092$). Similarly, in the RTPP, ChAT-ChR2 animals also preferred the stimulus-associated ON chamber in comparison to OFF (Fig. 4s; Bonferroni's *post hoc* t test, $t(16)=9.507$, $p=0.0092$) or to eYFP (Bonferroni's *post hoc* t test, $t(16)=4.878$, $p=0.0003$). Concordantly, the difference of time spent between chambers was increased in the ChAT-ChR2 group (Fig. 4t; *post hoc* t test, $t(8)=2.814$, $p=0.0227$).

Discussion

The LDT has been linked with different behaviours, including locomotion, sleep and lately with reward-related behaviours (Mena-Segovia, 2016; Mena-Segovia and Bolam, 2017). LDT contribution for reward and reinforcement has been initially proposed based on its modulatory role of VTA dopaminergic activity, indirectly influencing dopamine release in the NAc (Forster and Blaha, 2000; Grace et al., 2007; Lodge and Grace, 2006). Indeed, recent studies showed that optogenetic stimulation of LDT-VTA neurons enhances conditioned place preference (Lammel et al., 2012) and operant responses (Steidl and Veverka, 2015; Steidl et al., 2017b). However, the discovery that the LDT also sends inputs to the NAc indicates that it can also directly control accumbal activity and influence reward behaviours (Dautan et al., 2014, 2016). In agreement, here we show that LDT sends inputs of different natures to NAc and that optogenetic modulation of LDT (cholinergic) terminals alters different dimensions of reward-related behaviours.

We found that the majority of LDT-NAc projections were cholinergic, with additional contribution of glutamatergic and GABAergic inputs, in concordance with a previous study suggesting that cholinergic projections accounted from 41-78% of all inputs (Dautan et al., 2014). Additionally, we show that these projections are functional and provide a predominantly excitatory input to NAc MSNs. Though some accumbal neurons also present evoked inhibitory responses, the net firing rate of this region was increased. These results also highlight the need to perform additional anatomical studies to characterize to which type of NAc cells different populations of LDT neurons project to.

In an effort to understand the role of LDT-NAc neurons in reward-related behaviours, we modulated these inputs during different reward-related behaviours. Stimulation of LDT terminals focuses and amplifies a reward previously paired with laser activation over an otherwise identical reward. In laser extinction conditions, animals still display preference for the stim+ lever, suggesting that this lever has indeed increased value for the animal. Interestingly, animals decreased lever pressing as soon as the associated reward was omitted, regardless of being stimulated or not. Moreover, optogenetic inhibition of LDT-NAc terminals shifted preference for the non-stimulated lever. This suggests that LDT-NAc activation/inhibition is able to add/diminish value to a laser-associated reward, respectively.

In addition to this increase in reward saliency, we found that LDT-NAc stimulation amplified motivation/"wanting" towards the laser paired reward, given by a 40% increase of the breakpoint.

One could argue that these effects were due to an increase in "liking" the reward, however, stimulation during free feeding behaviour did not induce any differences in the free feeding test of chow or palatable food. This enhancement in "wanting" and in the value of the laser-paired reward is reminiscent of another study in which they showed that optogenetic stimulation of central amygdala (CeA) was only observed if paired with an external food reward, and that CeA stimulation per se was not reinforcing since animals would not self-stimulate (Robinson et al., 2014).

Surprisingly, we found that in the ICSS test, optogenetic activation LDT axons was reinforcing, since animals would self-stimulate; in addition, stimulation induced place preference in the CPP and RTPP paradigms. So it appears that stimulation adds value and increases motivation of a reward, but is also reinforcing *per se*.

Because most of the LDT-NAc projections were cholinergic, we decided to selectively manipulate these inputs in mice. We showed that the majority (70%) of accumbal cells present an excitatory response to stimulation of LDT cholinergic terminals. We recapitulated the shift in preference to the laser-associated lever but again only when paired with an external reward, and the observed increase in motivational drive together with an increase in place preference. However, to our surprise, stimulation of cholinergic LDT-NAc inputs was not sufficient to drive self-stimulation, suggesting that other LDT neurons, likely glutamatergic, contribute for the reinforcing effect we observed with general stimulation. In this line of evidence, it has been shown that electrical stimulation of LDT elicits dopamine release in the NAc via a complementary cholinergic and glutamatergic action in the VTA (Forster and Blaha, 2000).

It is known that acetylcholine release in the striatum can have a plethora of outcomes, and has a differential effect in MSNs or interneurons, and also influences neurotransmitter release via presynaptic mechanisms (Calabresi, Picconi, Tozzi, Ghiglieri, & Filippo, 2014; Z. Wang et al., 2006). However, one caveat is that most of published studies were based on the assumption that the only source of striatal acetylcholine was originated from cholinergic interneurons. For example, M2 and M4 receptors in cholinergic interneurons were often considered autoreceptors (Bernard, Normand, & Bloch, 1992; Ding et al., 2006; Oldenburg & Ding, 2011), but we cannot exclude that they also convey signals from LDT cholinergic inputs. One can also envisage that in addition to CINs, LDT-acetylcholine can also regulate corticostriatal glutamatergic or VTA dopaminergic release via pre-synaptic muscarinic and nicotinic receptors, respectively (Calabresi et al., 2014; Oldenburg & Ding, 2011; Z. Wang et al., 2006).

Importantly, we found that during the PR test, a proxy of motivational drive, animals that were stimulated presented a significant positive correlation with the number of c-fos^{+}D1^{+} cells, whereas LDT-NAc cholinergic projections inhibition preferentially activated D2-MSNs. This may suggest that the reinforcing properties of the LDT-NAc circuit may depend downstream on a preferentially activation of D1-MSN pathway, which is known to be tightly associated with positive reinforcement (Calabresi et al., 2014; Ikemoto, Glazier, Murphy, & McBride, 1997; Kravitz, Tye, & Kreitzer, 2012; Lobo et al., 2010; Soares-Cunha et al., 2016; Volman et al., 2013).

In summary, our findings provide the first evidence about the role of LDT-NAc in reward-related behaviours. Further anatomical and mechanistic studies are now needed to characterize LDT-NAc circuit, and to understand the relevance of different LDT populations in the control of NAc activity and in behaviour.

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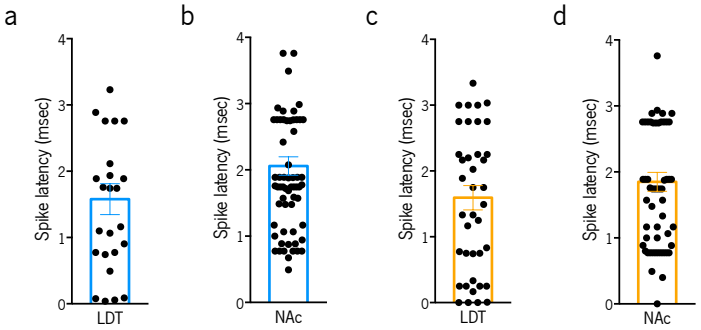
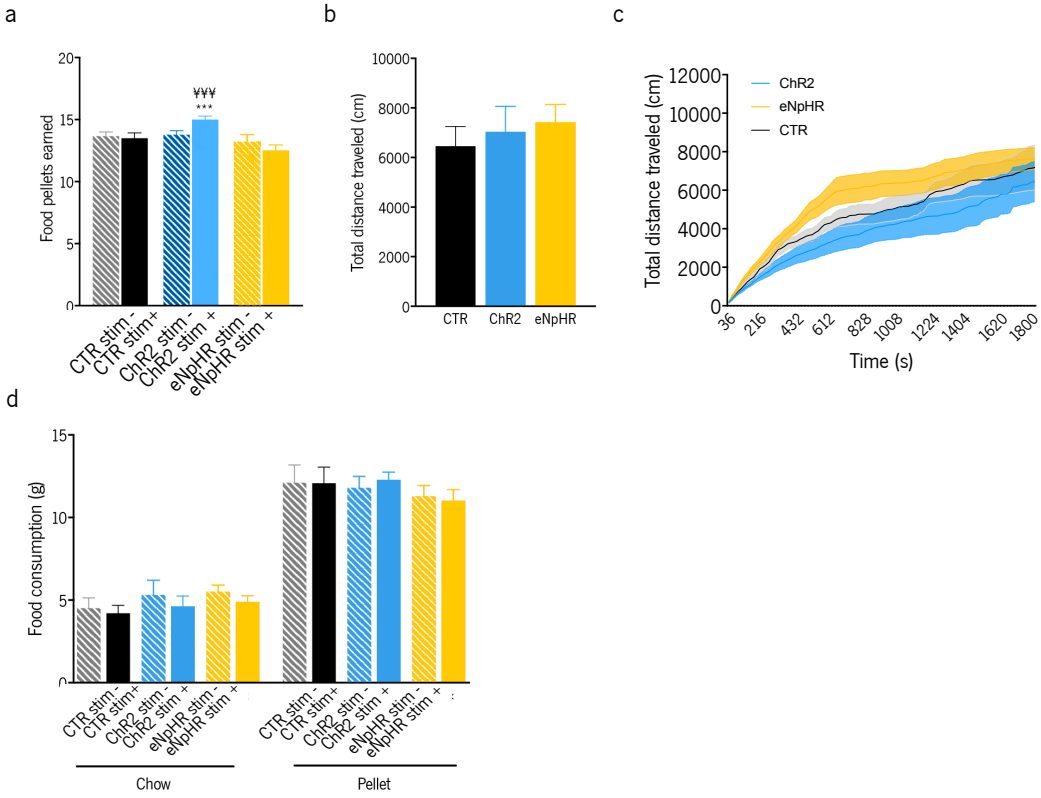
Supplementary Information

Laterodorsal tegmentum projections to NAc are essential for reward and motivation

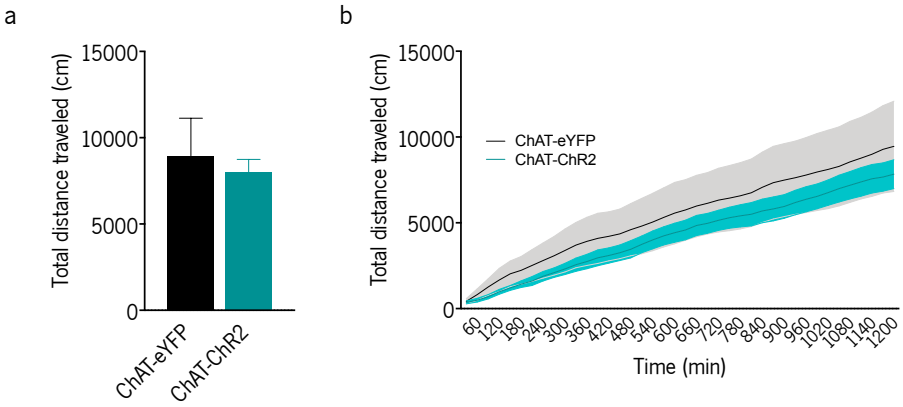
Manuscript in preparation



Supplemental Figure 1. Latencies from evoked optical stimulation responses. (a) Spike latency after optical activation and (c) inhibition in the LDT. (b) Spike latency after optical activation and (d) inhibition of LDT terminals in the NAC (c) cumulative distance travelled. Values are shown as mean ± SEM.



Supplemental Figure 2. Effect of LDT-NAC stimulation on food consumption and locomotion. (a) Food pellets earned during the PR sessions on the stim + and stim – levers. (b) No differences were observed in the locomotor activity between groups, as assessed by total and (c) cumulative distance travelled. (d) No effect of optical stimulation in free-feeding behaviour for either normal chow or palatable food pellets. *Significant difference between ChAT-ChR2 stim+ and stim– lever; ¥Significant difference between ChR2 stim+ and CTR stim+ lever. Values are shown as mean ± SEM.



Supplemental Figure 3. Effect of cholinergic stimulation of LDT terminals in the NAc on locomotion. (a) No differences were observed in the locomotor activity between groups, as assessed by total and (b) cumulative distance travelled. Values are shown as mean ± SEM.

Chapter 4

General Discussion

General discussion

In the present work we took advantage of different approaches to better dissect the role of laterodorsal tegmentum (LDT) modulation on the mesoaccumbens pathway (specifically the ventral tegmental area (VTA) and the nucleus accumbens (NAc), with emphasis on:

1) The impact of *in utero* exposure to high levels of GC (iuGC model) on LDT-VTA projections
2) Role of LDT-VTA inputs in reward-related behaviours
3) Anatomical and electrophysiological characterization of LDT-NAc projections
4) Role of LDT-NAc inputs in reward-related behaviours

Reward deficits in iuGC model: a focus on LDT-VTA inputs

In our first study, we aimed to understand the impact of early life exposure to GC in the LDT-VTA circuit. For this purpose, we used iuGC animals, which are exposed *in utero* to the synthetic GC dexamethasone (DEX) on gestation days 18 and 19. This model is of special interest considering that synthetic GCs are frequently prescribed to pregnant woman at risk of preterm labour (~7% of pregnancies) to ensure foetal lung maturation (Crowley, 1995; Oliveira et al., 2006). This treatment reduces the morbidity and mortality associated with premature birth; however, some deleterious effects have been reported in animal models and humans. For example, children exposed to DEX showed to develop social withdrawal, impairments in verbal working memory and increased emotionality (Hirvikoski et al., 2007, 2012; Trautman et al., 1995). Others showed that this treatment resulted in reduced head circumferences and attention deficits (French et al., 2004). Thus, it seems that exposure to iuGC/stress potentiates the appearance of cognitive, mood, affective and addictive disorders (French et al., 2004; Heim and Nemeroff, 2002; McArthur et al., 2005; Sinha, 2001). Yet, it is important to refer that others revealed no significant impact of GCs whatsoever (Boersma and Tamashiro, 2014; Holson et al., 1995).

The iuGC model has been extensively characterized by our team. iuGC animals develop anxious and depressive-like behaviour, present increased emotional reactivity, social deficits, as well as drug-seeking behaviour and decreased motivation towards natural rewards ((Benesová and Pavlík, 1985; Borges et al., 2013a; DeKosky et al., 1982; Nagano et al., 2008; Oliveira et al., 2006, 2012; Rodrigues et al., 2012; Roque et al., 2011; Soares-Cunha et al., 2014). These prominent behavioural alterations observed in iuGC model render opportune advantages for the study of reward dysfunction and motivation deficits.

The most comprehensively studied circuit involved in reward and motivation is the mesolimbic pathway (Berridge, 2007, 2012; Berridge and Robinson, 1998), encompassing dopaminergic projections from the VTA to the NAc. Manipulations of the mesolimbic dopaminergic system modifies motivation for natural rewards, such as food, as well as for drugs (Berridge, 2007; Kelley and Berridge, 2002; Koob and Le Moal, 2008; Koob and Volkow, 2010). Importantly, we found that iuGC animals present stable and long-lasting changes in the reward/mesolimbic circuit, namely decreased dopaminergic VTA-NAc innervation and reduced dopamine levels in the NAc (Fig.1) (Leão et al., 2007; Rodrigues et al., 2012).

Interestingly, the LDT has the ability to control the dopaminergic tone of the mesolimbic circuit, by directly tuning VTA dopaminergic neurons activity (Blaha et al., 1996; Forster and Blaha, 2000; Grace et al., 2007; Lodge and Grace, 2006). Apart from the molecular changes previously observed in the mesolimbic circuit, iuGC animals also presented increased number of cholinergic cells in the LDT (Borges et al., 2013b), suggesting that iuGC exposure could affect LDT projections and function.

Given the later, in Chapter 2.1, we decided to evaluate how iuGC exposure impacted the LDT-VTA network. We found that iuGC animals have increased expression of choline acetyltransferase (ChAT) and, conversely, decreased levels of acetylcholine esterase (AChE) in the LDT (Fig. 1). This suggests that iuGC can induce long-lasting gene expression changes. In fact, other studies have shown that at critical developmental periods, exposure to GCs can induce persistent effects in specific genes that may lead to increased susceptibility for emotional disorders in adulthood. For example, exposure to iuGC induced long-lasting epigenetic and gene expression changes of dopamine receptor D2 (D2R) in different brain regions, which seem to underlie part of the behavioural deficits observed in these animals (Rodrigues et al., 2012; Soares-Cunha et al., 2014). Interestingly, and in line with the molecular data, it has been shown that increased expression of D2R in the NAc increases operant performance and incentive motivation in rodents (Trifilieff et al., 2013).

Our molecular data suggested that the LDT could be affected by iuGC exposure. The projections originating in the LDT are heterogeneous and consist of cholinergic, glutamatergic and GABAergic components (Bevan et al., 1995; Charara et al., 1996; Omelchenko and Sesack, 2005). LDT modulates VTA activity mainly through cholinergic signalling, though other types of inputs may also contribute for this regulation (Blaha et al., 1996; Forster and Blaha, 2000; Forster

et al., 2002; Grace et al., 2007; Lester et al., 2008; Lodge and Grace, 2006). Using *in vivo* singe-cell electrophysiological recordings, we found that LDT basal activity was decreased in iuGC-exposed animals, while VTA basal activity was unaffected. However, we observed a bidirectional effect in VTA upon LDT stimulation, since iuGC animals present a decrease in the magnitude of excitation and an increase in the magnitude of inhibition in this region. Importantly, we found an increase of inhibition of VTA dopaminergic neurons and simultaneous decrease of inhibition of VTA GABAergic neurons. This suggests that VTA dopaminergic neurons are less active, which is in accordance with the observed decreased basal levels of dopamine in the NAc of iuGC animals (Fig.1) (Leão et al., 2007; Rodrigues et al., 2012). Considering this, we hypothesize that these neurophysiological alterations could underlie reward deficits in iuGC animals.

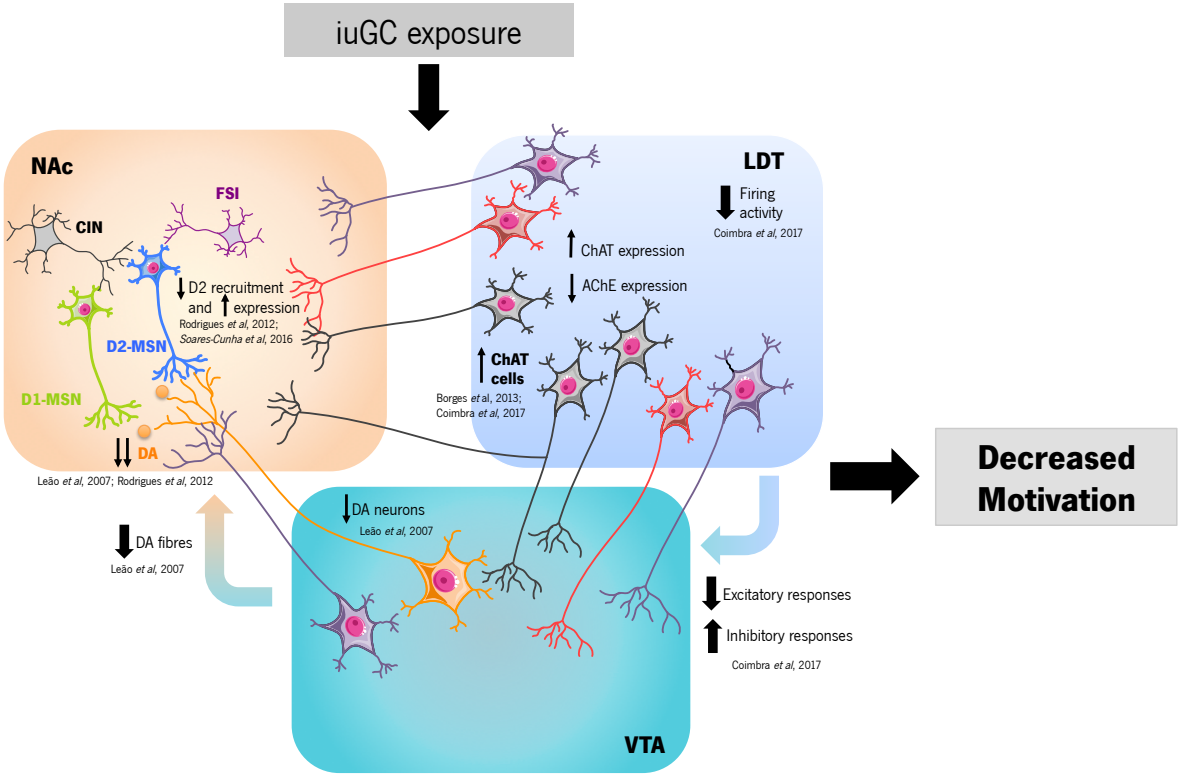


Figure 1. Schematic representation of iuGC exposure effects in the LDT-VTA-NAc circuit. iuGC exposure causes a significant impairment in the firing activity of the LDT, resulting in decreased excitatory and increased inhibitory responses in the VTA. Decreased dopaminergic cells in VTA and fibres in the NAc resulted in an overall decrease in dopamine levels in the latter. In the NAc of iuGC-exposed animals, an increase in the expression of D2R was observed; in the LDT, an increase in ChAT and a decrease in AChE expression were observed. Overall these changes culminated in decreased motivation.

D1-MSN: dopamine receptor D1-expressing medium spiny neuron; D2-MSN: dopamine receptor D2-expressing medium spiny neuron; CIN: cholinergic interneuron; FSI: fast spiking interneuron; iuGC: in utero glucocorticoid exposed rats; NAc: nucleus accumbens; VTA: ventral tegmental area; LDT: laterodorsal tegmentum; DA: dopamine; ChAT: choline acetyltransferase; AChE: acetylcholine esterase ↑: increased; ↓: decreased.

Motivated behaviour is determined both by biological needs and by learned associations between environmental cues and the value associated with the outcome of particular actions, comprising both appetitive behaviour and consummatory behaviours (Koob et al., 2013). We evaluated motivation using the progressive ratio (PR) test, as motivation depends on the contingency between a response and a reinforcer, as well as the strength of the reinforcer. In this paradigm, animals learn arbitrary instrumental actions (lever press), to gain access to positive reinforcers such as food. Animals will vary their level of activity depending on the value of the reinforcer and the likelihood that their behaviour will result in the reinforcer, thus, assessing its capacity to work for a goal. In line with previous observations (Soares-Cunha et al., 2014, 2016), we found that iuGC animals present significant motivational deficits to work for food in the PR test. In addition, we showed that brief optogenetic activation of LDT-VTA terminals during cue exposure was sufficient to rescue this behavioural phenotype (Chapter 2.1).

Another measure of reinforcer value involves assessing whether animals manifest preference for a particular context associated with a rewarding stimulus - the conditioned (CPP) and the real-time place preference (RTPP) tests. Activation of LDT axons in the VTA results in place preference in both control and iuGC animals. Importantly, iuGC animals appear to be more susceptible to the reinforcing properties of LDT-VTA stimulation, in line with our previous findings showing enhanced morphine-induced CPP (Rodrigues et al., 2012). The increase in place preference and motivation by stimulation of LDT terminals in the VTA observed here is consistent with previous studies showing that optogenetic burst-stimulation of brainstem neurons in the VTA increases operant self-stimulation and place preference, by activating mesoaccumbens dopaminergic neurons (Lammel et al., 2012; Steidl and Veverka, 2015; Steidl et al., 2017a; Xiao et al., 2016).

Our results suggest that early life exposure to GC induces long-lasting molecular and neurophysiological alterations in the LDT-VTA network, which may lead to reward/motivational deficits later in life.

LDT inputs to VTA are important for distinct phases of reward behaviour

To further evaluate the role of LDT-VTA projections in rewarding behaviours, we decided to use optogenetics to manipulate the circuit during different stages of reward behaviours in control animals (chapter 2.2).

Activation of the LDT-VTA circuit made unequal an equal reward, i.e, induced preference for a lever that delivers phasic optical stimulation + reward (pellet) versus a lever that only delivers the reward. Laser stimulation of LDT terminals amplified the motivational attractiveness or incentive value of its associatively paired reward representation, raising that incentive value. Such intense and narrow enhancement of learned motivation for a single associated reward by LDT terminal stimulation complements previous demonstrations of broader motivation effects by this region (Coimbra et al., 2017; Ikemoto and Wise, 2002; Kofman et al., 1990; Lammel et al., 2012; Steidl et al., 2017b; Yeomans et al., 1985). Laser extinction did not abolish preference, but omission of reward completely eliminated preference and motivation to work for that lever, proposing that LDT-VTA stimulation added value/salience to the reward rather than being rewarding itself in this paradigm.

Contrary to what we expected, in another behavioural test, LDT-VTA stimulation was rewarding since animals would self-stimulate and prefer the laser-associated chamber in the CPP and RTPP tests. This suggests that excitation of these inputs can increase value of rewards but also act as an independent reward/reinforcer. But it is important to refer that the two tests are based on different premises. First, in the PR test, animals are food deprived and learn to associate an instrumental action to get a food pellet to meet a physiological need of the animal. When the pellet is removed, the instrumental action is no longer reinforced, so animals quit responding because the outcome is absent. In the self-stimulation paradigm and CPP/RTPP, there is no learned association, and animals are not performing any action based on a need. These findings highlight the complexity of reward behaviours, and the importance of the context and associative learning in the process. Other studies have originated similar results - optogenetic stimulation of CeA to ventromedial PFC induces place preference in the RTPP, but is not able to induce self-stimulation behaviour (Seo et al., 2016).

But how can LDT(-VTA) neurons encode motivational value or act as reinforcers?

Sensory stimulation triggers the activation of PPN and LDT cholinergic neurons (Mena-Segovia et al., 2008). In turn, these inputs activate their targets (Steriade et al., 1991) and increase the responsiveness of their target neurons enabling them to bind other modalities of stimuli (Munk et al., 1996). This seems to suggest a role in salience, where cholinergic neurons signal the presence of potentially relevant cues that in turn increase the level of behavioural arousal (Pan and Hyland, 2005). Activation of LDT axons was shown to change burst firing of dopaminergic neurons in VTA, switching the discharge mode (Dautan et al., 2016a). This suggests that, by increasing the number of spikes within bursts, the LDT increases the amount of information contained within each burst, equivalent to increasing the value associated with a reward prediction.

Our findings thus demonstrate the importance of the LDT inputs for the modulation of VTA neuron function and impact on reward-related behaviour. It remains to be established how brainstem cholinergic neurons work in concert with brainstem glutamatergic or GABAergic neurons in the midbrain (and the NAc) to shape behaviour and to determine an organism's response to reward-related stimuli.

LDT sends inputs of different nature to the NAc and these are important modulators of reward-related behaviours

Studies from our lab have suggested that the LDT provided a direct innervation to the NAc, which was lately confirmed in an anatomical study (Dautan et al., 2014). In the NAc, cholinergic LDT neurons that innervate this region were shown to also send collaterals that innervate the midline thalamus and the VTA; in turn, these also project to the NAc (Dautan et al., 2014). This suggests that the cholinergic LDT neurons that modulate mesolimbic dopaminergic neurons also target postsynaptic structures in the NAc and potentially converge with the axons of the same neurons that they modulate within the VTA.

However, to date, nothing was known about how LDT controls neither NAc activity nor the impact of these projections in behaviour. Most of the projections were cholinergic in nature, but we also found a substantial proportion of glutamatergic and GABAergic inputs, that can work together to control striatal activity and shape behaviour.

We observed that stimulation of LDT inputs to the NAc largely activates medium spiny neurons (MSNs), though to date we are uncertain if this effect is observed in dopamine receptor 1

(D1-) and/or dopamine receptor 2 (D2-) MSNs (Chapter 3). We are currently performing anatomical tracing studies to map to which types of accumbal neurons LDT projects to.

Optogenetic stimulation of the LDT-NAc terminals focused towards a reward previously paired with laser activation over an otherwise identical reward, conversely to optogenetic inhibition, which shifted motivation to the opposite reward. These findings were replicated by selective modulation of cholinergic inputs, although we cannot exclude that the other neuronal populations might also contribute for the observed behavioural effects. In fact, stimulation of cholinergic inputs did not induce self-stimulation, contrary to stimulation of all LDT inputs, suggesting that other neuronal populations also contribute for the observed behavioural changes.

In vivo, MSNs exhibit short response latencies to salient stimuli in the range of 100-150ms (Reig and Silberberg, 2014; Schulz et al., 2009). However, whether the thalamo-striatal or mesostriatal projections could mediate these short-latency responses of striatal neurons directly is not clear. Latencies of thalamic neuron responses to salient stimuli are in the range of 150-200ms and for dopaminergic neuron in the range of 70-100ms (Doig et al., 2014; Schultz, 1998, 2015). Brainstem neurons response latencies to salient stimuli are always shorter than the ones observed in dopaminergic, thalamic and striatal neurons (Kobayashi and Okada, 2007). So, one could propose that the LDT is one source of short-latency salient stimuli input that drives NAc neuron responses. However, further experiments are needed to evaluate how LDT mediates salience/value.

Acetylcholine released in the striatum was traditionally thought to arise from a single source: cholinergic interneurons (CINs) (Ding et al., 2010; Wang et al., 2006). However, we, in accordance with the study by Dautan and colleagues, demonstrated that the LDT provides an extrinsic source of acetylcholine to the striatum (Dautan et al., 2014, 2016b). These cholinergic axons make synaptic contact with spines and shafts, suggesting that MSNs and interneurons are receiving direct cholinergic inputs from the brainstem. These LDT direct projections may constitute supplementary level of cholinergic modulation of the striatum activity in addition to the brainstem-midbrain-striatum and the brainstem-thalamus-striatum pathways (Dautan et al., 2016a).

Acetylcholine from LDT could act directly on MSNs, which express M1, M4 muscarinic receptors (mAChRs) and nicotinic receptors (nAChRs) nAChRs. M1 is present in all MSNs, while M4 is present in all direct pathway MSNs and less than half of indirect pathway MSNs (Fig. 2) (Bernard et al., 1992; Yan et al., 2001). Moreover, it can also act on CINs, which express M2 and M4 receptors, leading to a strong inhibition (Ding et al., 2006; Smiley et al., 1999). Almost all CINs

express the β2 subunit, while only half of the CINs present α7 and less than 10% present α3 and α4 subunits (Zhou et al., 2002).

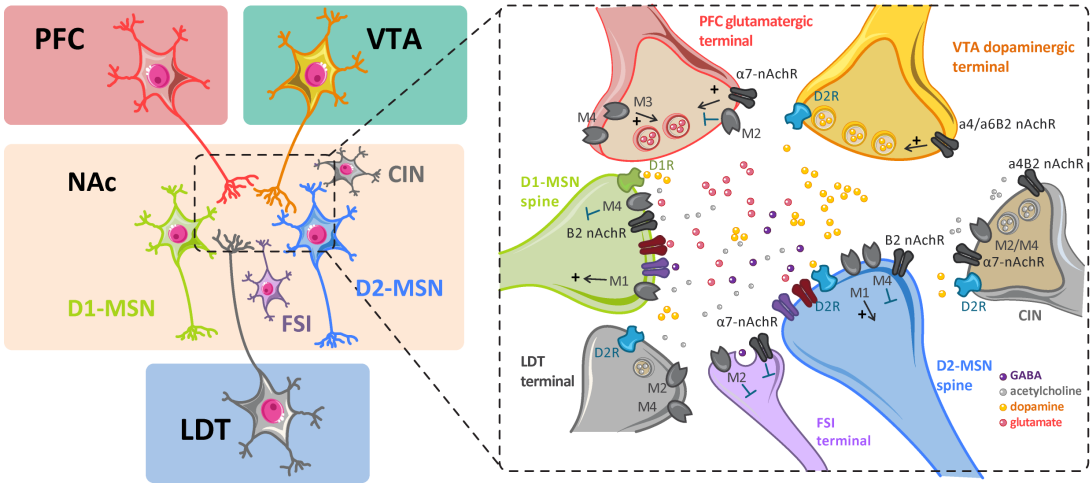


Figure 2. Cholinergic receptors distribution in the NAc microcircuitry. Simplified schematic representation of NAc microcircuit. Left: The NAc receives excitatory glutamatergic (red) inputs from cortical regions (PFC) and dopaminergic (yellow) inputs from the VTA. The LDT sends external cholinergic (grey) inputs to the NAc (as well as GABAergic and glutamatergic – not represented). Besides MSNs, the NAc contains cholinergic interneurons and GABAergic interneurons of different natures, including fast-spiking interneurons that tightly regulate striatal activity. Right: expression of different neurotransmitter receptors, focusing on cholinergic receptors, in striatal neurons and terminals. Of relevance to mention that CINs also express dopamine receptor D2R, inducing dopamine release from VTA terminals in a α4β2–nAChR or α6β2–nAChR dependent manner. MSNs present both excitatory and inhibitory, M1-mAChR or M4-mAChR. (This scheme was adapted from Soares-Cunha et al., 2018).

PFC: prefrontal cortex; NAc: nucleus accumbens; VTA: ventral tegmental area; D1-MSN: D1-expressing medium spiny neuron; D2-MSN: D2-expressing medium spiny neuron; CIN: cholinergic interneuron; FSI: Fast-spiking interneuron; nAchR: nicotinic (ionotropic) cholinergic receptors; M1/M4: muscarinic (metabotropic) cholinergic receptors.

In addition, one can hypothesize that besides a direct effect in local accumbal neurons, following salient stimuli, LDT-released acetylcholine could control dopaminergic and glutamatergic release at the presynaptic level in corticothalamic or VTA inputs respectively, since they express cholinergic receptors. Activation of nAChRs on glutamatergic terminals modulates the release of glutamate and the excitatory drive onto MSNs. The presynaptic modulation of glutamate release was suggested to be mainly controlled by the α7 subunit (Campos et al., 2010; Carpenedo et al., 2001; Gray et al., 1996). Although α4β2 receptors are present on glutamatergic terminals in the Str, their activation has a weaker effect on the release of glutamate than α7 receptors activation

(Xiao et al., 2009). Glutamate release is also negatively modulated by M2 receptors and positively modulated by M3 receptors (Hersch et al., 1994; Levey et al., 1991). NAc dopamine levels are important for performing active instrumental responses that are elicited or maintained by conditioned stimuli (Salamone, 1992), effort in instrumental responding over time (Salamone and Correa, 2002; Salamone et al., 2001), and for regulating the allocation of behavioural resources by setting constraints on the instrumental responses (Hernandez et al., 2010; Salamone and Correa, 2012; Salamone et al., 2007). Nicotinic receptors are expressed on dopaminergic nerve terminals in the Str (Zoli et al., 2002) and their activation potently increases dopamine release (Zhou et al., 2001): ACh, acting via presynaptic nAChRs, triggers release of dopamine; dopamine acting via D2R produces a pause in firing and inhibits ACh release from CINs (Yan et al., 1997). Studies using real time electrochemical detection of DA in striatal slices indicate that ACh released from tonically active CINs (Bennett and Wilson, 1999; Zhou et al., 2001, 2003) acts at β2-subunit-containing (β2*)-nAChRs on striatal DA axons contributing to the high probability of DA release evoked by a single pulse (Rice and Cragg, 2004; Zhou et al., 2001). Thus, LDT inputs may control the dopaminergic tone directly in the striatum by acting VTA terminals, since for example, acetylcholine can elicit dopamine release via α4β2* nAChRs located on dopamine terminals in a manner that is independent of VTA dopamine neuron firing (Cachope et al., 2012; Threlfell et al., 2012).

Additional studies are now needed to understand how LDT modulates accumbal local neurons and terminals in a concerted manner to drive rewarding behaviours.

Challenges and future directions

First, we believe that it is crucial to characterize in detail to which cells in the NAc the LDT projects to using tracing strategies. One possibility would be monosynaptic circuit tracing with glycoprotein (G)-deleted rabies viruses (RVdG). The RVdG does not contain the G protein, which is essential for its replication, and is pseudo-typed with EnvA (an avian virus envelope protein), which uses TVA receptor in order to enter the host cells. Since TVA is not expressed in mammalian cells, only the cells that express TVA and G protein will allow the replication of the virus (Callaway, 2008). By having Cre-dependent expression of TVA and G protein in specific Cre transgenic lines, we are able to selectively infect particular cell types with the RVdG. This strategy is monosynaptic in nature

because the rabies virus cannot spread upstream due to the lack of expression of TVA and G protein in other neurons beside the starter cells (Callaway and Luo, 2015a). In using this, one could identify in detail to which accumbal cells the LDT projects to. Furthermore, although in our work we show that activation of LDT cholinergic terminals in the NAc are sufficient to increase motivation (Chapter 3), we could not fully exclude a possible influence of other types of terminals from this region to the NAc. So, in addition to behavioural interpretation of manipulating specifically cholinergic, glutamatergic or even GABAergic, it would be very interesting to record the activity of these neural circuits. For example, an optogenetic approach together with a freely moving electrophysiological setup could allow a deeper understanding on the electrophysiological modifications caused by optical stimulation of specific neurons and how these modifications influence behavioural performance during reward-related behaviours. This could help solving one of the major caveats of optogenetic experiments, which is the importance of choosing the right stimulation/inhibition parameter close to *in vivo* neuronal functioning. Moreover, patch clamp experiments coupled with pharmacological manipulation could complement these *in vivo* data since it could provide some clues on which accumbal cells are being activated/inhibited by LDT neurons, and by which type of neurotransmitter signalling.

Apart from electrophysiological recordings, *in vivo* imaging could also be an interesting technique to better dissect this circuit since it would allow the identification of the recruitment of different types of accumbal neurons in precise moments of reward-related behaviours. For example, we could use Cre-dependent viral expression of the genetically encoded calcium indicator (GECI) GCaMP3 together with an *in vivo* photometry method using time-correlated single-photon counting (TCSPC)-based optic fibre optics to monitor neuronal activity (Akerboom et al., 2012; Chen et al., 2013; Gunaydin et al., 2014; Lammel et al., 2015; Lerner et al., 2015; Tian et al., 2009). Calcium ions play an important role in neuron function, acting as intracellular signals that can elicit responses such as altered gene expression and neurotransmitter release from synaptic vesicles. Calcium imaging takes advantage of intracellular calcium flux to directly visualize calcium signalling in living neurons. This technique is sensitive enough to track real-time dynamics of genetically and topologically specified subsets of neuronal projections in freely moving mice (Akerboom et al., 2012; Chen et al., 2013), measuring the coordinated activity of neuronal afferents projecting to a particular downstream target in the brain of a behaving animal. Thus, we would be able to identify the recruitment of either D1R- and D2R-expressing neurons in the NAc by each type of input from the LDT in precise moments of reward-related behaviours.

Conclusions

We found that prenatal GC exposure impacts the LDT-VTA circuit, leading to motivational deficits later in life. Selective activation of LDT-VTA inputs rescues behavioural deficits, highlighting the importance of this circuit in reward/motivation-related behaviours. In line with this, we show that activation of LDT-VTA projections is reinforcing but is also able to increase salience of an external reward in control animals.

In addition, we show for the first time that LDT-NAc projections are functional, are mainly excitatory, and play an important role in motivation, saliency and reinforcement. Manipulation of this circuit shapes animal reward-related behaviour and LDT cholinergic inputs appear to be the most determinant for the observed behavioural effects.

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