Universidade do Minho
Escola de Ciências da Saúde

Carina Isabel Soares da Cunha

Role of Nucleus accumbens dopamine D1- and D2-expressing neurons in reward and motivation

Papel dos neurónios do núcleo accumbens que expressam recetores de dopamina D1 e D2 na recompensa e motivação

setembro de 2016

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Carina Isabel Soares da Cunha
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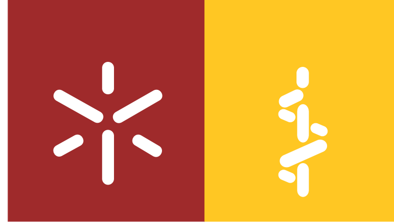

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Trabalho efectuado sob a orientação da
Prof. Doutora Ana João Gomes Rodrigues
e do
Professor Doutor Nuno Jorge Carvalho de Sousa

setembro de 2016

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"The brain is a world consisting of a number of unexplored continents and great stretches of unknown territory..."

Ramon Y Cajal (1852-1954)

Role of Nucleus accumbens dopamine D1- and D2-expressing neurons in reward and motivation

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Abstract

The reward circuit, mainly comprised by dopaminergic fibres arising from the ventral tegmental area (VTA) to the nucleus accumbens (NAc), is one of the most widely studied brain circuits due to its role in mediating reward perception and reinforcement. Dopamine signals from the VTA are essentially decoded by two major populations of GABAergic medium spiny neurons (MSNs) in the NAc, which are canonically segregated into those expressing dopamine receptor D1 (D1R) (D1-MSNs), representing the *direct* pathway, and those expressing the dopamine receptor D2 (D2R) (D2-MSNs), comprising the *indirect* pathway. For many years a functional dichotomy between the direct and the indirect pathways has been assumed in motor control, and lately the same was proposed for valenced stimuli: whereas D1-MSNs mediate reward and reinforcement, D2-MSNs have been associated with aversion and punishment.

Interestingly, previous work from our team has shown that *in utero* exposure to high levels of the synthetic glucocorticoid (GC) dexamethasone (iuGC), induces a decrease in dopaminergic innervation of the NAc together with epigenetic and expression changes of D2R (but not other dopamine receptors). At a behavioural level, iuGC animals presented anhedonia and increased drug-seeking behaviour, suggestive of an imbalance of the reward circuit.

In this thesis we show that iuCG exposure negatively affects motivational drive towards natural rewards and induces prominent D2R changes in different brain regions. Importantly, normalization of dopamine levels by systemic administration of levodopa rescues this phenotype, and this seems dependent on D2R but not D1R signalling.

To get further insight on the role of D1- and D2-MSNs in behaviour, we used optogenetic tools to selectively activate (and inhibit) these neuronal populations during reward-related behaviours. Contrary to the dominant perspective in the field, our results suggest that the classic view of D1-D2 functional antagonism does not hold true for all dimensions of reward-related behaviours, and that D2-MSNs may play a more prominent pro-reward role than originally anticipated.

We demonstrate that both D1- and D2-MSNs are recruited during tasks requiring different degrees of motivation, and that optogenetic activation of either neuronal population enhances motivation in mice. Because data regarding D2-MSNs was paradoxical, we used a different methodological approach in rats, and further show that activating NAc D2-MSNs increases

motivation and is able to induce behavioural conditioning, i.e, is reinforcing. Conversely, optogenetic inhibition of D2-MSNs decreases motivation and induces aversion. These results were extended to the iuGC model, in which we show that brief D2-MSNs activation rescues their motivational deficits. *In vivo* electrophysiological recordings in downstream target regions confirmed the selectivity of the pathway activation and further supported the behavioural data.

We next performed hybrid experiments in which optogenetic activation of D2-MSNs was coupled with pharmacological manipulation, and showed that the increase in motivation required acetylcholine-induced dopamine release from VTA dopaminergic terminals into the NAc, and that both D1R and D2R are essential for this behavioural effect.

Lastly, we show for the first time that NAc D2-MSNs can bi-directionally modulate valenced behaviours depending on their neuronal activation pattern: brief stimulation of these neurons is reinforcing whereas prolonged D2-MSNs stimulation is aversive.

In conclusion, with this work we prove for the first time that the classic view of D1- and D2-MSNs functional antagonism is not true for all dimensions of reward behaviours and that D2-MSNs play a more pro-motivation/reward role than initially anticipated.

Papel dos neurónios do núcleo accumbens que expressam recetores de dopamina D1 e D2 na recompensa e motivação

Resumo

O sistema de recompensa, constituído principalmente por fibras dopaminérgicas originárias da área tegmental ventral (VTA) para o núcleo accumbens (NAc), é um dos circuitos do cérebro mais estudados devido ao seu papel na percepção de recompensas e no reforço. Os sinais dopaminérgicos do VTA são principalmente descodificados por duas populações GABAérgicas de neurónios espinhosos médios (MSNs) no NAc, que são tipicamente segregados nos que expressam o recetor de dopamina D1 (D1R) (D1-MSNs), que constituem a via direta, e aqueles que expressam o receptor de dopamina D2 (D2R) (D2-MSNs), representando a via indireta. Ao longo de vários anos foi proposta uma dicotomia funcional entre a via direta e a via indireta no que respeita ao controlo motor, e, recentemente, o mesmo foi sugerido para estímulos com valência: enquanto que os D1-MSNs medeiam recompensa e reforço positivo, os D2-MSNs têm sido associados a aversão e punição ou reforço negativo.

Trabalho realizado anteriormente pelo nosso grupo demonstrou que a exposição *in utero* a elevados níveis do glicocorticóide (GC) sintético dexametasona (iuGC), induzia uma diminuição da inervação dopaminérgica do NAc, bem como alterações epigenéticas e de expressão do D2R (mas não de outros recetores de dopamina). A nível comportamental, os animais iuGC apresentavam anedonia e aumento de *drug-seeking behavior*, o que sugeria um desequilíbrio do sistema de recompensa.

Nesta tese mostrámos que a exposição a iuCG afeta negativamente a motivação para obter recompensas naturais, e induz alterações proeminentes nos D2R em diferentes regiões do cérebro. A normalização dos níveis de dopamina através da administração sistémica de levodopa, foi capaz de resgatar este fenótipo, dependendo da sinalização via D2R (mas não D1R).

De forma a compreender melhor qual o papel dos D1- e D2-MSNs no comportamento, usamos a técnica de optogenética para seletivamente ativar (ou inibir) populações neuronais durante tarefas de recompensa. Os nossos resultados sugerem que a visão clássica de antagonismo funcional entre D1-D2, não é válida para todas as dimensões do comportamento de recompensa, e que os D2-MSNs podem desempenhar um papel *pró-recompensa* mais saliente do que inicialmente previsto.

Demonstramos que tanto os D1- como os D2-MSNs são recrutados durante tarefas que requerem diferentes graus de motivação, e que a ativação optogenética de qualquer uma destas populações neuronais aumenta a motivação em murganhos. Uma vez que os dados sobre os D2-MSNs eram paradoxais, usámos uma abordagem metodológica diferente em ratos. Confirmamos que a ativação dos D2-MSNs no NAc aumenta a motivação e, além disso, mostramos que induz condicionamento, ou seja, é recompensador. Por outro lado, a inibição optogenética dos D2-MSNs diminuiu a motivação e induziu aversão. Mostramos ainda que ativação breve dos D2-MSNs durante a tarefa reverte os défices motivacionais dos animais iuGC. Gravações electrofisiológicas *in vivo* em regiões alvo a jusante do NAc confirmaram a seletividade da ativação da via.

De seguida, realizámos experiências *híbridas* em que a ativação optogenética dos D2-MSNs foi emparelhada com manipulação farmacológica. O aumento na motivação induzida pela activação dos D2-MSNs depende da libertação de dopamina mediada por acetilcolina no NAc pelos terminais dopaminérgicos do VTA. Tanto os D1R como os D2R são essenciais para este efeito comportamental.

Por fim, mostrámos que os D2-MSNs do NAc podem modular bidireccionalmente comportamentos de valência, dependendo do seu padrão de ativação neuronal: a estimulação breve destes neurónios é recompensadora, enquanto que uma estimulação mais prolongada é aversiva.

Em conclusão, com este trabalho provámos pela primeira vez que a visão clássica de antagonismo funcional dos D1- e D2-MSNs não é verdade para todas as dimensões de comportamentos de recompensa e que os D2-MSNs desempenham um papel mais pró-motivacional do que o antecipado.

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ABBREVIATIONS

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

5HIAA: 5-hydroxyndoleacetic acid

5HT: serotonin

A

A_2aR: adenosine receptor 2a

AAV: adeno-associated virus

AC: adenyl cyclase

ac: anterior commissure

ACC: anterior cingulate cortex

ACh: acetylcholine

ACTH: adrenocorticotropic hormone

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

Amy: amygdala

ANOVA: analysis of variance

AP: anteroposterior

AVP: arginine vasopressin

B

BLA: basolateral amygdala

bp: base pair

BPI: baseline performance interval

C

cAMP: cyclic adenosine monophosphate

CaMK II: calcium-dependent protein kinase II

Cav: calcium channel

CeA: central amygdala

ChAT: choline acetyltransferase

ChR2: channelrhodopsin 2

CIN: cholinergic interneuron

CNS: central nervous system

CONT: control

CPP: conditioned place preference

CR: calretinin

CREB: cAMP response element-binding protein

CRF: continuous reinforcement

CRH: corticotropin-releasing hormone

CS: conditioned stimulus

D

D1R (Drd1 or D1): dopamine receptor D1

D2R (Drd2 or D2): dopamine receptor D2

DA: dopamine

DARPP-32: Dopamine- and cAMP-regulated phosphoprotein, Mr 32 kDa

DAT: dopamine transporter

DEX: dexamethasone

DIO: *double-flox*ed *inverted* open reading frame

DLS: dorsolateral striatum

dlVP: dorsolateral ventral pallidum

DMS: dorsomedial striatum

DNA: deoxyribonucleic acid

dOFC: dorsal orbitofrontal cortex

DOPAC: 3,4-Dihydroxyphenylacetic acid

DPSS: diode-pumped solid-state

DREADD: Designer Receptors Exclusively Activated by Designer Drugs

DV: dorsoventral

E

EFA: exploratory factor analysis

eNpHR: enhanced halorhodopsin

ERK: extracellular signal-regulated kinase

eYFP: enhanced yellow fluorescent protein

F

FBS: foetal bovine serum

FELASA: federation for laboratory animal science associations

FR: fixed ratio

FS: fast spiking GABAergic interneuron

FSCV: fast scan cyclic voltammetry

G

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

GABA: gamma-aminobutyric acid

GC: glucocorticoid

GECI: genetically encoded calcium indicator

GFP: green fluorescent protein

GIRK: G protein-coupled inwardly-rectifying potassium channel

GluR: glutamate receptor

GPCR: G protein-coupled receptor

GP: globus pallidus

GPe: glubus pallidus external

GPi: globus pallidus internal

GR: glucocorticoid receptor

GRF: guanine nucleotide-releasing factor

H

Hipp: hippocampus

HPA: hypothalamus-pituitary-adrenal

HPLC/EC: high pressure liquid chromatography/electrochemical detection

I

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

IF: Immunofluorescence

ILC: infralimbic cortex

INTRSECT: INTronic Recombinase Sites Enabling Combinatorial Targeting

IP3: Inositol triphosphate

ITI: inter-trial interval

iuGC: in utero exposure to glucocorticoids

K

Kir: Inwardly rectifying potassium channel

Kv: voltage-gated potassium channel

KO: knockout

L

L-DOPA: levodopa

LCN: lipocalin

LDT: laterodorsal tegmentum

LH: lateral hypothalamus

LHb: lateral habenula

lOFC: lateral orbitofrontal cortex

LTS: low threshold spiking GABAergic interneuron

M

mAChR: muscarinic acetylcholine receptor

MDT: mediodorsal thalamus

MEK: mitogen-activated protein kinase

MFB: medial forebrain bundle

mGluR: metabotropic glutamate receptor

ML: mediolateral

mPFC: medial prefrontal cortex

MR: mineralocorticoid receptor

MSN: medium-sized spiny neuron

MV: magazine visit

N

NA: numeric aperture

NAc: nucleus accumbens

NAcc: nucleus accumbens core

nAChR: nicotinic acetylcholine receptor

NAcs: nucleus accumbens shell

Nav: voltage-gated calcium channel

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

NOS: nitric oxide synthase

NPY: neuropeptide Y

O

OFC: orbitofrontal cortex

P

PCR: polymerase chain reaction

PFA: paraformaldehyde

PFC: prefrontal cortex

PIT: Pavlovian-to-instrumental transfer

PKA: protein kinase A

PLC: prelimbic cortex

PLC: Phosphoinositide phospholipase C

PP1: Protein phosphatase

PPN: pedunculopontine nuclei

PR: progressive ratio

PTSD: post-traumatic stress disorder

PVN: paraventricular nucleus of the hypothalamus

R

RMT: rostromedial tegmental nucleus

RR: random ratio

RTPP: real-time place preference

S

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

SN: substantia nigra

SNc: substantia nigra pars compacta

SNr: substantia nigra pars reticulata

STN: subthalamic nuclei

T

t-test: Student's t-test

TAN: tonically active cholinergic interneuron

TCSPC: time-correlated single-photon counting

TH: tyrosine hydroxylase

U

UCS: unconditioned stimulus

UNC: University of North Carolina

V

vmVP: ventromedial ventral pallidum

vOFC: ventral orbitofrontal cortex

VP: ventral pallidum

VTA: ventral tegmental area

Y

YFP: yellow fluorescent protein

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

THESIS OUTLINE

The present dissertation is organized in 4 Chapters. Chapter 1 is the General Introduction, the chapters concerning the experimental work are presented in Chapters 2 and 3 (in the form of research articles) and Chapter 4 is the general discussion of the work. The manuscript of Chapter 2 has been published in Translational Psychiatry. The manuscript in Chapter 3.1 is also published in Nature Communications. Chapters 3.2 and 3.3 include manuscripts that are in preparation for submission.

In Chapter 1, a general introduction to the theme of this dissertation is presented. We describe the brain reward circuit and the role of dopamine in reward processing. Next, we provide a more extensive description of the Nucleus accumbens (NAc) region, discussing its different neuronal populations, and the role of medium spiny neurons (MSNs) expressing either dopamine receptor D1 (D1R) (D1-MSNs) or dopamine receptor D2 (D2R) (D2-MSNs) on 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 ("The motivational drive to natural rewards is modulated by prenatal glucocorticoid exposure"), we show that *in utero* exposure to high levels of the synthetic glucocorticoid dexamethasone (iuGC model) decreases motivation towards natural rewards and that systemic administration of levodopa, a dopamine precursor, or treatment with a D2R agonist normalizes this behavioural deficit.

In Chapter 3, we investigate the function of NAc D1- and D2-MSNs in reward behaviours using optogenetics.

In Chapter 3.1 ("Activation of D2 dopamine receptor-expressing neurons in the nucleus accumbens increases motivation"), we show that activation of either NAc D1- or D2-MSNs enhance motivation. We further show that D2-MSNs activation rescues motivational deficits of iuGC-exposed rats.

In Chapter 3.2 we show that the motivational enhancement caused by NAc D2-MSN stimulation appears to be dependent on cholinergic-mediated release of dopamine from VTA terminals in this region and that it requires both D1R and D2R signalling.

In Chapter 3.3 we show that D2-MSNs can bi-directionally modulate valenced behaviours, depending on the type of activation protocol: brief optogenetic activation of this population is reinforcing whereas longer activation periods induce aversion.

Chapter 4 comprises the general discussion of the present dissertation. We reflect about the main findings of this work in the context of the available literature, critically discuss its challenges and drawbacks, and briefly present some future perspectives.

CHAPTER 1

General Introduction

1 General introduction

One of the best-characterized systems in the human and rodent brain is the reward circuit that comprises dopaminergic projections from the ventral tegmental area (VTA) to the nucleus accumbens (NAc). Importantly, this specific circuit has been implicated in the recognition, processing and consumption of rewards in the environment[1–3]. The NAc conveys not only dopaminergic, but also glutamatergic and gamma-aminobutyric acid (GABA)ergic signalling from various brain regions, and serves as a limbic-motor interface in the mediation of motivational and emotional processes toward rewarding (or aversive) stimuli. Because of this, disruption in this brain region has been implicated in numerous neuropsychiatric disorders namely depression and addiction[4]. In the last years, evidence has shown that both prenatal and postnatal exposure to stress or stress hormones (glucocorticoids – GCs) can modify the reward circuit at a molecular, morphological and functional level, which in turn may promote the appearance of these disorders (nicely reviewed by Lupien[5]).

In the following sections, we will provide an overview of the reward system, with a particular focus on the NAc inputs and outputs, and the contribution of different types of accumbal neurons for reward-related behaviours. We will mainly focus on the role of NAc medium spiny neurons (MSNs) expressing either dopamine receptor D1 (D1R) or dopamine receptor D2 (D2R) (D1- and D2-MSNs, respectively), emphasizing the need to re-evaluate the common idea that they mediate opposing aspects of valence behaviours. We finalize this section by providing an overview about the impact that stress/glucocorticoids have in the reward circuit, and how this is translated into altered behaviour.

1.1 The brain reward circuit

Reward is a complex construct that has been the subject of intense debate over the years by psychologists and neurobiologists. One of the possible definitions of reward is “a positive emotional stimulus”, i.e., a reward is able to induce reinforcement since it promotes repeated responding toward the same stimulus. Moreover, rewards allow the maintenance of learned behaviour, elicit approach and consummatory behaviour and may even elicit motivational value in objects that

signal its delivery/presence[6–8], ultimately helping the establishment of value systems for behaviour.

Although there are no specific peripheral receptors for rewards, neurons in several brain regions seem to be particularly sensitive to rewarding events. Remarkably, the most well characterized anatomical component of the reward circuit comprises dopaminergic projections from the VTA to the NAc (Fig. 1)[1,3,9], which are crucial for the detection and perception of rewards and the initiation of their consumption. In addition to this very strong connection with the NAc, VTA dopaminergic neurons also innervate other reward-related regions, such as the medial prefrontal cortex (mPFC), central nucleus of the amygdala (CeA), basolateral amygdala (BLA) and hippocampus[10,11] (Fig. 1).

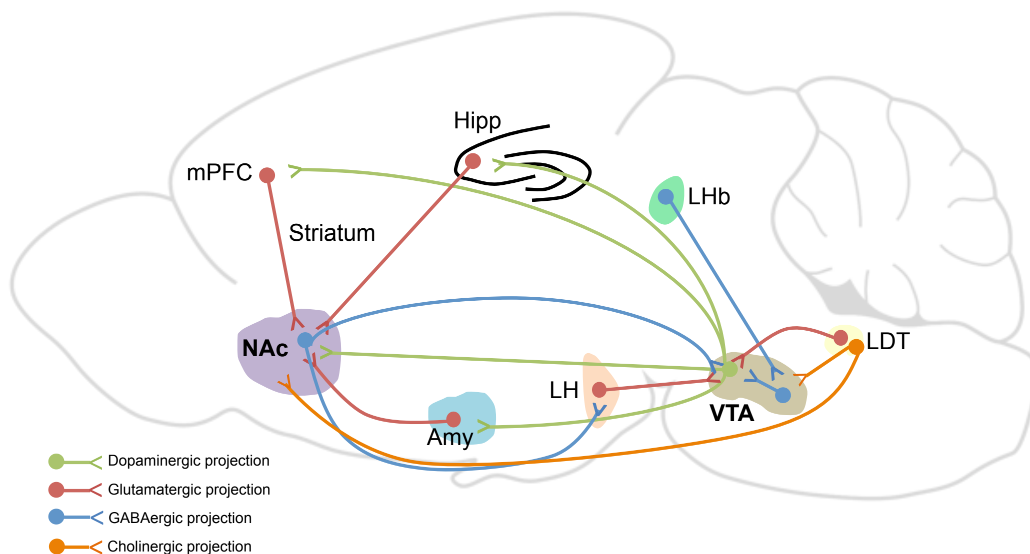

Figure 1. The brain reward system. Simplified scheme of the classical projections comprising the rodent brain reward circuit: a focus on dopaminergic, glutamatergic, GABAergic and cholinergic connections to and from the VTA and NAc. The core of the reward circuit is composed by dopaminergic projections from the VTA to the NAc. In addition, glutamatergic innervation arising from the mPFC, hippocampus and amygdala (and thalamic nuclei – not represented) also modulates accumbal activity. In turn, the NAc is able to modulate VTA through GABAergic projections (either direct or indirect through basal ganglia nuclei such as VP, not depicted). Dopaminergic neurons of the VTA receive direct glutamatergic inputs from the mPFC, LDT and LH, which leads to increased release of dopamine, thus promoting reward. On the other hand, GABAergic neurons of the VTA receive glutamatergic inputs from the LHb, which function to promote aversion. Lastly, both VTA and NAc receive direct cholinergic innervation from the LDT, which are thought to act on positive reinforcement. (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: lateral dorsal tegmentum ; LHb: lateral habenula; LH: lateral hypothalamus; VP: ventral pallidum.

Interestingly, most VTA dopamine neurons that project to the NAc show short, phasic activation in the context of several behavioural situations that involve exposure either to rewards or reward-predicting cues (e.g. Pavlovian conditioning paradigm)[12–15]. Although it is generally recognized that VTA dopamine projections are crucial to sensorimotor function associated with reward processing[16–20], the nature of the contribution of this particular neuronal system to reward processing is still debated.

In the following section we will reflect on theories regarding the specific function of dopamine in reward processing.

1.2 The role of dopamine in reward processing

Dopaminergic neurons originated in the VTA fire in an asynchronous tonic pacemaker manner that can switch to transient synchronous bursts (consisting of a series of consecutive spikes) upon exposure to unexpected or anticipated rewards[21,22]. Accumulating evidence shows that striatal dopamine release occurs in two dissociable temporal modes, "tonic" and "phasic", both of which are highly regulated by glutamatergic forebrain inputs and by cholinergic and GABAergic inputs within the striatum[23–25].

Tonic dopamine transmission constitutes the extra-synaptic pool of steady-state dopamine levels. These levels are strongly regulated and maintained within a narrow concentration in the striatum[26]. Phasic dopamine, which is proposed to be the signal that mediates rapid behaviourally relevant activation of the dopamine system by relevant stimuli, is a high-amplitude transient signal that results from a cell-burst firing and consequent uptake of high levels of dopamine by the synapse[27].

To date, several theories have emerged on what is the role of these dopamine transients in the reward system (extensively reviewed by Berridge[28]):

1. Dopamine is crucial for the process of *learning*;
2. Dopamine mediates the hedonic impact – or the *liking* – of the reward;
3. Dopamine signals *incentive salience* towards the reward – i.e. *wanting* the reward – that is translated into motivation to obtain the reward;

4. Dopamine signals a *reward prediction error* signal – i.e, signals the discrepancy between the predicted and currently experienced reward – which is crucial for reinforcement learning.

Learning

For several years dopamine was pinpointed as a mediator of associative learning between a stimulus and a reward. However, several studies have proven otherwise. Early studies using mutant mice that lack the enzyme tyrosine hydroxylase (TH) showed that absence of dopamine in the brain does not inhibit learning for a location where a sucrose solution was delivered over a location that delivered water[29]. In addition, mice with chronically elevated dopamine levels (dopamine transporter (DAT) knockout (KO) mice) have normal learning in both Pavlovian conditioning and operant responding for food[30]. Others have also shown that depletion of NAc dopamine does not alter learning about rewards during goal-directed behaviours[31]. Hence, these data provide sufficient evidence to support that dopamine is not needed for the learning process.

Liking

Studies that focused on the hedonic ("liking") properties of rewards indicated that dopamine in the striatum is essentially a pleasure neurotransmitter[32,33]. Supporting evidence for the effect of dopamine in the process of liking came from neuroimaging studies that showed that subjective pleasure ratings were often correlated with dopamine receptor occupancy in the ventral striatum/NAc[34]. Nonetheless, neither chemical lesions on medial forebrain neurons or pharmacological blockage of dopaminergic neurotransmission had an effect on the preference of animals toward more pleasurable food rewards[35–38]. Importantly, dopamine depletion associated with Parkinson's disease does not have an impact on subjective pleasure ratings for food rewards[39], thus suggesting that dopamine is neither sufficient nor necessary for hedonic processing of rewards[28].

Incentive salience

Later studies proposed that dopamine primarily mediates the incentive salience (or wanting) component of the reward circuitry[28,40]. Berridge showed that dopamine is capable of controlling the dynamic attribution of incentive salience to reward-related stimuli, causing them and their associated reward to become motivationally "wanted"[28]. In fact, dopamine shapes incentive salience of reward conditioned stimulus (CS) or unconditioned stimulus (UCS) in several ways –

dopamine activation or suppression is able to modulate responses towards CSs when they occur spontaneously and unexpectedly or when the individual is working for a rewarding UCS. Thus, conditioned "wanting" can be seen as a stimulus that will be reflected in increased effort to obtain the reward[28,41,42]. Importantly, dopamine receptor blockage during the performance of a Pavlovian-to-instrumental transfer (PIT) task resulted in reduced cue-triggered "wanting" for the reward CS without suppressing baseline lever pressing, supporting a crucial role of dopamine neurotransmission in generating conditioned incentive motivation[42].

Although this theory is behaviorally consubstantiated, it is still debatable since it contains at least 3 explanatory gaps, according to Colombo: "First, it does not precisely identify the relevant anatomical location of the dopaminergic components; second, it is uncommitted as to possible different roles of phasic and tonic dopaminergic signals; finally, it is not formalized by a single computational model that could yield quantitative predictions"[43].

Reward prediction error

Over the past decades several theoretical and empirical evidences have associated dopamine with reinforcement learning or associative learning[6,44–48], which enables the individuals to anticipate the occurrence of important outcomes. Interestingly, several studies showed that the activity of dopaminergic neurons (and NAc neurons) when animals undergo Pavlovian or instrumental conditioning is compatible with the notion that these neurons encode a reward prediction error signal of the type required by theoretical models of reinforcement learning[46,49–51]. This reward prediction error can be calculated by the difference between the predicted value and the actual value of the reward/punishment after an action is completed.

Pioneering work by Schultz and colleagues using electrophysiological recordings in the primate ventral striatum revealed excitations in response to cues that predict reward[52–56], that depended strongly on the predictive value of the cue[54], the magnitude of the predicted reward[53] and the temporal proximity to the reward[57].

A unifying hypothesis: dopamine signals incentive salience and error prediction signal

Interestingly, recent evidence suggests that both competing hypothesis may indeed be *correct*, and that the answer may rely on dopamine release in distinct sub-regions of the NAc[58]. Experiments that measured real-time dopamine release within the NAc core suggest that this release is consistent with the reward prediction error theory whereas in the shell, dopamine

dynamics is correlated with motivation-based theories (e.g., incentive salience)[58], suggesting that parallel dopamine signals support different dimensions of appetitive behaviour.

1.3 The Reward circuit – not just dopamine

Although dopamine plays a crucial role in reward related behaviours, it is still important to highlight that all of the brain regions that integrate the reward circuit form inter-connections, which are not exclusively of dopaminergic origin (Fig. 1). For example, apart from receiving dopaminergic projections from the VTA, the NAc also receives glutamatergic projections from the prefrontal cortex (PFC), amygdala and hippocampus; the PFC, amygdala and hippocampus also form glutamatergic connections with each other[1,59]. The VTA and NAc also receive cholinergic innervation from the laterodorsal tegmentum (LDT)[60–62].

The functional output of these complex brain signals is modulated by GABAergic neurons of the striatum[63–65] that project to basal ganglia nuclei, such as the ventral pallidum (VP), VTA, subtalamic nucleus (STN)[66], substantia nigra pars reticulata (SNr)[67] and lateral hypothalamus (LH)[68]. Finally, striatal activity is also intricately controlled by the tonic activity of cholinergic and GABAergic interneurons that constitute its microcircuit, further increasing the complexity of reward processing[1,59–61,69].

1.4 The Reward circuit – also encoding for aversion

Besides the traditional role of VTA-NAc dopamine neurons in reward and appetitive behaviours, these can also mediate/signal aversive stimuli[70–72]. Interestingly, studies suggested that neurons that are excited or inhibited by aversive stimuli are anatomically distinct[73], but the projection targets of these neurons can also separate their function: VTA-NAc shell neurons respond to rewarding stimuli whereas VTA-mPFC respond to aversive stimuli[70]. Moreover, dopamine can activate different receptors that are clustered into two families, D1R-like (D1R and D5R) and D2R-like (D2R, D3R and D4R) families, which are believed to lead to distinct (and opposing) cellular outcomes[74,75].

Optogenetic activation of dopaminergic neurons in the VTA, and in particular its projections to the NAc, facilitates the development of positive reward-related behaviours[76–78], whereas stimulation of GABAergic neurons in the same brain region induces avoidance/aversive behaviours[79–81].

Other studies showed that activation of glutamatergic projections to the NAc arising form both BLA and PFC are rewarding[82,83], but activation of GABAergic neurons arising from the rostromedial tegmental nucleus (RMT) to the VTA induce aversive behaviours[84].

Interestingly, and despite the fact that several brain nuclei (e.g PFC, amygdala, or hippocampus) are, in some aspects, responsible for reward processing, the NAc, a major component of the striatum, has long been thought to be a major component in mediating rewarding[85–87,4,88] and motivational processes[20,89–91]. Because of this, impairment in NAc has been implicated in several neuropsychiatric disorders with distinctive disruption in motivation, such as depression, obsessive-compulsive disorder and anxiety, and in drug abuse and addiction.

As a result, a great deal of interest has been given to a deeper understanding of the role of specific accumbal neuronal sub-populations in reward processing, both in the context of disease and health.

2 Focusing on the striatum – *the case of nucleus accumbens*

In the following section we will give a general overview on the relevance of the NAc anatomy, structure and function in reward processing and motivated behaviours.

2.1 Neuroanatomy of the striatum: primate *vs.* rodent

Despite several bridges that are commonly made between rodent and human striatal anatomy, it is important to highlight some differences between these two phylogeneticaly distinct species (Fig. 2a,b). The human dorsal striatum is subdivided into caudate nucleus and putamen by the internal capsula[92] in its most dorsal portion (Fig. 2a). The caudate nucleus receives inputs mostly from cortical regions (prefrontal and orbitofrontal cortices) and the putamen receives mainly excitatory inputs from sensorimotor areas[93,94]. The human ventral striatum is composed by the NAc and olfactory tubercle (Fig. 2a), which receive projections from both prefrontal and limbic structures, and act as motor-limbic interface, being involved in motor, emotional and motivational processes[94,95].

The rodent striatum is also composed by the dorsal and ventral striatum[96] (Fig. 2b). The dorsal striatum is sub-divided into dorsomedial striatum (DMS), which receives inputs mainly from the most ventral region of the PFC (infralimbic cortex(ILC))[97], and the dorsolateral striatum (DLS, homologue to the primate putamen), that receives projections mainly from the motor cortex[96] (Fig. 2b).

The rodent ventral striatum is composed by the NAc[98,99], subdivided into core (NAcc) and shell (NAcs) portions (Fig. 2b), which receives cortical projections from the prelimbic cortex (PLC) and ILC[96,97,100] (Fig. 2b). More detail on these two sub-divisions of the NAc will be given hereafter.

Importantly, recent studies using transgenic animals, novel viral tools and anterograde/retrograde tracers have provided a comprehensive map of direct inputs and outputs of the striatal neurons and interneurons, showing a far more complex network than initially anticipated, and which may be determinant for future functional investigation of striatal circuits[101,102]. Moreover, striatal regions strongly project to different regions of the brain, including the basal ganglia and different cortical and thalamic regions (Fig. 2c)[101,103–105].

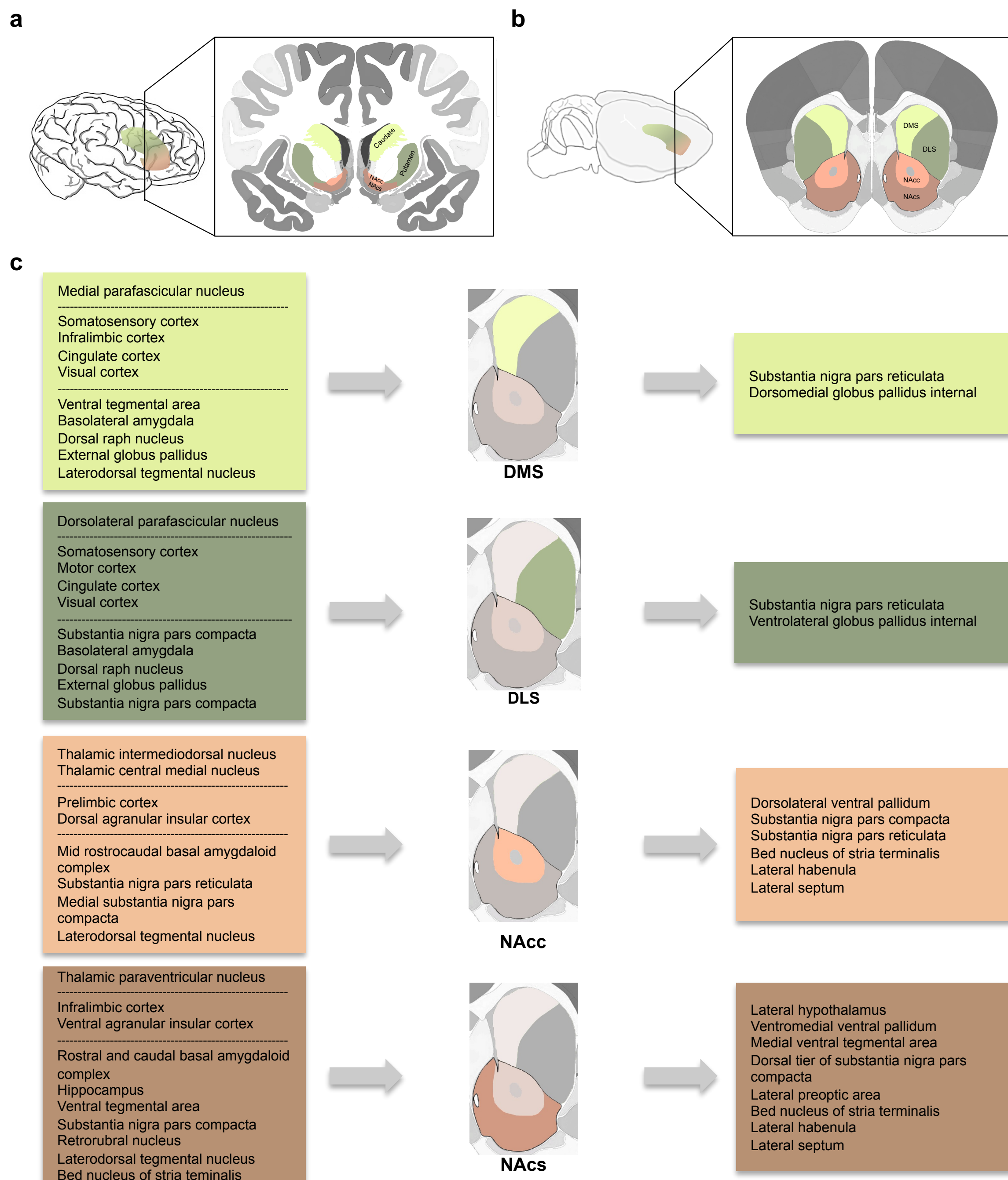

Figure 2. Simplified neuroanatomy of human and rodent striatum. **(a)** Human dorsal striatum includes the caudate and putamen and ventral striatum includes the nucleus accumbens, which is subdivided in core and shell portions and the olfactory tubercle. **(b)** The rodent striatum is divided in dorsal striatum and ventral striatum. The dorsal striatum is subdivided in DMS and DLS portions, while the ventral striatum is comprised by the nucleus accumbens (NAcc and NAcs portions). **(c)** Summary of all brain regions projecting to the striatal sub-divisions, and targets of these striatal sub-divisions.

NAcc: nucleus accumbens core; NAcs: nucleus accumbens shell; DMS: dorsomedial striatum; DLS: dorsolateral striatum

The NAc core and shell

The core is the central portion of the ventral striatum, beneath and continuous with the dorsal striatum and surrounding the anterior commissure (ac). The NAcs is localized in the most ventral and medial portions of the ventral striatum[106,107].

Although the cellular composition of these two NAc sub-divisions is the same, they differ in terms of morphology, projection patterns and function. For example, molecules like substance P[108], calretinin[108], dopamine[109], serotonin[109] and serotonin receptors[110] are more enriched in the NAcs portion, while other molecules, such as calbindin[108,111,112], encephalin[113–115] and $GABA_A$ receptors[116] are more prominently expressed in the NAcc.

There are noteworthy differences between NAcc and NAcs projections (shown in detail in Fig. 2c), which might essentially contribute to the differences in their role in behaviour output. The NAcs division, and particularly its medial portion, is more significantly related with reward processing[91,117–119], while the NAcc appears to be more important to cue-conditioned motivated behaviors[120,121]. In addition, the striatum possesses two complementary chemical compartments: the patch and the matrix, each of this portions enriched in different markers[122–125].

Although the division of the striatum in a patch/matrix compartmental organization has been recognized, an accurate explanation of the function of this division is still lacking.

2.2 Snapshot of striatal neurons

Striatal neurons represent a homogeneous neuronal population, constituted mainly by GABAergic MSNs, which represent about 95% of all neurons.

Dorsal striatal MSNs are traditionally subdivided into two distinct subtypes based on their axonal targets: striatonigral MSNs which *directly* project to output nuclei of the basal ganglia, namely to the globus pallidus internal (GPi), substantia nigra (SN) and VTA (direct pathway; Fig. 3a); and the striatopallidal MSNs, which reach output nuclei *indirectly*, by projecting to the globus pallidus external (GPe) and STN (indirect pathway; Fig. 3b)[126,127]. Activation of the direct pathway briefly supresses pallidal activity, allowing corticothalamic activation and facilitation of movement, whereas activation of the indirect pathway further inhibits corticothalamic neurons and inhibits movement[128–130]. These two MSN populations express different molecules; striatonigral/direct MSNs express D1R, substance P and dynorphin, and striatopallidal MSNs express D2R, adenosine

receptor 2a (A_2aR), and enkephalin[126,131–133]. Despite the distinctive molecular fingerprint of these two MSN subpopulations, it has been suggested that 5-15% of dorsal striatum MSNs can express both D1R and D2R[134], although not much is known about their role in striatal function.

In the NAc, there is a similar direct/indirect dichotomy; although, evidence gathered in the past years shows that the discrete separation of D1-MSNs and D2-MSNs in direct/indirect pathway is not precise. The direct pathway involves NAc projections to the ventral mesencephalon (SN and VTA) and from there to the mediodorsal thalamus (MDT) (Fig. 3c). The indirect circuit travels through the VP and STN before reaching the ventral mesencephalon. Whereas NAcc projects to dorsolateral VP (dlVP) and SN, NAcs projects to ventromedial VP (vmVP) and VTA[99] (Fig. 3d). Akin to dorsal striatum, in the *direct* pathway, NAc-SN/VTA connections are entirely mediated by D1-MSNs; however, *indirect* pathway NAc-VP projections have major contributions of both types of MSNs[135,136]. Because the VP serves as an output nucleus, sending projections outside of the basal ganglia to the MDT, this suggests that both D1R- and D2R-expressing populations can inhibit/disinhibit thalamic activity, contrary to dorsal striatum[135].

The remaining 5% of striatal neurons are interneurons[137]. Interneurons play a very important role in basal ganglia function, by directly or indirectly controlling MSN excitability, and affecting behaviour (reviewed in several studies[138–141]).

Interneurons include large tonically active cholinergic neurons (CIN/TAN). CIN have been implicated in the control of the activation/modulation of the direct and indirect striatal pathways[142,143] and constitute the main cholinergic source of this brain region (at least proven to be functional so far)[144]. Yet, it is important to refer that a neuroanatomical study showed that brainstem cholinergic neurons also provide a direct innervation of the striatal complex [145].

CINs are tonically active *in vivo* and usually pause their activity upon exposure to a behavioural-associated stimulus[143]. Interestingly, specific stimulation of CINs evokes dopamine release in a β2 nicotinic acetylcholine (ACh) receptor (nAChR)-dependent manner[146]. Interestingly, if CINs are selectively activated or inhibited in the striatum it will result in a reduction or increase in the electrophysiological activity of MSNs, respectively[143].

The second class of interneurons are the fast spiking GABAergic (FS) interneurons, which characteristically express parvalbumin and are similar to the FS interneurons present in the cortex and hippocampus[147,148]. These interneurons contain wide axonal processes that can innervate a

large number of striatal MSNs, providing strong feed-forward inhibition that shapes the firing patterns of MSNs[149–151].

Another population of interneurons are the low threshold spiking (LTS) GABAergic interneurons that express somatostatin, neuropeptide Y (NPY) and nitric oxide synthase (NOS), and are involved in long-term plasticity[152,153]. NPY-expressing neurogliaform neurons are the second major class of projecting interneuron in the striatum and, similarly to MSNs, can be activated by cholinergic interneurons[154,155]. Less studied are the TH and calretinin (CR) striatal interneurons, which present sparse connections with MSNs.

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*[156–158].

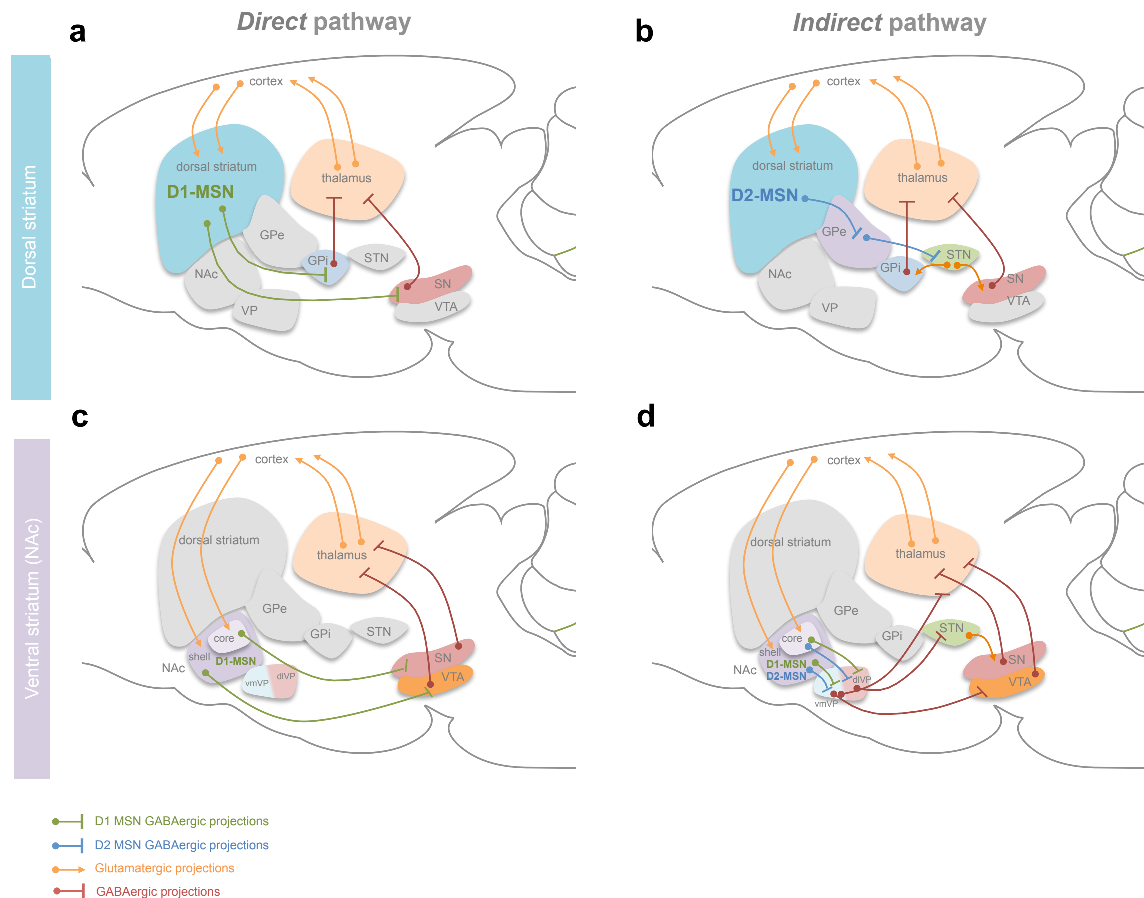

Figure 3. Direct and indirect striatal pathways. **(a)** In the dorsal striatum, MSNs from the direct/striatonigral/D1-MSN pathway project *directly* to the basal ganglia output nuclei, the SNr and the GPi. The inhibitory GABAergic effect leads to disinhibition of the thalamus, which in turns projects to the cortex. MSNs from the direct pathway express D1R, but also express muscarinic 4 (M4) cholinergic receptors, dynorphin, and substance P. **(b)** Activation of indirect/striatopallidal/D2-MSN MSNs, which project *indirectly* to the SNr via the GPe and the STN, inhibits thalamic output to cortex. Dopaminergic input from the SNc modulates corticostriatal transmission by exerting a dual effect on MSNs, depending on their nature (D1R-expressing or D2R-expressing). Indirect pathway MSNs co-express D2R, A_2aR, enkephalin, and neurotensin. There is a part of MSNs that co-express both receptors (<15%), although the functional role of these is still unknown. **(c)** In the ventral striatum, more specifically within the NAc, there is a similar direct/indirect dichotomy akin the dorsal striatum. The direct pathway involves NAc projections to the SNr/VTA and from there to the mediodorsal thalamus. The direct NAc innervation of the SN/VTA is entirely mediated by D1-MSNs. **(d)** The indirect circuit projects to the VP and STN before reaching the output nuclei. In the NAc, while the core projects to the dlVP, the shell innervates the vmVP. The dlVP projects to the STN and SN, whereas the vmVP projects to VTA and out of the basal ganglia to the MDT. Indirect pathway NAc-VP projections have major contributions of both types of MSNs.

SNr: substantia nigra pars reticulata; GPi: globus pallidus pars interna; GPe: globus pallidus pars externa; STN: subthalamic nucleus; SNc: substantia nigra pars compacta; NAc: nucleus accumbens; VP: ventral pallidum; dlVP: dorsolateral pallidum; vmVP: ventromedial pallidum; D1R: dopamine receptor 1; D2R: dopamine receptor 2; A_2aR: adenosine receptor 2a; MDS: mediodorsal thalamus.

2.3 Differences and commonalities between D1R and D2R signalling

Dopamine activation of G-protein coupled dopamine receptors (D1R-D5R) excites or inhibits MSNs by modulating the gating and trafficking of voltage-dependent and ligand-gated (ionotropic) ion channels located at the cell membrane[159]. The separation of the two major classes of dopamine receptors is based on intrinsic structural, pharmacological, and signalling properties: D1R and D5R are grouped in the D1R-like receptor subfamily, while the D2R, D3R and D4R are clustered in the D2R-like receptor subfamily[75].

D2R-like receptors present a higher affinity towards dopamine when compared with the D1R subfamily, which can reach 10- to 100-fold[75]. Because of this differential affinity to dopamine, phasic dopamine transients that result from synchronous burst firing are mainly thought to activate the low-affinity striatal D1R, while tonic dopamine levels arising from pacemaker firing maintain steady-state activation of high-affinity D2R[160–164]. However, recent studies show that high-affinity D2R are also activated by phasic dopamine transients[165].

Both dopamine receptor subfamilies are G-protein coupled receptors (GPCRs), although the second messengers and effector proteins activated by both receptor classes vary greatly and often mediate opposite effects[166]. Briefly, D1R stimulate $G_{s/olf}$ which are coupled to adenyl cyclase (AC) and cause increase in activation of protein kinase A (PKA)[167]. On the other hand, D2R are coupled to $G_{i/o}$ proteins, inhibiting AC and limiting PKA activity[168].

In general, D1R signalling increases the responsiveness of striatonigral neurons to sustained release of glutamate, but it also reduces the responsiveness to transient neurotransmitter release (glutamate and GABA)[167,169–176]. On the other hand, D2R-dependent signalling changes the electrophysiological properties of neurons and generally causes a transient decrease in neuronal excitability[177–181] (summary in Table 1).

D2R are also found pre-synaptically, where they can modulate neurotransmitter release[182–185]. Found at both somatodendritic and axonal sites of the VTA, D2R autoreceptors regulate the firing patterns of dopamine neurons and control the timing and amount of dopamine released within the striatum[186].

It is important to keep in mind that D2R have a mechanism of alternative splicing, and thus, the same gene encodes two distinct isoforms of the same receptor, D2S and D2L[187]. Interestingly, these two alternatively spliced receptors have distinct functions *in vivo*; while D2L acts mainly at postsynaptic sites, D2S serves presynaptic autoreceptor functions[188].

Table 1. Dopamine receptor-dependent modulation of synaptic channels in striatal MSNs.

Channel	Modulation	References
D1R		
Nav1.1	Decrease peak current; negative shift of voltage-dependent inactivation	Carr *et al.*, 2003; Hallett *et al.*, 2006; Hernández-López *et al.*, 1997; Scheuer and Catterall, 2006; Snyder *et al.*, 2000; Vilchis *et al.*, 2000
Kir2	Decrease peak current	
Kv1	Decrease peak current	
Kv4	Decrease peak current	
Cav1.2	Increase peak current	
Cav1.3	Increase peak current	
Cav2	Decrease peak current	
AMPA	Increase peak current and trafficking	
NMDA	Increase peak current and trafficking	
GluR1	Increase surface expression	
D2R		
Nav1	Negative shift of voltage-dependent inactivation and increase in slow activation	Greif *et al.*, 1995; Håkansson *et al.*, 2006; Hernandez-Lopez *et al.*, 2000; Nair and Sealfon, 2003; Olson *et al.*, 2005; Stoof and Kebabian, 1984.
Kir3	Increase peak current	
Cav1.2	Decrease peak current	
Cav1.3	Decrease peak current	
Kir3	Increase channel opening	
AMPA	Decrease in current and surface expression	
GluR1	Decrease in current and surface expression	

2.4 Role of D1- and D2-MSNs in reward/reinforcement

Despite the known cytoarchitecture of the striatum, its heterogeneity poses a major challenge for the attribution of specific biological roles to each type of MSN in behaviour. Traditionally, D1-MSNs are thought to mediate reward and positive reinforcement whereas D2-MSNs encode aversion and negative reinforcement[189–191]; however, recent data questions this dichotomy[135,192–194].

Having this in mind, in the following sections, we will discuss pharmacological, genetic and optogenetic studies that have been applied to try to clarify the biological role of each type of striatal MSNs in reward behaviours.

2.4.1 Pharmacological approaches

Initial pharmacological manipulations using selective agonists and antagonists allowed a breakthrough in the understanding of the role of D1R and D2R in reward behaviours. Because systemic administration of these compounds challenges the interpretation of the data, here we will only focus on intra-striatal administration of D1R and D2R agonists/antagonists.

Early studies have shown that NAc administration of D1R and D2R agonists is reinforcing[195]. Conversely, others have shown that intra-accumbal administration of D1R or D2R agonists is not reinforcing *per se*, because animals do not readily self-administer these compounds. However, concurrent activation of both D1R and D2R in the NAcs had a cooperative effect, inducing in self-administration protocols[196]. In accordance, recent studies showed that both D1R and D2R in the NAc are required for mediating the reinforcing properties of optogenetic self-stimulation of VTA-NAc projections[197].

Regarding the effects of these receptors in mediating natural reward-related responses, it was shown that in hungry rats, pharmacological blockade of D1R or D2R in the NAc affects the amount and duration of feeding, but it does not reduce the amount of food consumed[198]. In agreement, others showed that blockade of D1R or D2R within the NAcc or NAcs decreased lever pressing for food reinforcers, but rats remained directed towards the acquisition and consumption of food[199]. This data indicates that both D1R and D2R signalling are relevant for the motivational drive to work to get a food pellet. In fact, using PIT, which indirectly measures "wanting"/incentive salience, it was shown that selective D1R and D2R blockade in the NAc reduced behavioural performance[200,201]. In agreement, others showed that microinjection of D1R or D2R antagonists into the NAc reduces the proportion of cues to which the animal responded to get a food pellet[202].

Pharmacological targeting of NAc D1R and D2R signalling has also been used to tackle the role of these neurons in the response to drugs of abuse. Most of the studies pinpoint for a *pro-rewarding/reinforcing* role of D1R and a null or attenuating effect of D2R. Accordingly, rodents pre-treated with a D1R (but not D2R) antagonist showed an attenuated locomotor response to an acute cocaine challenge[203], and intra-accumbal administration of D1R antagonist prevents cocaine[204] and ethanol place preference[205,206]. On the contrary, others showed that both D1R and D2R antagonists significantly block the development of amphetamine place preference[207]. Later studies suggested that activation of accumbal D2R does not cause primary rewarding effects, but it can facilitate the

previously associated rewarding effects of environmental stimuli[208,209]. In fact, administration of D2R agonists have prominent reinforcing and enhancing effects in cocaine self-administration in animals with extensive experience in this behaviour[210]. Of course that the observed discrepancies in the studies may be related with drug-specific effects, the use of different dosages of antagonists or subtle differences in the behavioural paradigms, but it is curious that such disparate effects were described.

Altogether, these studies seem to indicate that NAc D1R and D2R pathways promote reward/reinforcement, although dissimilar results were also found. Off course that the use of agonists/antagonists raises different questions: i) the existence of D1/D2-MSNs is not taken into consideration, ii) other types of striatal cells namely interneurons also possess D1R and D2R, iii) the existence of pre-synaptic D2R in dopaminergic terminals that strongly modulate striatal neuronal activity, iv) drugs are rarely specific to one type of receptor and v) pharmacological approaches have poor temporal resolution and diffusion to other brain regions can occur, challenging the interpretation of the data.

2.4.2 Genetic models

The use of genetic methods can provide an alternative for understanding the importance of a specific receptor/pathway. However, it also poses some problems: conventional KOs often present compensatory mechanisms, and the deletion of D1R or D2R throughout the brain defies the identification of the biological role of striatal neurons *per se* in behaviour. Later studies include specific striatal ablation of these receptors, providing a more elegant approach.

D1R knockout models and reward/reinforcement

The first constitutive D1R KO transgenic mice lines were obtained by deletion of either part[211] or most of the D1R coding region[212] from the genome. D1R KO mice have abnormal locomotor behaviour that ranges from hypoactive to hyperactive depending on the experimental conditions. Although in several studies it was reported that these mice were hyperactive in a novel environment and during the dark phase of the light/dark cycle[212,213], others reported an increased latency to move in an open field consistent with a hypoactive phenotype[214].

Although these animals failed to feed normally after weaning[215], this phenotype could be rescued by providing KO mice free access to a palatable food, suggesting that the absence of D1R is more related to a motor deficit than to a reward deficit[212,216]. However, more recent studies showed that D1R KO animals present greatly reduced motivation to work for a food reward in a sucrose preference test, which demonstrates that dopamine signalling through D1R-expressing neurons possibly plays an important role in the modulation of motivational processing[217,218]. D1R KO mice also failed to perform a Pavlovian conditioning task[219].

The response to drugs of abuse is also compromised in D1R KO animals (nicely reviewed by Lobo and Nestler, 2011)[220]. D1R KO mice performing a cocaine self-administration task presented decreased preference for the active lever and reduced overall responses[221]. These animals also presented diminished ethanol consumption[222].

D2R knockout models and reward/reinforcement

Constitutive D2R KO mice display decreased body weight and reduced spontaneous locomotion[223–225]. These animals also respond less for food rewards in instrumental tasks, which could either be attributed to their hypolocomotion or to impairment in motivated responses[226].

The effects of D2R deletion in the response to drugs of abuse resemble those for natural rewards. D2R KOs have blunted response to the rewarding[227] and reinforcing properties of morphine[228], ethanol[229] and cocaine[230].

Other genetic studies have targeted different aspects of D2-MSNs intracellular signaling. Lobo and co-workers knocked down the expression of the sphingosine-1-phosphate receptor Gpr6 (which can stimulate cyclic adenosine monophosphate (cAMP) production - in opposition to D2R activation) in striatopallidal cells (indirect pathway)[231]. These animals have a faster acquisition of operant lever pressing for sugar pellets, suggesting enhanced motivation in a situation that partially mimics increased D2R activity[231]. Also, overexpression of D2R in the NAc using viral gene transfer, selectively increased motivation for food (in a progressive ratio (PR) schedule) without altering consummatory behaviour, the representation of the value of the reinforcer, or the capacity to use reward-associated cues in flexible ways, contrary to dorsal striatum D2R overexpression, which did not alter performance in any of the tasks[232].

In summary, data from genetic models suggest that whereas the response to natural rewards

appears to be modulated by both types of receptors in a similar/synergistic manner, the same does not seem to be true regarding drugs of abuse, with D1R promoting reward and sensitizing responses to psychostimulants and D2R dampening these behaviors. This paradoxical finding is far from being understood and highlights the importance of distinguishing natural vs non-natural rewards and associated responses.

Specific striatal manipulations

An elegant approach using a reversible neurotransmission blocking technique showed that the striatonigral pathway is critical for reward learning whereas the striatopallidal pathway is important for aversive learning[233]. Later, these researchers combined this methodology with local infusion of a receptor-specific agonist or antagonist in the NAc to show that bilateral blockade of the direct pathway in the NAc, but not that of the indirect pathway, significantly reduced cocaine-induced place preference and decreased expression of appetitive reward learning. In addition, mice lacking the D2L receptors (postsynaptic) present a significant impairment in aversive learning[234]. Interestingly, this data was supported by the fact that specific inhibition of D2R-dependent neurotransmission within the NAc also causes impairment in aversive learning in a one-trial inhibitory avoidance task[234].

Others showed that ablation of NAc A_2aR-expressing neurons (A_2aR is co-expressed with D2R receptor in striatum) induced an increase in amphetamine conditioned place preference, suggesting that these neurons inhibit drug reinforcement[235].

2.4.3 Pharmacogenetic studies

Recent studies used viral-mediated expression of a designer receptor exclusively activated by a synthetic drug (DREADDs); activation of the DREADD hM4D receptor potently reduces striatal excitability. With this technique, researchers found that transient disruption of striatopallidal (D2R) neuronal activity facilitated behavioural sensitization to amphetamine, whereas decreasing excitability of striatonigral (D1R) neurons impaired its persistence[236].

2.4.4 Optogenetic studies

Optogenetic studies provided an elegant tool to manipulate different populations of striatal neurons in a fast, specific and reversible manner in order to overcome some caveats of earlier studies. However, the use of these tools also present clear limitations: i) viral expression of opsins relies on the use of *specific* promoters that sometimes are not fully characterized, ii) different stimulation protocols may activate/inhibit distinctive neuronal populations, leading to idiosyncratic behavioural outcomes (this is particularly important considering the unique role of phasic vs tonic dopamine signalling), iii) the use of transgenic cre strains to drive opsin expression may present ectopic expression of cre protein[237,238] and last, but not the least, iv) optical neuronal activation does not necessarily mimic physiological *activity.*

Optogenetic activation of D1-MSNs: NAc versus dorsal striatum

Most of the studies use cre-dependent opsins packed in viral particles in combination with cre transgenic models. Optogenetic activation of NAc D1-MSNs wass sufficient to increase cocaine sensitization and cocaine place preference[190]. In agreement, optogenetic inhibition of D1-MSNs supressed cocaine sensitization[239].

Dorsal striatum D1-MSNs activation was sufficient to induce an operant behaviour in order to receive optic stimulation of these neurons, which confirms its reinforcing properties[240]. Researchers showed that unilateral stimulation of D1-MSNs shifts responses for reward toward the contralateral side of the stimulation[241]. The authors attributed this bias to the positive effects of activation of the direct pathways within the dorsal striatum on motivated responses[241]. Interestingly, stimulation of D1-MSNs of the dorsal striatum also resulted in an increase in cocaine place conditioning[189] emphasizing the role of D1-MSNs in reinforcement. Recently it was shown that although both activation of D1- and D2-MSNs within the DLS resulted in increased self-stimulation, optical stimulation of D1-MSNs was shown to be involved with actions that support goal-directed behaviours, while activation of D2-MSNs resulted in actions that support the formation of stimulus-response habits[242].

Optogenetic activation of D2-MSNs: NAc versus dorsal striatum

Less concordant is the contribution of NAc D2-MSNs in valenced behaviours (Table 2).

Stimulation of NAc D2-MSNs attenuated cocaine conditioned place preference (CPP)[190] and

stimulation of A_2aR-containing neurons (supposedly D2-MSNs) supressed cocaine self-administration[243]. This *anti-reward/reinforcement* role was challenged by evidence showing that stimulation of NAc D2R-expressing neurons affected neither the acquisition nor the expression of cocaine-induced behavioral sensitization[244]. Moreover, the reinforcing properties of optogenetic activation of VTA-NAc dopaminergic terminals requires both D1R and D2R signalling[197]. The discrepancy in the results might be attributed to technical differences in the experimental protocols. For example, Vicente and co-workers found that, in the DLS, D2-MSNs is sufficient to maintain instrumental responding[242]. On the other hand, optogenetic stimulation of DMS D2R-expressing neurons shifted operant responses for food rewards toward the ispilateral side of the stimulation, resembling a decrease in the value for the reward[241,245] (summary in Table 2[246–252]).

In summary, optogenetic studies seem to support the established view of pro-reward/reinforcement role of D1-MSNs both in the ventral and dorsal striatum. Less explicit is the role of D2R-expressing neurons in reward since conflicting findings have been described, which suggests that further studies are needed to understand the contribution of these MSNs for valenced dimensions.

Table 2. Optogenetic modulation of striatal neurons or striatal projections.

Target/ Promoter	Manipulation	Behavioural outcome	Reference
Nucleus accumbens			
D1-MSNs	Activation	Enhances cocaine CPP Induces locomotion after cocaine exposure	Lobo *et al.*, 2010
D2-MSNs	Activation	Prevents cocaine CPP	
GABAergic neurons	Activation	Enhances cocaine CPP	
GaBAergic neurons	Inhibition	Suppresses reinstatement after cocaine self-administration	Stefanik *et al.*, 2013
ChAT (cholinergic cells)	Inhibition	Blocks cocaine CPP	Witten *et al.*, 2010
D2-MSNs	Activation	Does not affect initiation or expression of cocaine-induced behavioural sensitization Attenuates expression of cocaine-induced behavioural sensitization during withdrawal period	Song *et al.*, 2014
VGAT (GABAergic cells)	Activation	Attenuates the learning of cocaine-induced reinforcement Inhibits the expression of cocaine	Wang *et al.*, 2014
ChAT (cholinergic cells)	Activation	Is not sufficient to drive cocaine CPP	Witten *et al.*, 2010
ChAT (cholinergic cells)	Inhibition	Inhibits cocaine CPP	
D2-MSNs	Activation	Inhibits the occurrence of risky decision-making	Zalocusky *et al.*, 2016
Nucleus accumbens – to – Ventral Pallidum			
GABAergic neurons	Inhibition	Reduces cocaine-plus-cue-primed reinstatement	Stefanik *et al.*, 2013
Nucleus accumbens – to – Substantia Nigra			
GABAergic neurons	Inhibition	Fails to block cocaine-plus-cue-primed reinstatement	
Basolateral amygdala – to – Nucleus accumbens			
CaMKII (general neuronal marker)	Activation	Induces optical self-stimulation	Stuber *et al.*, 2011
CaMKII	Inhibition	Reduces cue-evoked intake of sucrose	
CaMKII	Inhibition	Inhibits cocaine cue-induced reinstatement	Stefanik and Kalivas, 2013
CaMKII	Activation	Reinforcing in operant behaviour	Britt *et al.*, 2012

Table 2 (continued). Optogenetic modulation of striatal neurons or striatal projections.

Target/ Promoter	Manipulation	Behavioural outcome	Reference
Ventral tegmental area – to – Nucleus accumbens			
GAD (GABAergic cells)	Activation	Enhances associative learning Unable to disrupt reward consumption	Brown *et al.*, 2012
TH (dopaminergic cells)	Activation	Sufficient to drive intra-cranial self-stimulation	Steinberg *et al.*, 2014
Medial prefrontal cortex – to – Nucleus accumbens			
CaMKII	Activation	Does not elicit optical self-stimulation	Stuber *et al.*, 2011
Dorsal striatum			
D1-MSNs	Activation	Increases spontaneous locomotion	Kravitz *et al.*, 2010
D2-MSNs	Activation	Decreases motor initiation Increases feexing and bradykinesia	
D1-MSNs	Activation	Shifts responses for reward toward contralateral side of the stimulation	Tai *et al.*, 2012
D2-MSNs	Activation	Shifts responses for reward toward the ipsilateral side of stimulation	
D1-MSNs	Activation	Induces self-stimulation Preference in the CPP	Kravitz *et al.*, 2012
D2-MSNs	Activation	Induces transient punishment Decreases locomotion No preference in the CPP	
D1-MSNs	Activation	Positive reinforcement	Vicente *et al.*, 2016
D2-MSNs	Activation	Positive reinforcement	

2.5 D1- and D2-MSNs beyond reward: role in aversion

Although less explored, it is known that the NAc is involved in the processing of aversive stimuli. One of the possible reasons why the role of the NAc in aversion is not so extensively explored might be because an aversive event induces a negative hedonic state, and the capacity of evaluating this state in animals is very limited. So, to the purpose of aversion studies, many researchers frequently use avoidance behaviour as a representation of aversion[253]. Using the two-way active avoidance task, researchers showed that D2R antagonist infusion into the NAc (but not

in dorsal striatum) slightly decreases the number of conditioned avoidance responses[254]; a similar effect was observed with D1R antagonists[255]. Such effects may be partially due to D1R and D2R contribution for early consolidation of aversive memory[256].

Additionally, studies using genetic models showed that D1R- and D2R-expressing striatal neurons greatly respond to aversive stimuli[234,257]. D1R blockade prevented the expression of conditioned aversive stimuli, which may indicate that D1R receptor activation is also required for the acquisition and expression of taste aversion learning[257]. In addition, inactivation of post-synaptic D2R controlled aversive learning[234]. Interestingly, it was shown that mice lacking the D2L receptors (post-synaptic) present a significant impairment in aversive learning[234]. This data was supported by the fact that specific inhibition of D2R-dependent neurotransmission within the NAc also caused impairment in aversive learning in a one-trial inhibitory avoidance task[234].

3 Striatal D1R- and D2R-expressing neurons in human behaviour

Similarly to findings in animal models, earlier human imaging studies showed that dopamine is released in the NAc in anticipation to monetary or taste rewards[258,259]. Later studies have refined these paradigms and were able to show that the striatum responds (albeit differently) to monetary outcomes of different value (reward and punishment)[260]. In addition to anatomical specificities, in non-human primates, separate neural striatal populations coding for actions that are probabilistically associated with positive and negative outcomes exists[261]. Several works supported the co-existence of appetitive and aversive prediction-error signals within the dopaminergic mesolimbic system, which shifts the view of the canonical "reward pathway" to a value-based decision making system (reviewed by others[262,263]).

D1- and D2-MSNs also seem to contribute differentially for reward-related behaviors. For example, individual differences in human approach and avoidance learning are predicted by variability in striatal D1R and D2R binding, respectively[264]. Adding extra complexity to the interpretation of these findings, it was shown that striatal (and frontal) asymmetries in D2R binding (rather than absolute binding levels) predict individual differences in learning from reward versus punishment[265].

In addition to the prevalent view that (phasic) dopamine encodes prediction error signals that mediate reinforcement learning[266,267], there is also evidence that (tonic) dopamine may play a role in response vigor and motivational aspects of reinforcement, and this may rely on the activation of different dopamine receptors at the striatum level [268,269]. For example, higher D2R availability in the left striatum was associated with greater positive incentive motivation in healthy controls[270].

The study of the effect of negative experiences throughout life on accumbal function has shown to be highly relevant to unravel its physiological properties. For instance, exposure to stress or stress hormones has shown to alter accumbal activity, which is translated in the manifestation of specific behavioural traits[5]. Post-traumatic stress disorder (PTSD) patients have decreased activity of accumbal neurons, which results in a decrease in motivation toward more valuable rewards (loss of salience)[271]. Also, patients with stress-related depression exhibit a large decrease in accumbal inhibitory tone[272]. Interestingly, deep brain stimulation of this region was sufficient to revert the anhedonic feature of depressed and anxious patients[86,272]. In addition, aversive stressful events, correlated with poor early life maternal care, were shown to induce a significant increase in

dopamine release in the ventral striatum[273], supporting the thesis that stress-dependent modulation of dopamine activity in the NAc can have a profound effect in behaviour.

4 Prenatal stress as a model of reward dysfunction

Adverse events occurring during early prenatal or postnatal life, can induce long lasting negative effects on mental health[274,275]. In particular, stress or high levels of GCs during prenatal period are believed to induce deleterious programming effects in the reward system that may increase the risk for development of several neuropsychiatric disorders later in life, such as depression, anxiety or even addiction (nicely reviewed by others[5,276,277]).

In the following section the effects of early life exposure to stress, or GCs, on the reward circuitry (and in particular on the NAc) and its behavioural consequences will be discussed.

4.1 Stress/Glucocorticoid programming effects in the brain

Exposure to stress or stress hormones (GC) in different developmental windows and in adulthood has been proposed to trigger different neuropsychiatric disorders including depression, anxiety and addiction disorders[5,275,278–280]. Strikingly, eight in every ten adults who suffered any type of abuse or neglect during infancy or childhood are predicted to develop some type of neuropsychiatric disorder in adulthood[278,281–283]. For example, it was reported that physical abuse or negligent care occurring during childhood increases the risk for the occurrence of several neuropsychiatric disorders, such as depression[284,285], anxiety[278], schizophrenia[286,287] or even addictive disorders[288].

Stress triggers the activation of the hypothalamus-pituitary-adrenal (HPA) axis[289–292], culminating in the production of GCs by the adrenals (Fig. 4). In summary, whenever the organism detects a stressor, the hypothalamus produces corticotropin-releasing hormone (CRH), which in turn binds to specific receptors of the pituitary stimulating the production of adrenocorticotropic hormone (ACTH). ACTH is then translocated to the adrenal glands, that will produce and release GCs (cortisol in humans and corticosterone in rodents) to the blood circulation. In turn, GCs act through negative feedback to both hypothalamus and pituitary in order to cease further release of GCs and prevent deleterious effects of prolonged exposure to these hormones (Fig. 4). However, if GC exposure occurs at sensitive developmental periods it can *program* the HPA axis, leading to maladaptive GC secretion[292]. For example, prenatal stress modifies circadian rhythm of the HPA axis and, consequently, hippocampal corticosteroid receptor expression in the rat[323]. Importantly,

compelling evidence show that this is also true in humans, since maternal stress during gestation alters HPA axis responsiveness of infants and adults[293,294].

GCs act by binding to either mineralocorticoid receptors (MR) or glucocorticoid receptors (GR), which will induce genomic and non-genomic (signalling cascades) effects. For example, GR translocation to the nucleus[295] results in the induction of expression of anti-inflammatory genes[296]. In addition, GCs modulate several metabolic[297–300], cardiovascular[301,302] and immune[303303–305] functions.

At the central nervous system (CNS) level, physiological levels of GC mainly activate MR, while GR receptor-binding is only observed in the presence of increased levels of GCs[306]. Interestingly, MR and GR present distinct anatomical distributions in rodents: MR distribution extends mainly to the hippocampus, hypothalamus, amygdala, septum and cerebral cortex[307] whereas GR is mainly present in the hippocampus, hypothalamus and NAc[307].

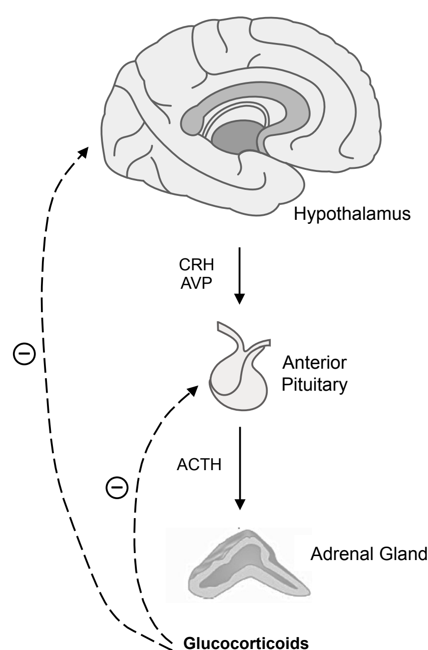

Figure 4. The hypothalamus-pituitary-adrenal (HPA) axis. The activation of the HPA axis occurs in the neurons of the medial parvocellular region of the PVN, which release CRH and AVP. These hormones will then trigger the secretion of ACTH from the pituitary gland, which leads to the production of GCs by the adrenal cortex, and its release to circulation. GCs are capable of regulating ACTH and CRH release by binding to two corticosteroid receptors, the GR and the MR receptors. GCs exert a negative feedback at various levels to regulate the HPA axis and return to a homeostatic state.

CRH: corticotropin releasing hormone; AVP: arginine vasopressin; ACTH: adrenocorticotropic hormone; GR: glucocorticoid receptor; MR: mineralocorticoid receptor; PVN: paraventricular nucleus of the hypothalamus.

4.2 A model of *in utero* exposure to glucocorticoids – implications for the reward system

As mentioned before, exposure to either stress or GCs during prenatal period can have long lasting deleterious effects in the offspring[5,276]. Nevertheless, synthetic GCs, such as dexamethasone (DEX), are often used in the clinics to ensure foetal lung maturation during late pregnancy[308], drastically reducing neonatal morbidity and mortality. Synthetic GCs are preferred to endogenous ones because they are not efficiently degraded by the placental enzyme 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), contrary to endogenous cortisol, of which 80% is oxidized to its inactive metabolite[309]. In addition, DEX is 25 times more potent than cortisol/corticosterone[310], and acts mostly through GR signalling, contrary to physiological levels of GCs that act through MR[306,311,312]. In this perspective, although more efficient than endogenous GCs, the administration of synthetic GCs poses additional risks for the developing brain.

In order to further understand the impact of this treatment in the developing brain, our laboratory developed a model of *in utero* exposure to GCs in the last part of the rat pregnancy – iuGC (subcutaneous administration of DEX 1mg kg^{-1} on gestation days 18 and 19). iuGC animals are hyper-anxious[313,314], present depressive-like behaviour[314,315], deficits in social behaviours[314], and enhanced drug-seeking behaviour[316], but no cognitive deficits[317]. A remarkable feature is that these animals present a notorious impairment in the reward system development, exhibiting a significant decrease in the number of TH-positive fibres in the VTA[318]. There is a significant increase in the ratio of apoptotic to proliferative cells in the VTA[318] that largely contributes to the impoverishment in dopaminergic innervation of the NAc. These results are not surprising considering the fact that the dopaminergic system in primates is only fully mature during puberty[319,320]. Similarly, in rodents, midbrain dopamine neurons are formed during early life development, according to a rostrolateral to caudomedial gradient[321], and are only fully maturated after the first two weeks post birth[322].

In addition to midbrain changes, we also observed an overall decrease in NAc volume[318], accompanied by significant changes in spine density and neuronal morphology of accumbal MSNs[316]. In addition, iuGC animals present a significant decrease in dopamine levels in the NAc and amygdala[313,316], two key regions of the reward system. Importantly, peripheral administration of dopamine precursor levodopa (L-DOPA) rescued all of the reward deficits observed in these animals, i.e, anhedonia, social deficits and drug-seeking behaviour[314,316].

Interestingly, iuGC treatment caused alterations in the pattern of expression of the D2R in the NAc (but not other dopamine receptors), due to the occurrence of epigenetic phenomena[316], suggesting a specific programming effect of GCs in this receptor.

In summary, iuGC animals present prominent reward deficits that *partially* occur due to impairments in the dopaminergic innervation to the NAc, and seem to pinpoint for a specific epigenetic programing of D2R in these animals.

OBJECTIVES

In this thesis we intended to evaluate the impact of prenatal GC exposure in the reward network, and to further dissect the role of different NAc populations in reward and motivation.

The main objectives of the present thesis were:

1. Assess the impact of *in utero* exposure to high levels of DEX (a synthetic glucocorticoid; iuGC model) on the motivational drive towards natural rewards (food);

2. Evaluate the effects of NAc D1R- and D2R-expressing neurons modulation in motivation-dependent behaviours, in *naïve* animals and in iuGC-exposed animals;

3. Assess the behavioural and electrophysiological effects of selective optogenetic manipulation of NAc D2R-expressing neurons.

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CHAPTER 2

The motivational drive to natural rewards is modulated by prenatal glucocorticoid exposure

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ORIGINAL ARTICLE

The motivational drive to natural rewards is modulated by prenatal glucocorticoid exposure

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Exposure to elevated levels of glucocorticoids (GCs) during neurodevelopment has been identified as a triggering factor for the development of reward-associated disorders in adulthood. Disturbances in the neural networks responsible for the complex processes that assign value to rewards and associated stimuli are critical for disorders such as depression, obsessive–compulsive disorders, obesity and addiction. Essential in the understanding on how cues influence behavior is the Pavlovian–instrumental transfer (PIT), a phenomenon that refers to the capacity of a Pavlovian stimulus that predicts a reward to elicit instrumental responses for that same reward. Here, we demonstrate that *in utero* exposure to GCs (iuGC) impairs both general and selective versions of the PIT paradigm, suggestive of deficits in motivational drive. The iuGC animals presented impaired neuronal activation pattern upon PIT performance in cortical and limbic regions, as well as morphometric changes and reduced levels of dopamine in prefrontal and orbitofrontal cortices, key regions involved in the integration of Pavlovian and instrumental stimuli. Normalization of dopamine levels rescued this behavior, a process that relied on D2/D3, but not D1, dopamine receptor activation. In summary, iuGC exposure programs the mesocorticolimbic dopaminergic circuitry, leading to a reduction in the attribution of the incentive salience to cues, in a dopamine-D2/D3-dependent manner. Ultimately, these results are important to understand how GCs bias incentive processes, a fact that is particularly relevant for disorders where differential attribution of incentive salience is critical.

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INTRODUCTION

Early life stress or exposure to elevated levels of glucocorticoids (GCs) may increase the risk for the development of neuropsychiatric disorders, including those associated with reward deficits such as depression, obesity and addiction.[1–3] Although the neural circuits involved in their pathophysiology are complex, it is strongly believed that they are tightly linked to mesolimbic dopaminergic dysfunction.[4,5] Mesolimbic dopamine (DA) signaling has long been implicated in reward processing, but its precise contribution remains a topic of intense debate, in particular the role of accumbal DA.[6,7] Apart from the classical role in mediating the hedonic impact of a reward,[8] this circuit also seems crucial for reinforcement learning, being responsible for establishing stimulus–response associations (associative stamping) and, eventually, to enhance habit formation.[9] It is also hypothesized that changes in the activity of DA neurons encode a quantitative prediction error.[10] In addition, Berridge[6] has suggested that DA is responsible for the attribution of incentive salience to (otherwise neutral) cues that predict a reward, which triggers a motivational state of 'wanting' for both the cue and its associated reward. Regardless of the mechanism, mesolimbic DA seems to stamp in response–reward and stimulus–reward associations that are essential for the expression of motivated behaviors.[7]

Previous work from our lab has shown that prenatal exposure to GCs leads to morphological adaptations within the nucleus accumbens (NAc) and amygdala, together with a significant reduction in dopaminergic innervation arising from the ventral tegmental area (VTA).[11–13] As a result, these animals displayed persistent anhedonia but enhanced vulnerability for drug-seeking behavior in adulthood.[13,14] These symptoms may result from a complex differential attribution of incentive salience to natural versus non-natural rewards and their associated cues. In fact, individual differences in incentive salience attribution/'wanting' have been linked with propensity for addiction-like behaviors.[15] In this framework, we sought to further dissect if and how prenatal GC exposure alters the attribution of incentive salience to a cue predicting a natural reward (food). To do so, we used the Pavlovian–instrumental transfer (PIT) paradigm, which relies on the well-described phenomenon of a Pavlovian stimulus to invigorate an ongoing instrumental action.[16] The associative value of the cue and its motivational significance are crucial for proper transfer, a phenomenon that resembles cue-mediated increased drive for drugs seen in addicted individuals.[17,18] This test is often seen as a reflex of motivation for a specific reward, as it measures the ability of a cue to trigger the drive for a reward in the absence of both primary and secondary reinforcements.[16] In addition, we further evaluated the role of DA, and the impact of specific activation of either D1 or D2/D3 DA receptors in this type of behavior.

MATERIALS AND METHODS

Detailed explanation of all procedures is provided in the Supplementary Material.

Animals

All manipulations were conducted according to current regulations (European Union Directive 2010/63/EU). Pregnant Wistar Han rats were

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subcutaneously injected with the synthetic GC dexamethasone (DEX; *in utero* exposure to GC (iuGC) animals), at 1 mg kg^{-1} or with vehicle (CONT; control animals), on days 18 and 19 of gestation. Male offspring aged 3–4 months were used.

Behavioral procedures

Subjects and apparatus. Subjects were 6–10 iuGC and CONT experimentally naive rats, 3 months old at the start of the experiment. Rats had restricted access to water, with the bottles being removed from the home cages 90–60 min before the trainings and replaced 30–60 min after. The access to food was restricted to maintain the rats at ±85% of their free feeding weight.

Two identical operant chambers (Med associates, St. Albans, VT, USA) housed in light- and sound-attenuating boxes, were used in the experiment. Each chamber contained a central, recessed magazine that provided access to 45-mg food pellets (Bio-Serve, Frenchtown, NJ, USA) or 100 μl of sucrose solution (20% wt/vol in water) delivered by a pellet dispenser and a liquid dipper, respectively, and two retractable levers that were located on each side of the magazine. Magazine entries were measured automatically by an infrared beam located at the mouth of the magazine. A 1-kHz tone and an amplified white noise, each with a sound of 80 dB, where available as discrete auditory cues. A 2.8 W, 100 mA house light positioned at the top-center 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.

General PIT

The behavioral procedure was adapted from protocols previously described.[16,18,19]

Both groups of animals were given 10 sessions of Pavlovian training. Each training session included eight presentations of the conditioned stimulus (CS) - four presentations of the positive stimulus (CS+) and four presentations of the innocuous/negative stimulus (CS−). During each 2-min presentation of the CS+, the reward (food pellet) was delivered. The average intertrial interval (ITI) between CS presentations was 2 min. For half the rats in each group, the CS+ was the tone; and for the remaining rats, the CS+ was the amplified white noise. Magazine visits (MVs, number of times that the animal introduced the nose in the food magazine) were recorded during the CS period and during the ITI. Data are shown as the number of MVs performed during the CS+ period (8 min) and the number of MVs performed during the CS− period (8 min). Rats received one Pavlovian extinction session identical to the training, but under extinction (without reward).

For the instrumental training, animals were trained on random ratio (RR) schedule of reinforcement. During the training sessions, animals learned to press the lever (left and right levers counterbalanced) to receive the outcome (food pellet). Animals first received 2 days of continuous reinforcement (CRF) and were then shifted to an RR5 schedule (that is, each action delivered an outcome with a probability of 0.2). After 2 days of training, this was changed to an RR10 schedule (or a probability of 0.1) for two additional days. The training ended after 30 min of testing or after the animals earned 30 pellets. The number of times that the animals pressed the lever during the time of testing was registered. Data are presented as the number of lever presses per time of training. After training, animals were given a Pavlovian reminder, which was identical to the training.

Twenty-four hours later all rats were tested for PIT under extinction. The lever, which the animals learned to press was inserted into the chamber. In the first 8 min, the lever was available but no stimuli were presented; this period corresponding to a baseline performance interval (BPI). Each of the stimuli was then presented four times in a pseudorandom order. Each CS lasted for 2 min, separated by a 2-min ITI. The number of lever presses during both CS+ and CS− was assessed and plotted.

Selective PIT

The behavioral procedure used was adapted from protocols previously described.[20]

Pavlovian training comprised nine daily sessions in which each of two auditory CS (tone and white noise) was paired with a different outcome (pellet or sucrose solution). Each of the CS exposure that lasted for 2 min was presented four times per session using a pseudorandomized order, with an ITI of 2 min in average. Data were plotted as the number of MVs performed during both CS presentations (16 min in total) and the number of MVs performed during the ITI (pre-CS period).

Animals were then trained for the instrumental conditioning. Training was performed in two separate sessions per day (one session for each lever) and the order of training was alternated during days (average interval between the two sessions was 3 h). Each session finished after 30 rewards were delivered or 30 min had elapsed. In the first 2 days, lever pressing was in a CRF order. The probability of getting a reward decreased according to the following sequence: days 3–4, RR5; days 5–6, RR10. The number of lever presses per session was registered and plotted. After training, animals were given a Pavlovian reminder, which was identical to the training.

Twenty-four hours later, subjects were placed in the operant chamber to test for PIT transfer with both levers inserted. After a BPI that lasted for 8 min, four blocks of each auditory CS were presented randomly and lever presses were registered. During each stimulus presentation, lever presses were considered correct if it encoded the same reward as the audible sound. When encoding was different, the actions were considered incorrect. The number of lever presses performed during the test is plotted.

Operant behavior

Training and the devaluation test were described in previous work.[21] Briefly, animals were exposed to increasing difficulty schedules of reinforcement: 2 days of CRF, 2 days of RR5, 2 days of RR10 and finally 7 days of RR20. Animals were tested, using a reversible devaluation paradigm, at two different phases of training: after the first day of RR20 (early devaluation) and after the last training day (late devaluation). The devaluation test commenced 24 h after the previous training day and lasted 2 days. On each day rats were given *ad libitum* for 1 h, either the reinforcer earned by lever pressing (devalued condition) or the one received for free in the home cage (valued condition), so devaluation was achieved by sensory-specific satiety. Immediately after, rats were given a 5 min test in extinction.

Locomotor behavior

Locomotion was assessed using the open field test. Briefly, rats were injected with the drug and immediately after placed in the center of an arena (Med associates). The ambulation was monitored online over a period of 90 min. Total distance traveled was used as an indicator of locomotor activity.

Macrodissection

Rats were anesthetized, decapitated and the heads were immediately snap-frozen in liquid nitrogen. Brain areas of interest were rapidly dissected using Paxinos landmarks,[22] and stored at −80 °C until use.

Western blot

Samples were treated as previously described.[13] Briefly, tissue was mechanically homogenized, centrifuged and the supernatant was quantified using the Bradford method. Fifty micrograms of the protein were run in SDS-polyacrylamide gel and transferred to nitrocellulose membranes. Membranes were incubated with the primary antibodies: rabbit anti-DA receptor D1, rabbit anti-DA receptor D2, rabbit anti-DA receptor D3 and mouse anti-α-tubulin. The secondary antibodies were incubated at a 1:10 000 dilution (anti-rabbit and anti-mouse). Detection was performed using ECL kit and bands were quantified using ImageJ (http://rsbweb.nih.gov/ij/).

Immunohistochemistry

For c-fos activation analysis, all animals were submitted to the selective PIT protocol described above (nine sessions of Pavlovian training; six sessions of instrumental training; one session of PIT test). On the PIT test day half of the animals of each group (five CONT test animals and five iuGC test animals) performed the PIT test, whereas the other half did not perform the test (five CONT animals and four iuGC animals).

Rats that performed the test were sacrificed 110 min after initiation of PIT testing and the rats that did not perform the test were killed on the same day. Both groups were anesthetized with pentobarbital and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde. Brains were processed and sectioned coronally on a vibratome at a thickness of 50 μm.

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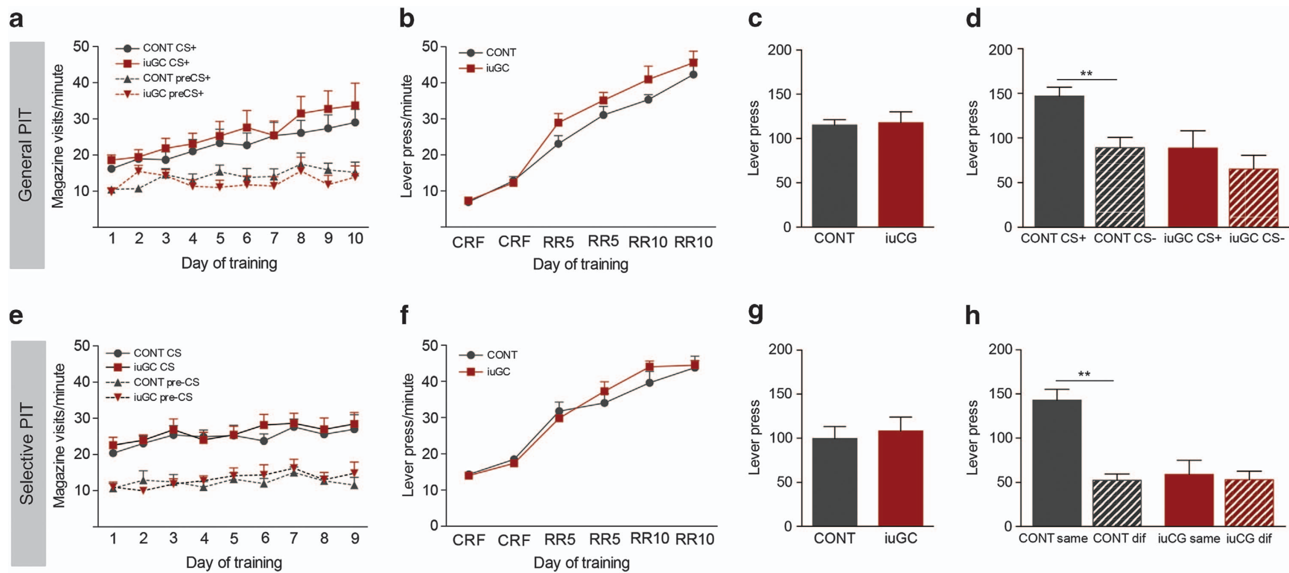

Figure 1. iuGC exposure disrupts Pavlovian–instrumental transfer (PIT). (**a**) Pavlovian conditioning of the general PIT protocol, represented as the average of magazine visits (MVs) performed during conditioned stimulus (CS), was similar between groups. For each group, mean MVs per min of the CS+ and mean MV per min of the CS− are plotted. (**b**) Instrumental conditioning of the general PIT protocol occurred at the same rate in both CONT and iuGC groups. Data are represented as the mean lever press per min for CONT and iuCG animals. (**c**) Baseline performance interval (BPI) of CONT and iuGC animals in the general PIT test session. For each group, the total number of lever presses performed during the 8 min of BPI is presented. (**d**) General PIT outcome is represented as the total number of lever presses performed during the CS+ and CS− periods. iuGC animals present a robust transfer impairment. (**e**) Pavlovian conditioning of the selective PIT paradigm was similar between groups. For each group, mean MVs per min of the CS period presentations and intertrial interval (pre-CS) period presentations are plotted. (**f**) Instrumental conditioning of the selective PIT paradigm revealed no differences between controls and iuGC animals. The number of lever presses per min performed in each day of the training is represented for each group. (**g**) BPI of CONT and iuGC animals in the selective PIT test session. For each group, the total number of lever presses performed during the BPI is presented. (**h**) iuGC animals present an impairment in selective PIT performance. The outcome of the selective PIT paradigm is shown as the total responses on the same lever or the different (dif) lever pressed, according to the CS presented. Same - lever pressing on the lever that originates the same reward as the CS presented; dif - lever pressing on the lever that originates a different reward as the CS presented. Graphs represent the total number of lever presses during the CS. All graphs are presented as mean ± s.e.m. CONT, control animals; CRF, continuous reinforcement; iuGC, *in utero* glucocorticoid exposed animals; RR, random ratio. $n = 8$–10 per group. $^{*}P \leqslant 0.05$, $^{***}P \leqslant 0.001$.

Briefly, free-floating sections were pretreated with 3% H_2O_2, rinsed in phosphate-buffered saline, blocked with 2.5% fetal bovine serum for 2 h at room temperature and incubated overnight at 4 °C with rabbit anti-c-fos polyclonal antibody. Afterwards, sections were washed and incubated with the secondary polyclonal swine anti-rabbit biotinylated for 1 h and processed with an avidin–biotin complex solution and detected with 0.5 mg ml^{-1} 3,3′-diaminobenzidine. Sections were washed and mounted on glass slides, air-dried, counterstained with hematoxylin and cover-slipped with Entellan.

Neurochemical analysis

Levels of catecholamines were evaluated by high-performance liquid chromatography, combined with electrochemical detection (HPLC/EC) as previously described.[13]

Three-dimensional dendritic analysis

Animals were transcardially perfused with 0.9% saline under deep pentobarbital anesthesia and processed as described previously.[23] In summary, brains were immersed in Golgi–Cox solution[24] for 14 days, processed and cut on a vibratome at 200 μm thick coronal sections. For each selected neuron, all branches of the dendritic tree were reconstructed at × 1000 magnification, using a motorized microscope (Zeiss, Thornwood, NY, USA) attached to a camera (Sony, Tokyo, Japan) and Neurolucida software (Microbrightfield, Williston, VT, USA). A three-dimensional analysis of the reconstructed neurons was performed using NeuroExplorer software (Microbrightfield). Ten neurons were analyzed for each animal.

Stereological analysis

Perfused cerebral hemispheres were separated by a longitudinal cut in the midsagittal plane. The outline of the medial prefrontal cortex (mPFC)—infralimbic cortex (ILC), prelimbic cortex (PLC), anterior cingulate cortex (ACC)—and the orbitofrontal cortex (OFC)—dorsal OFC (dOFC) and ventral OFC (vOFC), was defined in each section using established landmarks.[22] Cavalieri's principle estimates of volumes and cell numbers were obtained using Integrator System software (Visiopharm, Copenhagen, Denmark) and a camera (PixeLINK, Ontario, Canada) attached to a motorized microscope (Olympus, Tokyo, Japan).

Drugs and treatment

All treatments started on the first day of the Pavlovian training and continued throughout all behavioral procedures. Levodopa (L-DOPA)/carbidopa (Sinemet, Merck, NJ, USA) was administrated orally at a dose of 24.0/6.0 mg kg^{-1} 3 h before behavioral procedures. Quinpirole hydrochloride (Biogen Scientifica, Madrid, Spain) was administered intraperitoneally at a dose of 0.15 mg kg^{-1} 30–40 min before the procedure. SKF82958 hydrobromide (Sigma, Seelze, Germany) was administered subcutaneously at a dose of 0.05 mg kg^{-1} 15 min before the procedure. All animals performed a shorter version of the selective PIT protocol described above - 4 days of Pavlovian training, 3 days of instrumental training (1 day of CRF, 1 day of RR5 and 1 day of RR10) and finally 1 day of PIT test.

Statistical analysis

Statistical analysis was performed in GraphPad Prism 5.0 (La Jolla, CA, USA) and SPSS Statistics (Armonk, NY, USA). Parametrical and nonparametrical analyses were used when appropriate. Statistical comparisons are presented throughout the results section and in Supplementary Tables or in the legends of Supplementary Figures.

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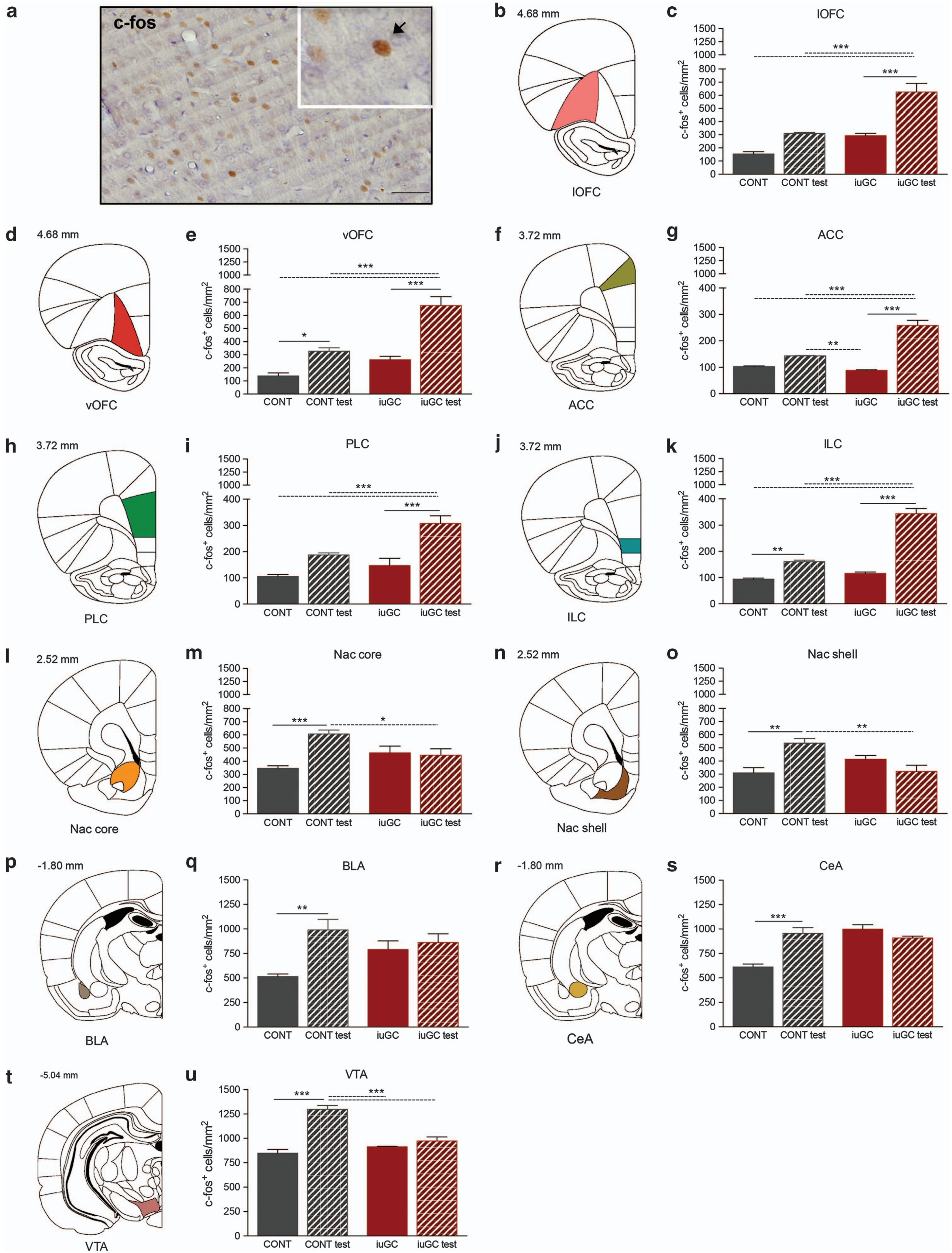

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RESULTS

iuGC exposure impairs PIT in adulthood

Herein, we characterized, in the PIT protocol, an animal model of GC exposure at gestation days 18 and 19 (iuGC animals) that presents marked mesolimbic hypodopaminergia, anhedonia and drug-seeking behaviors.[11–13,25]

In the Pavlovian conditioning phase of the general PIT, both groups increased the number of MVs throughout the 10 days of training (Figure 1a, CONT: $F_{(1,144)}=8.3$, $P=0.013$; iuGC: $F_{(1,144)}=11.2$, $P=0.004$), with a significant effect of training day (CONT: $F_{(9,144)}=7.9$, $P<0.000$; iuGC: $F_{(9,144)}=5.6$, $P<0.000$). Also, results demonstrate that there was no significant interaction between the groups and day of training ($F_{(9,369)}=0.2$, $P=0.998$) or in the response to the cues ($F_{(1,360)}=1.4$, $P=0.174$). In the Pavlovian extinction test, animals from both groups conditioned to the CS+ (Supplementary Figure 1a). In the instrumental phase, both groups acquired the conditioning for lever pressing throughout the days of training (Figure 1b, $F_{(5,90)}=134.6$, $P<0.000$), at the same rate ($F_{(5,90)}=1.2$, $P=0.339$). In the test day, the BPI of CONT and iuGC animals did not differ (Figure 1c, $t_{18}=0.2$, $P=0.844$). While the CONT group showed an increase on the lever press when the CS+ was presented in comparison with the CS− (Figure 1d, $t_9=4.7$, $P=0.001$) as expected; contrarily, the iuGC animals did not differ in the lever pressing between the CS+ and the CS− presentation periods ($t_9=1.1$, $P=0.300$), indicating a disruption in the general PIT transfer. The impairment was further confirmed by the absence of interaction between the groups and the cues ($F_{(1,36)}=1.4$, $P=0.244$).

Besides the effect that cues paired with a reward can have in invigorating responses, they can also bias response selection,[26,27] a phenomenon evaluated by a selective version of the PIT paradigm, in which different rewards are paired with different cues. Pavlovian training for the selective PIT showed a significant increase in the MVs throughout the 9 days of training (Figure 1e; CONT: $F_{(1,104)}=46.6$, $P<0.000$; iuGC: $F_{(1,104)}=28.2$, $P<0.000$) with a strong effect of training day (CONT: $F_{(8,104)}=1.8$, $P=0.080$; iuGC: $F_{(8,104)}=0.4$, $P=0.001$). The analysis of interaction showed that both groups had the same performance through the days ($F_{(8,252)}=0.001$, $P=0.977$) and in response to the cues ($F_{(1,252)}=0.155$, $P=0.996$). In instrumental conditioning, both groups increased lever pressing throughout training (Figure 1f, $F_{(5,70)}=118.0$, $P<0.000$), with identical rate of acquisition ($F_{(5,70)}=1.2$, $P=0.320$). In the PIT transfer, the BPI of both groups did not differ (Figure 1g, $t_{14}=0.4$, $P=0.694$). On the same test, CONT animals showed evidence for outcome-specific transfer (Figure 1h, $t_7=5.3$, $P=0.001$), whereas iuGC animals showed no discrimination between levers, presenting a marked impairment in the transfer ($t_7=0.36$, $P=0.731$). The absence of response by iuGC group was further confirmed by the absence of interaction between the groups and the response to the cues ($F_{(1,28)}=3.0$, $P=0.097$).

iuGC exposure does not shift goal-directed actions to habit formation

Importantly, we have shown that stress biases decision-making strategies, potentiating the transition from goal-directed to habitual actions.[21,28] Moreover, it has been suggested that a Pavlovian CS can potentiate habitual responding more than it can potentiate goal-directed actions,[29] and that habits are particularly sensitive to general transfer effects as they are not associated with detailed sensory representations of the outcome.[30] Considering this, we decided to further explore the impact of prenatal GC exposure in the development of instrumental habit, using the operant behavior test. Animals increased lever pressing throughout training (Supplementary Figure 1b, $F_{(15,195)}=89.7$, $P<0.000$), with identical rate of acquisition between groups ($F_{(1,195)}=0.8$, $P=0.392$). To ascertain devaluation effects in action–outcome contingency, we analyzed two different periods: after moderate (early test) and extensive (late test) training. In both tests, we observed a decrease in lever pressing in the devalued condition versus valued condition (early test, Supplementary Figure 1c; CONT: $t_{12}=4.1$, $P=0.002$; iuGC: $t_{14}=2.9$, $P=0.124$; late test, Supplementary Figure 1d; CONT: $t_{11}=4.6$, $P=0.001$; iuGC: $t_{11}=4.4$, $P=0.001$), suggesting that iuGC animals acquire habitual responding at a similar rate as CONT individuals.

iuGC exposure impairs neuronal activation upon PIT performance

With the intent to dissect the neuronal circuitry involved in the impaired PIT response, we evaluated the neuronal activation pattern (using c-fos, Figure 2a) during PIT transfer.

Results from the behavioral performance of the selective PIT protocol showed that although both CONT and iuGC animals acquired similar Pavlovian conditioning (Supplementary Figure 2a; interaction between groups and days of training: $F_{(8,305)}=0.466$, $P=0.880$; interaction between groups and cue: $F_{(1,305)}=0.969$, $P=0.326$) and instrumental conditioning (Supplementary Figure 2b, $F_{(5,85)}=0.05$, $P=0.998$), the iuGC animals had a clear impairment on the PIT test (Supplementary Figure 2d, CONT: $t_8=3.0$, $P=0.017$; iuGC: $t_8=0.6$, $P=0.558$; interaction between groups and cue: $F_{(1,16)}=1.0$, $P=0.331$). The BPI of CONT and iuGC animals on PIT test day did not differ (Supplementary Figure 2c, $t8=1.5$, $P=0.164$).

A marked neuronal activation after PIT performance was found in the mPFC and OFC. Results showed a significantly different pattern of activation between groups that performed PIT and groups that did not (lateral OFC: $F_{(1,15)}=5.3$, $P=0.037$; vOFC: $F_{(1,15)}=7.2$, $P=0.017$; ACC: $F_{(1,15)}=40.4$, $P<0.000$; PLC: $F_{(1,15)}=3.8$, $P=0.071$; ILC: $F_{(1,15)}=13.5$, $P<0.000$). As anticipated, *post hoc* analysis showed that several regions of the OFC, namely lateral OFC and vOFC, were recruited after PIT performance in CONT animals ($P=0.062$, $P=0.029$, respectively) and in iuGC animals (Figures 2b–e; $P<0.000$). Similarly, mPFC subregions such as ACC, PLC and ILC were also activated in CONT animals ($P=0.066$, $P=0.059$, $P=0.003$, respectively) and iuGC animals (Figures 2f–k; $P<0.000$). These results are in accordance with previous data that

Figure 2. c-fos immunohistochemistry revealed that iuGC exposure leads to a differential neuronal activation pattern in iuGC animals after selective PIT performance. (**a**) Representative image of c-fos immunostaining in the PLC of an animal that performed the PIT test (black arrow indicates a c-fos+ cell). PIT transfer recruited all prefrontal cortical regions of both groups but at different extents, with the activation being more pronounced in the iuGC animals compared with controls in the lOFC (**c**), vOFC (**e**), ACC (**g**), PLC (**i**) and ILC (**k**). In the limbic regions, PIT testing increased c-fos staining in control animals in all regions analyzed—NAc core (**m**), NAc shell (**o**), BLA (**q**), CeA (**s**) and VTA (**u**); whereas iuGC animals presented no activation upon testing. Representative images of coronal brain sections of (**b**) lOFC, (**d**) vOFC, (**f**) ACC, (**h**) PLC, (**j**) ILC, (**l**) NAc core, (**n**) NAc shell, (**p**) BLA, (**r**) CeA and (**t**) VTA are shown; numbers represent distance in millimeters to bregma. Average numbers ± s.e.m. are plotted. ACC, anterior cingulate cortex; BLA, basolateral amygdala; CeA, central amygdala; CONT, control animals; ILC, infralimbic cortex; lOFC, lateral OFC; iuGC, *in utero* glucocorticoid exposed animals; mPFC, medial prefrontal cortex; NAc, nucleus accumbens; OFC, orbitofrontal cortex; PLC, prelimbic cortex; vOFC, ventral OFC; VTA, ventral tegmental area. nCONT = 5, niuGC = 5, nCONT test = 5, niuGC test = 4. *$P\leqslant0.05$, **$P\leqslant0.01$, ***$P\leqslant0.001$. Scale bar, 50 μm.

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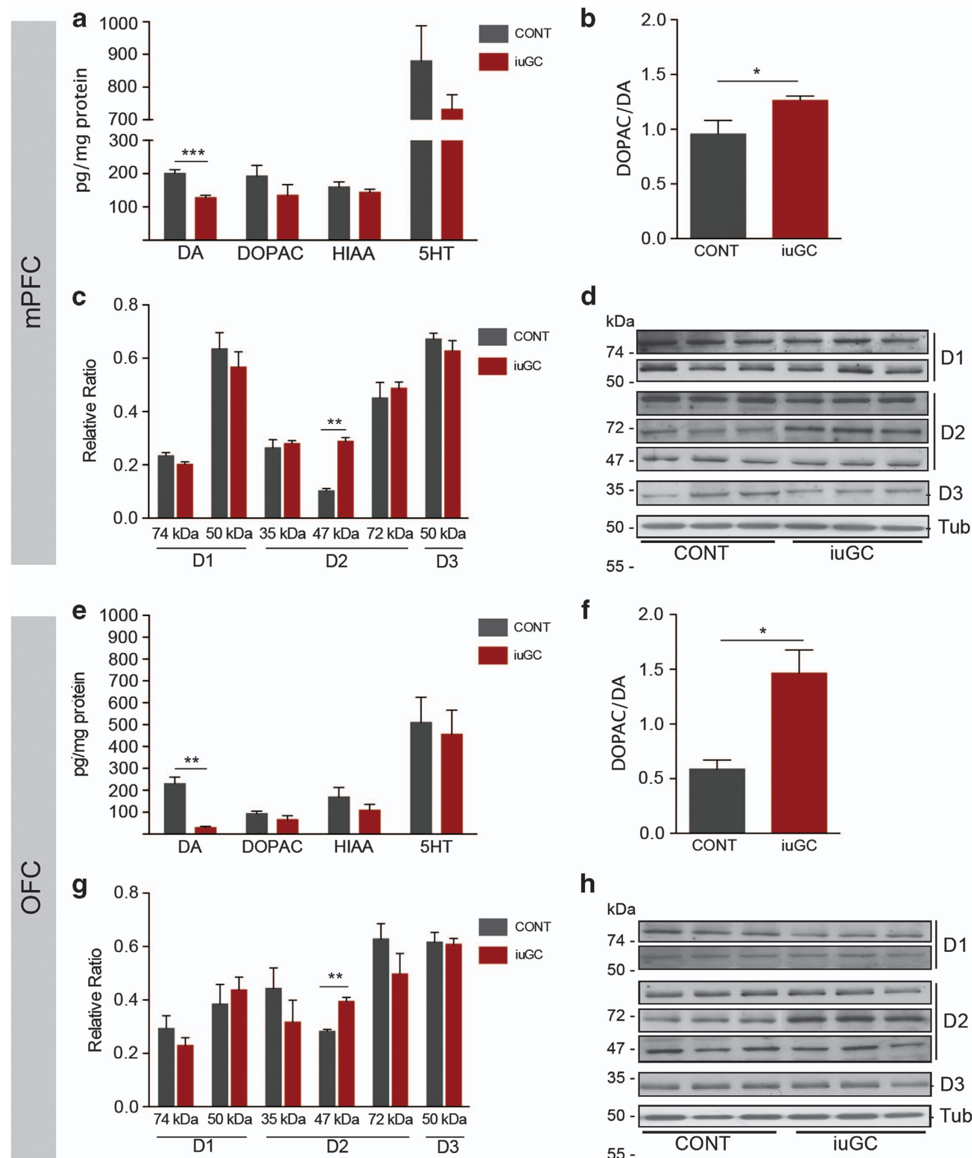

Figure 3. iuGC exposure affects cortical dopaminergic circuitry. (**a**) Dopamine (DA) was substantially reduced in the mPFC of iuGC animals in comparison with CONT animals in parallel with increased turnover (**b**), as measured by high-performance liquid chromatography with electrochemical detection. No differences in serotonin were found. (**c**) The levels of the nonglycosylated isoform of the dopamine receptor 2 (D2, ~47 kDa) were increased in the mPFC of iuGC animals, but no changes were observed regarding the D2 glycosylated isoform (~72 kDa), the putative D2 precursor (~35 kDa), the D1 glycosylated isoform (~74 kDa), the D1 nonglycosylated isoform (~50 kDa) or the D3 receptor (~50 kDa). (**d**) Representative immunoblot of D1 isoforms, D2 isoforms and D3 in the mPFC; tubulin was used as housekeeping protein. (**e**) In the OFC, we found reduced levels of DA in iuGC animals in comparison with CONT animals in parallel with an increased turnover of DA in this brain region (**f**). No differences in serotonin were found. (**g**) In the OFC, the nonglycosylated isoform of the D2 receptor (~47 kDa) was augmented in iuGC animals, whereas no changes were observed regarding the D2 glycosylated isoform (~72 kDa), the putative D2 precursor (~35 kDa), the D1 glycosylated isoform (~74 kDa), the D1 nonglycosylated isoform (~50 kDa) or the D3 receptor (~50 kDa). (**h**) Representative immunoblot of D1 isoforms, D2 isoforms and D3 in the OFC; tubulin was used as housekeeping protein. Average numbers ± s.e.m. are plotted. CONT, control animals; DOPAC, 3,4-dihydroxyphenylacetic acid; 5HIAA, 5-hydroxyindoleacetic acid; 5HT, serotonin; iuGC, *in utero* glucocorticoid exposed animals; mPFC, medial prefrontal cortex; OFC, orbitofrontal cortex. $n = 5$/group. $*P \leqslant 0.05$, $**P \leqslant 0.01$, $***P \leqslant 0.001$.

show the involvement of these regions in PIT.[16] Although iuGC animals did not present major differences in basal c-fos staining, iuGC animals that performed the PIT test presented a marked hyperactivation of these cortices in comparison with CONT animals (ACC, $P < 0.000$; PLC, $P = 0.003$; ILC, $P < 0.000$; lateral OFC, $P < 0.000$; vOFC, $P < 0.000$).

Results from the limbic regions - NAc core ($F_{(1,15)} = 13.5$, $P = 0.002$), NAc shell ($F_{(1,15)} = 16.5$, $P = 0.001$), basolateral amygdala ($F_{(1,15)} = 5.8$, $P = 0.029$), central amygdala ($F_{(1,15)} = 28.9$, $P < 0.000$) and VTA ($F_{(1,15)} = 27.8$, $P < 0.000$) also showed a significantly different pattern of activation between groups that have performed the PIT test or not. As anticipated, *post hoc* analysis showed that the NAc - core and shell (Figures 2l–o; $P = 0.001$, $P = 0.004$, respectively), the amygdala - basolateral amygdala and central amygdala (Figures 2p–s; $P = 0.005$, $P < 0.000$, respectively), and VTA (Figures 2t–u; $P < 0.000$) were strongly activated in CONT animals. On the contrary, no activation was observed in iuGC group, indicating a hypoactivation of the mesolimbic circuitry.

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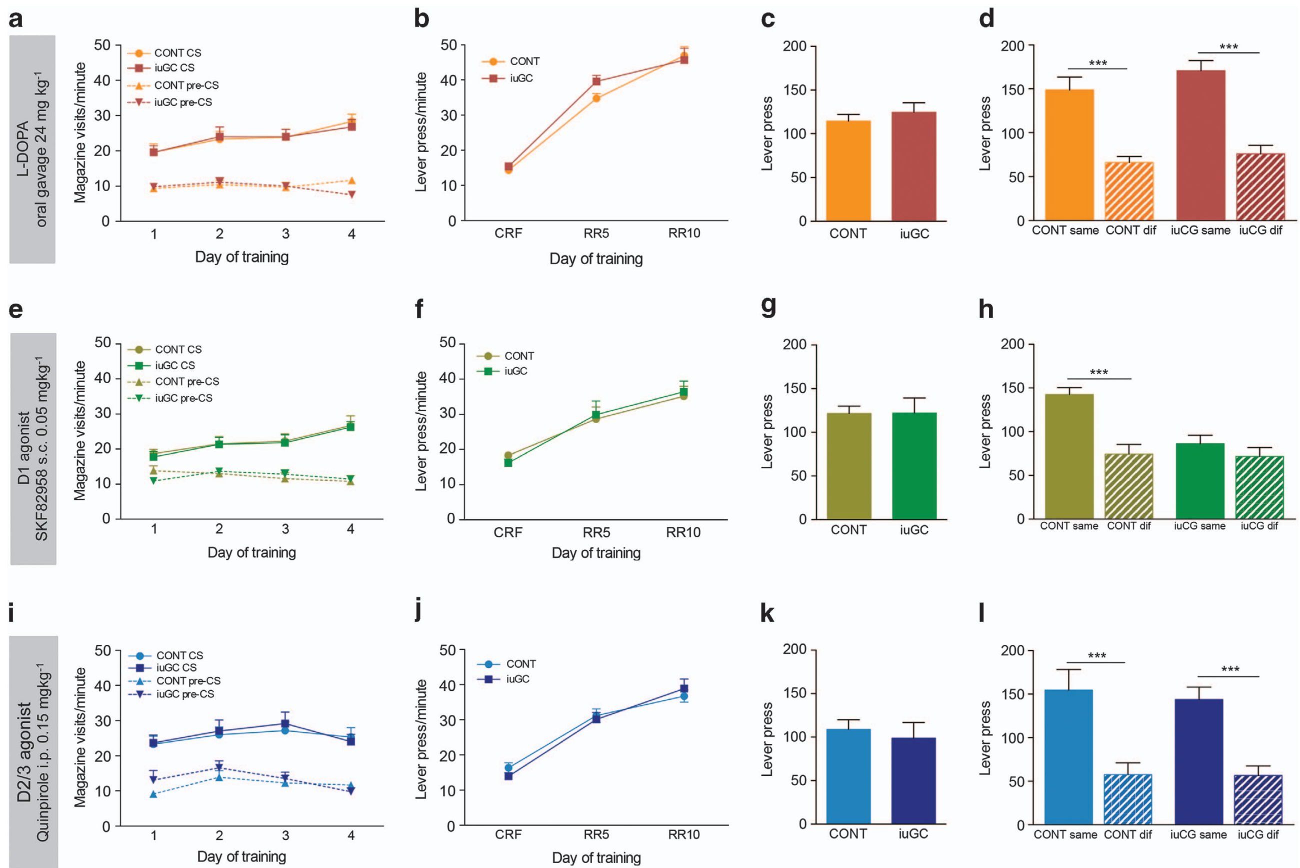

Figure 4. Modulation of dopaminergic transmission rescues impairment in PIT performance. Treatment with 24 mg kg^{-1} of L-DOPA, 3 h before behavior procedure did not alter the (**a**) Pavlovian conditioning and (**b**) the instrumental conditioning learning curves, or (**c**) the BPI. (**d**) L-DOPA treatment fully reverted the PIT impairment of iuGC animals. Administration of D2/D3 agonist, quinpirole at 15 mg kg^{-1} 30–40 min before behavior procedure, did not influence both (**e**) Pavlovian or (**f**) instrumental conditioning, as well as (**g**) the BPI. (**h**) D2/D3 agonist treatment fully reverted the PIT impairment in iuGC animals. Treatment with D1 agonist SKF82958 at 0.05 mg kg^{-1} 15 min before behavior procedure did not affect (**i**) Pavlovian and (**j**) instrumental conditioning or (**k**) the BPI. (**l**) The same treatment did not revert the PIT impairment observed in iuGC animals. Average numbers ± s.e.m. are plotted. CONT, control animals; CS, conditioned stimulus; iuGC, *in utero* glucocorticoid exposed animals; L-DOPA, levodopa. $^{*}P \leqslant 0.05$, $^{**}P \leqslant 0.01$, $^{***}P \leqslant 0.001$. $n = 8$ per group.

Dopaminergic dysfunction in iuGC animals

Several studies focused on the structural, morphological and neurochemical changes that iuGC exposure causes in limbic regions;[11,13,31] however, less is known about the cortical regions that also receive dopaminergic transmission arising from the VTA. In the current study we analyzed the neuronal number and volume of the OFC subregions - lateral OFC and vOFC, and the mPFC subregions - ACC, PLC and ILC (Supplementary Figure 3). In addition, we also performed three-dimensional morphological analysis of the pyramidal neurons of layer II/III of these regions. Interestingly, and despite slight differences in some of the mPFC and OFC subregions, it seems that iuGC exposure does not substantially affect the neuronal structure of these brain regions (Supplementary Figure 4).

To further evaluate the integrity of the above-mentioned regions, we quantified monoamine levels by HPLC/EC. DA levels were significantly reduced in the mPFC of iuGC animals (Figure 3a, $U = 0.0$, $P = 0.008$); this was accompanied by a significant increase in the DA turnover (Figure 3b, $U = 1.0$, $P = 0.016$). A similar pattern was found in the OFC (Figures 3e and f; DA: $U = 0.0$, $P = 0.008$; DA turnover: $U = 0.0$, $P = 0.016$). There were no major differences in serotonin levels in all the regions analyzed (Figures 3a and e).

We next quantified the expression levels of DA receptors in these brain regions by western blot analysis. D2 receptor (~47 kDa isoform) was increased in the mPFC (Figures 3c and d; $U = 0.0$, $P = 0.01$) and in the OFC (Figures 3g and h; $U = 0.0$, $P = 0.004$) of iuGC animals; these findings are in alignment with those previously reported for the NAc[13] and amygdala.[12] Conversely, we did not find changes in the D1 or D3 receptors in both the mPFC (Figures 3c and d) and the OFC (Figures 3g and h).

Manipulation of dopaminergic signaling improves PIT performance of iuGC animals

Since DA has a crucial role in reward-associated behaviors,[6] and in particular, in PIT performance,[16] we decided to normalize DA levels in the iuGC animals by performing a systemic treatment with the DA precursor L-DOPA. Administration of L-DOPA before training stages was effective in normalizing DA levels in the mesocorticolimbic circuit of iuGC animals (Supplementary Figure 5), and had no apparent effect on the performance on the Pavlovian conditioning, as both treated groups showed a significant increase in the MVs performed during the CS period compared with the pre-CS period (Figure 4a; CONT: $F_{(1,54)} = 28.6$, $P < 0.000$; iuGC: $F_{(1,54)} = 34.2$, $P < 0.000$). We further observed the absence of interaction between the treated groups and days of training ($F_{(3,136)} = 0.735$, $P = 0.533$) or the response to the cue ($F_{(1,136)} = 0.087$, $P = 0.768$). Also, the instrumental conditioning was identical between the groups (Figure 4b; $F_{(2,34)} = 0.820$, $P = 0.449$). On the test day, BPI of the CONT and iuGC animals was similar

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(Figure 4c, $t_{17} = 0.8$, $P = 0.442$). Yet, L-DOPA treatment fully rescued the impaired PIT response of the iuGC animals (Figure 4d; CONT: $t_{18} = 5.1$, $P < 0.000$; iuGC: $t_{16} = 6.3$, $P < 0.000$), which was further confirmed by a significant interaction between treatment and the response to the cues ($F_{(1,34)} = 63.67$, $P < 0.000$).

Because there was sparse evidence for the functional role of D1 and D2 receptors in PIT performance, and considering our molecular data showing divergent results in their expression levels, we decided to treat animals with either the D1-specific agonist SKF82958 or the D2/3-specific agonist quinpirole. To avoid the motor effects of the drugs, we monitored the locomotor behavior of the animals after treatment (Supplementary Figures 5b and c).

In the selective PIT, D1 agonist-treated groups did not differ in the Pavlovian conditioning as both treated groups increased significantly the MVs performed during the CS period compared with the pre-CS period (Figure 4e; CONT: $F_{(1,30)} = 18.7$, $P = 0.002$; iuGC: $F_{(1,30)} = 52.6$, $P < 0.000$). The absence of an effect of the treatment in the conditioning was confirmed by a nonsignificant interaction between the groups and the days of training ($F_{(3,80)} = 0.7$, $P = 0.512$) or the response to the cues ($F_{(1,80)} = ,0.8$ $P = 0.313$). The D1 agonist also had no effect in the instrumental conditioning of both the groups (Figure 4f; $F_{(2,20)} = 0.4$, $P = 0.666$). This treatment was not effective in reverting the PIT impairment observed in iuGC animals (Figure 4h; CONT: $t_{10} = 5.0$, $P = 0.035$; iuGC: $t_{10} = 1.0$, $P = 0.330$), with an absence of a significant interaction between the treated groups and the cue ($F_{(1,20)} = 2.3$, $P = 0.142$). However, the BPI of both groups in the PIT transfer was not different (Figure 4g, $t_{10} = 0.03$, $P = 0.973$). It is important to mention that the selected dosage of D1 agonist was biologically relevant as the animals were conditioned in a conditioned place preference paradigm (Supplementary Figure 6).

D2/3 agonist-treated animals did not present major changes in the Pavlovian conditioning as both CONT and iuGC animals treated with the agonist increased significantly the MVs performed during the CS period compared with the pre-CS period (Figure 4h; CONT: $F_{(1,54)} = 33.6$, $P < 0.000$; iuGC: $F_{(1,54)} = 19.9$, $P = 0.0004$). The absence of an effect of the D2/3 agonist treatment in the Pavlovian conditioning was further confirmed by the absence of interaction between the groups and the days of training ($F_{(3,136)} = 0.6$, $P = 0.597$) or the response to the cues ($F_{(1,136)} = 0.2$, $P = 0.660$). The D2/3 agonist treatment also failed to alter the instrumental conditioning of both the groups (Figure 4i; $F_{(2,34)} = 1.1$, $P = 0.330$). Importantly, D2/3 activation did not alter the BPI of CONT and iuGC animals ($t_{17} = 0.5$, $P = 0.632$), but it fully reverted the PIT impairment of iuGC animals (Figure 4k; CONT: $t_9 = 5.6$, $P = 0.0004$; iuGC: $t_8 = 11.9$, $P < 0.000$), which was confirmed by the significant interaction between the treatment and the response to the cues (Figure 4j, $F_{(1,34)} = 30.1$, $P < 0.000$).

Interestingly, the different treatments applied (L-DOPA, D1 agonist and D2 agonist) did not influence the baseline performance in the PIT test day ($F_{(2,44)} = 0.4$, $P = 0.696$).

Altogether, our results suggest that normalization of DA levels in the iuGC animals is crucial for the correct expression of PIT behavior, and this is dependent on the activation of D2/3, but not D1 receptors.

DISCUSSION

Herein, we show that iuGC animals present a significant impairment in the PIT performance that is dependent of DA levels. It is important to distinguish between general and selective PIT, as they are proposed to reflect distinct forms of incentive processing and to be mediated by somewhat different neural systems.[16,26,27] In general PIT, a Pavlovian cue generates an overall increase in the vigor of instrumental responding, independent of the specific sensory properties of the reward,[29,30,32] whereas in selective PIT, a CS elicits sensory-specific features of an outcome, biasing instrumental actions towards that outcome.[29,30,32] The disruption of both types of PIT suggests that iuGC exposure induces a general motivational deficit rather than alterations in bias action selection. Importantly, these findings are in accordance with human studies showing that childhood maltreatment is associated with blunted subjective responses to reward predicting cues in adulthood.[33] Evidence shows that injection of GCs after Pavlovian conditioning impairs transfer[34] and we recently demonstrated that chronic stress robustly decreases PIT performance.[20] This deficit is likely to occur as a result of programming effects of stress in dopaminergic neurons,[35,36] that ultimately leads to a decrease in the attribution of incentive salience.[11,37–39]

PIT is a complex behavior highly dependent on an intricate interaction between dopaminergic circuits of limbic (NAc, amygdala, dorsal striatum) and cortical (mPFC, OFC) structures.[16] The Pavlovian conditioning is highly dependent on limbic structures, particularly the NAc that is implicated in attaching motivational significance to Pavlovian stimuli.[40–42] Blockade of dopaminergic transmission in this region remarkably affects PIT behavior.[17,43,44] Yet, and despite a strong reduction in NAc dopaminergic signaling triggered by iuGC exposure, animals are able to acquire the Pavlovian conditioning, which may reflect compensation by other brain regions involved in this process, such as the central amygdala that encodes the affective value of the reward.[32] This is in line with studies showing that neurotoxic lesions of the NAc abolish PIT without affecting Pavlovian or instrumental conditioning separately.[45] The second phase of PIT encompasses instrumental conditioning that was apparently normal in the iuGC animals. This stage is mostly mediated by the dorsal striatum[46–48] with different functions ascribed to dorsolateral and dorsomedial subregions.[27,49] Importantly, it has been proposed that as behavior becomes habitual (dorsolateral subregion-dependent), it is also more susceptible for transfer of control.[50] Moreover, chronic stress can potentiate habit formation by inducing a hypertrophy of this region.[21] However, we found that iuGC exposure did not bias behavior towards habit, indicating a relatively preserved dorsal striatum.

Interestingly, the iuGC animals were unable to transfer, a phenomenon that can be the result of an abnormal neuronal activation of the regions that have an active role on PIT. For example, VTA lesions disrupt DA release in the NAc and produce an overall reduction in both general and selective PIT.[27] Thus, the observed hypoactivation of the VTA and NAc could (partially) explain the blunted response of the iuGC animals. On the contrary, as iuGC animals present hypotrophy and hypodopaminergia of cortical brain regions (mPFC and OFC), we also suggest that the hyperactivation observed during PIT testing reflects a compensatory adaptation. These changes could underlie PIT deficits as parallel phasic activation of mPFC and OFC neuronal subsets is required to integrate the transfer from Pavlovian incentives to instrumental actions,[51] contrary to the initial idea that each region acted on itself.[52–55]

The absence of transfer, with preservation of both Pavlovian and instrumental acquisitions, supports the premise that DA is crucial for the attribution of general incentive salience to reward-associated cues.[6,30] We found that normalization of DA levels was sufficient to fully rescue PIT disruption in the iuGC animals. It is well documented that boosting of DA by administration of amphetamines facilitates PIT performance.[56–58] Also, hyperdopaminergic mutant mice have higher incentive salience for sweet rewards.[59] Still, we failed to observe any detectable effect of L-DOPA in PIT of CONT animals, a discrepancy probably explained by an insufficient rise of DA levels with our treatment.

One other interesting finding supported by this work and our previous studies[12,13] was the selectivity of DA receptor D2 expression changes (putatively due to epigenetic alterations[13]) in the mesocorticolimbic system, particularly in the light of

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evidence associating D2 receptor with reward-associated disorders that present altered incentive salience attribution such as addiction and binge eating[60] as well as motivation perception.[61] Moreover, it was shown that the D2 antagonist pimozide abolishes PIT,[17] and NAc microinjections of D2 antagonist raclopride reduce transfer.[43] However, to our knowledge, no studies have evaluated the effect of specific activation of DA receptors in PIT performance. Here, we found that systemic administration of quinpirole (D2/3 agonist) completely reverted PIT disruption in the iuGC animals, whereas a D1 agonist had no effect. These results indicate that iuGC-induced D2 dysfunction underlies PIT impairment, although we cannot fully exclude a role for D1 receptor in this process.

The observed reduced cue motivational drive fits with the marked anhedonia for natural rewards (food and sex) of iuGC animals.[25,31] However, it is somewhat opposing to the enhanced drug-seeking behavior observed in iuGC animals,[13] considering the evidence showing that individual propensity to attribute incentive salience to food cues is predictive of addictive behavior and cue-induced reinstatement.[62,63] Yet, one hypothesis is that incentive salience amplifies 'wanting' in ways that can be specific to one motivational target.[6] For example, drugs that act on the dopaminergic system such as amphetamines can be more desired than natural rewards for some individuals.[6,64] However, due to the intrinsic complexity of this behavior, we believe that additional studies are needed to comprehend the link between incentive salience attribution and the development of aberrant behaviors such as addiction.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Translational Psychiatry website (http://www.nature.com/tp)

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Supplementary Information

The motivational drive to natural rewards is modulated by prenatal glucocorticoid exposure

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Supplementary Information 1

The following section includes detailed information of the Materials and Methods used.

Materials and Methods

Animals

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

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

Behavioural procedures

Subjects

Subjects were 6-10 iuGC and CONT experimentally naïve rats, 3 months old at the start of the experiment. For the operant behaviour, rats had restricted access to water, being the bottles removed from the home cages 90-60 minutes prior to the trainings and replaced 30-60 min after. The access to food was restricted to maintain the rats at ±85% of their free feeding weight.

Operant behaviour

Training and the devaluation test were based on previous work[1]. During training, one reinforcer was delivered in the operant chamber contingent upon lever pressing, and the other reinforcer was presented freely in the home cage and used as a control for the devaluation test. The reinforcer and lever used were counterbalanced across groups. Following one day for a magazine training session (30 min, on average 30 reinforcers delivered on a random time 60 s schedule), animals were trained (one session per day during 30 min or until 30 reinforcements) in increasing difficulty schedules of reinforcement: 2 days of continuous reinforcement (CRF), 2 days of random ratio-5

(RR5), 2 days of RR10 and finally 7 days of RR20 (on average one reinforcer every 20 lever presses). Using a reversible devaluation paradigm, animals were tested at two different phases of training: after the first day of RR-20 (early devaluation), and again after the last training day. The devaluation test commenced 24 h after the previous training day, and lasted 2 days. On each day rats were given ad libitum exposure to one of the reinforcers for 1 h in a separate cage. Rats were allowed to consume either the reinforcer earned by lever pressing (devalued condition), or the one they received for free in their home cage (valued condition), so devaluation was achieved by sensory specific satiety. Immediately after, rats were given a 5 min test in extinction with the training lever extended. The order of the valued and devalued condition tests (day 1 or day 2) was counterbalanced across groups.

Locomotor behavior

Locomotor behavior was investigated using the open field test. Briefly, rats were injected with the dopamine agonist and immediately after placed in the center of an arena (MedAssociates, St Albans, VT, USA) and their locomotion was monitored online over a period of 90 min. Total distances traveled and time spent moving were used as indicators of locomotor activity.

Conditioned Place Preference (CPP)

The place preference apparatus consisted of two compartments with different patterns on floors and walls, separated by a neutral area (MedAssociates). On the first day animals were placed in the central neutral area and allowed to explore both compartments, allowing definition of the preferred compartment. On days 2-9 rats were injected systemically with SKF 82958 hydrobromide (subcutaneously, $0.05 mgkg^{-1}$) or its vehicle, and then confined to one chamber of the place conditioning apparatus for 30 min. Drug was given on conditioning days 1, 3, 5, and 7 and always paired with one chamber; and the drug's vehicle on remaining conditioning days and paired with the other chamber. CPP was assessed on day 10 (20 min) when all compartments were accessible to the animal. Results are expressed as the difference of time spent in the drug-paired versus saline-paired side.

Macrodissection

Rats were anesthetized with pentobarbital, decapitated, and heads were immediately snap-frozen in liquid nitrogen. Brain areas of interest were rapidly dissected on ice under a stereomicroscope following anatomical landmarks[2]. Samples were frozen on dry ice and stored at -80ºC until use for western blot and assessment of catecholamine levels.

Western Blot

For western blotting analysis, samples were treated has previously described[3]. Briefly, tissue was mechanically homogenized on ice-cold lysis buffer (50mM Tris-HCl pH7.4, 50mM NaCl, 1mM phenylmethylsulfonyl fluoride, complete protease inhibitors (Roche, Basel, Switzerland)) and then 0.1% SDS and 1% Triton X-100 were added to each sample. After 1 hour on ice, samples were centrifuged at 13000 r.p.m. for 10 min at 4°C and the supernatant was quantified using the Bradford method. 50μg of the protein was run in SDS-polyacrylamide gel and then transferred to nitrocellulose membranes. Membranes were incubated with the primary antibodies: rabbit anti-Dopamine receptor D1 (1:2000, ab20066, Abcam, Cambridge, UK), rabbit anti-Dopamine receptor D2 (1:1000, ab21218, Abcam, Cambridge, UK), rabbit anti-Dopamine receptor D3 (1:2000, ab42114, Abcam, Cambridge, UK) and mouse anti-α-tubulin (1:200, AA4.3-s, DSHB, Iowa, USA). The secondary antibodies were incubated at a 1:10000 dilution (anti-rabbit and anti-mouse, Santa Cruz Biotechnologies, Santa Cruz, CA, USA). Detection was performed using ECL kit (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) and bands were quantified using ImageJ (http://rsbweb.nih.gov/ij/).

Immunohistochemistry (IHC)

For c-fos activation analysis, all animals were submitted to the selective PIT protocol described in the methods section of the main text. However, on the PIT test day only half of the animals of each group (5 CONT test animals and 5 iuGC test animal) performed the PIT test, while the other half did not perform the test (5 CONT animals and 4 iuGC animals).

Rats that performed the test were sacrificed 110 minutes after initiation of PIT testing and rats that did not perform the test were sacrificed on the same day. Both groups were anaesthetized with

pentobarbital and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde (pH 7.4 in 0.1M phosphate buffer). Brains were removed and post-fixed for 48 hours in 4% paraformaldehyde and then rinsed and stored in 30% of sucrose until sectioning. Brains were sectioned coronally, at a thickness of 50 µm, on a vibrating microtome (VT1000S, Leica, Germany) and stored in cryoprotectant solution at -20ºC until use.

Briefly, free-floating sections were pre-treated with 3% H_2O_2 in PBS for 20 min, thoroughly rinsed in PBS, blocked with 2.5% fetal bovine serum (FBS) in PBS-Triton 0.3% for 2 h at room temperature and then incubated overnight at 4ºC with primary antibody c-fos (1:1000; rabbit anti-c-fos Ab-5 polyclonal antibody, Calbiochem, USA). Afterwards, sections were washed and incubated with the secondary polyclonal swine anti-rabbit biotinylated (1:200, DAKO, Denmark) for 1 h, and processed with an avidin-biotin complex solution (ABC-Elite Vectastain reagent; Vector Lab., USA) and detected with 0.5 mg/ml 3,3´-diaminobenzidine including 12.5µl 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).

Neurochemical analysis

Levels of catecholamines in OFC and mPFC were evaluated by high-performance liquid chromatography, combined with electrochemical detection (HPLC/EC) using a Gilson Pump (Gilson, Middleton, WI, USA), fitted with an analytical column (Supleco Supelcosil LC-18 3mM, Bellefonte, PA, USA; flow rate: 1.0mlmin^{-1}). Samples were immersed in 0.2 N perchloric acid and kept at -20ºC for 2 hours. Afterwards, samples were mechanically homogenized, sonicated for 4 min on ice, and centrifuged at 5000 rpm for 3 min at 4°C. The supernatant was filtered through a Spin-X HPLC column (Costar, Lowell, MA, USA) to remove debris and 130µl aliquots were injected into the HPLC system, using a mobile phase of 0.7M aqueous potassium phosphate (pH 3.0) in 10% methanol, 1-heptanesulfonic acid (222mgl^{-1}) and Na-EDTA (40mgl^{-1}). On each day, a standard curve of fixed concentrations of all catecholamines detected was run. The catecholamine levels were then normalized for the protein levels present in the pellet (quantified using the Bradford method).

3D dendritic analysis

Rats (n=4) with 4 months old were transcardially perfused with 0.9% saline under deep pentobarbital anesthesia and processed as described previously[4]. Briefly, brains were immersed in Golgi–Cox solution[5] for 14 days and transferred to a 30% sucrose solution (7 days), before being cut on a vibratome. 200μm thick coronal sections were collected onto gelatin-coated microscope slides. The sections were then subsequently alkalinized in 18.7% ammonia, developed in Dektol (Kodak, Rochester, NY, USA), fixed in Kodak Rapid Fix (prepared as instructed with solution B omitted), dehydrated through a graded series of ethanol, cleared in xylene, mounted and coverslipped. For each selected neuron, all branches of the dendritic tree were reconstructed at x1000 magnification, using a motorized microscope with oil objectives (Axioplan 2, Carl Zeiss, Thornwood, NY, USA) that was attached to a camera (DXC-390, Sony, Tokyo, Japan) and Neurolucida software (Microbrightfield, Williston, VT, USA). A 3D analysis of the reconstructed neurons was performed using NeuroExplorer software (Microbrightfield, Williston, VT, USA). 10 neurons were analyzed for each animal (results from the same animal were averaged). The method for sampling dendritic branches for spine density was designed as follows: only branches that (1) were either parallel or at acute angles to the coronal surface of the section and (2) did not show overlap with other branches that would obscure visualization of spines were considered. Because treatment-induced changes in the apical dendritic branches varied with distance to soma, segments were randomly selected in the proximal parts of the tree; selection of apical dendrite segments was done at radial distances between 50 and 100μm for proximal and 150 and 200μm for distal to the soma. Spines in the selected segments were classified[6] as thin, mushroom, wide and ramified categories. Thin spines were considered immature, whereas the other spine types were considered to be mature spines.

Stereological analysis

Rats (n=4) from both experimental groups were deeply anesthetized and transcardially perfused with 4% paraformaldehyde (PFA) when 4 months old. Brains were removed and the cerebral hemispheres were separated by a longitudinal cut in the midsagittal plane. Blocks were washed in tap water and dehydrated through a graded series of ethanol solutions, and then embedded in

glycolmethacrylate (Tecnovit 7100, Heraeus Kulzer, Wehrheim, Germany). Microtome sections (30μm) were placed on slides coated with Entellan-new, before staining with Giemsa stain. The outline of the medial prefrontal cortex (mPFC) – Infralimbic cortex (ILC), prelimbic cortex (PLC), anterior cingulate cortex (ACC) – and orbitofrontal cortex (OFC) – dorsal OFC (dOFC) and ventral OFC (vOFC), was defined in each section using established landmarks[7]. Estimates of ILC, PLC, ACC and OFC region volumes and cell numbers were obtained using Visiopharm Integrator System software (Visiopharm, Copenhagen, Denmark) and a camera (PL-A622, PixeLINK, Ontario, Canada) attached to a motorized microscope (BX51TF, Olympus, Tokyo, Japan). Cavalieri's principle was applied to assess the volume of each region. Every 8th section was used, and the cross-sectional area was estimated by point counting (magnification of x400). The inter-point distance established, at the tissue level, was 75μm and the volume of the region of interest (ROI) was calculated from the number of points that fell within its boundaries and the distance between the systematically sampled sections. Average cell numbers were estimated using the optical fractionator method as described elsewhere[8]. Briefly, a grid of virtual 3D-boxes (30μm x 30μm x 20μm) was superimposed on every 8th section of the ROI, spaced as for the volumetric estimation. An estimate of total number of cells was then derived from the number of cells falling inside the boxes, the volume of each box, box spacing, and total number of boxes. Coefficients of error were automatically computed according to the formulas of Gundersen *et al*[9] for cell numbers and Gundersen *et al*[10] for volume estimations. Glial cells were not included in the estimations, and the discrimination between neuronal and glial cell bodies was based on previously described criteria[11,12].

Drugs and treatment

Levodopa (L-DOPA)/carbidopa (Sinemet, Merck, NJ, USA) was administrated orally at a dose of 24.0/6.0mgkg^{-1}. Animals were treated 3 hours prior to behavior. Quinpirole hydrochloride (D2 agonist, Biogen Scientifica, Madrid, Spain) was administered intraperitoneally (i.p.) at a dose of 0.15mgkg^{-1}, 30-40 minutes before. SKF82958 hydrobromide (D1 agonist, Sigma, Seelze, Germany) was administered subcutaneously (s.c.) at a dose of 0.05mgkg^{-1} 15 minutes before. In the PIT, rats performed 4 days of Pavlovian training, 3 days of instrumental training (1 day of CRF,

1 day of RR5 and 1 day of RR10) and finally 1 day of PIT testing. In order to reduce the duration of the treatment and to avoid possible side effects of the drugs we performed the original selective PIT protocol in all the animals, before the treatment began.

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

All statistical comparisons are presented in the results section or in the supplementary section (Supplementary Figures and Tables).

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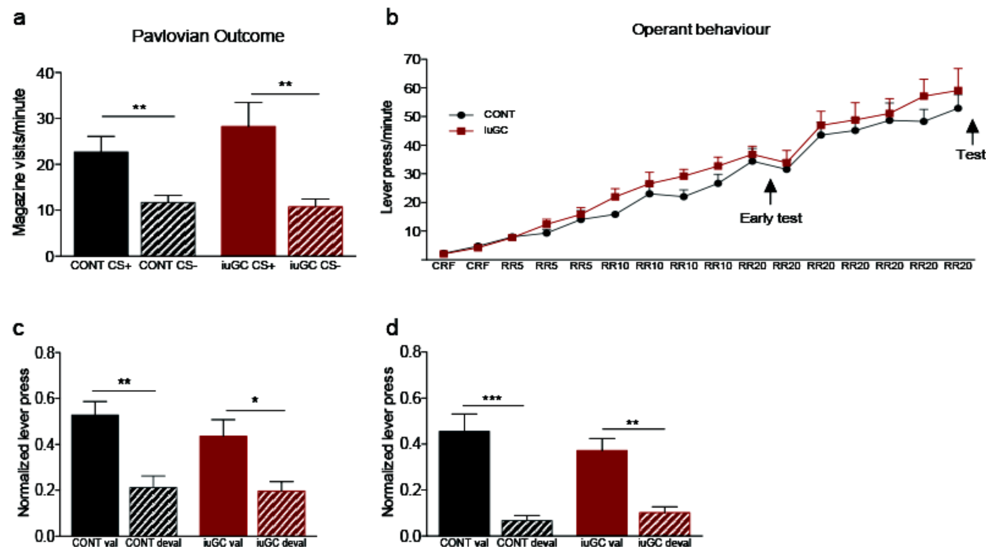
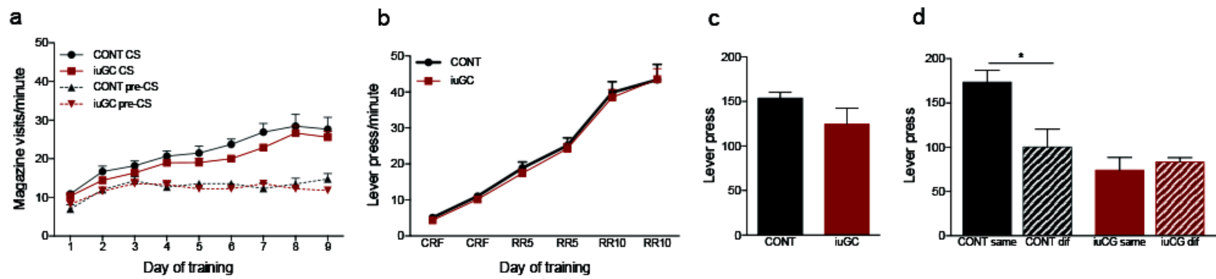
Supplementary information 2

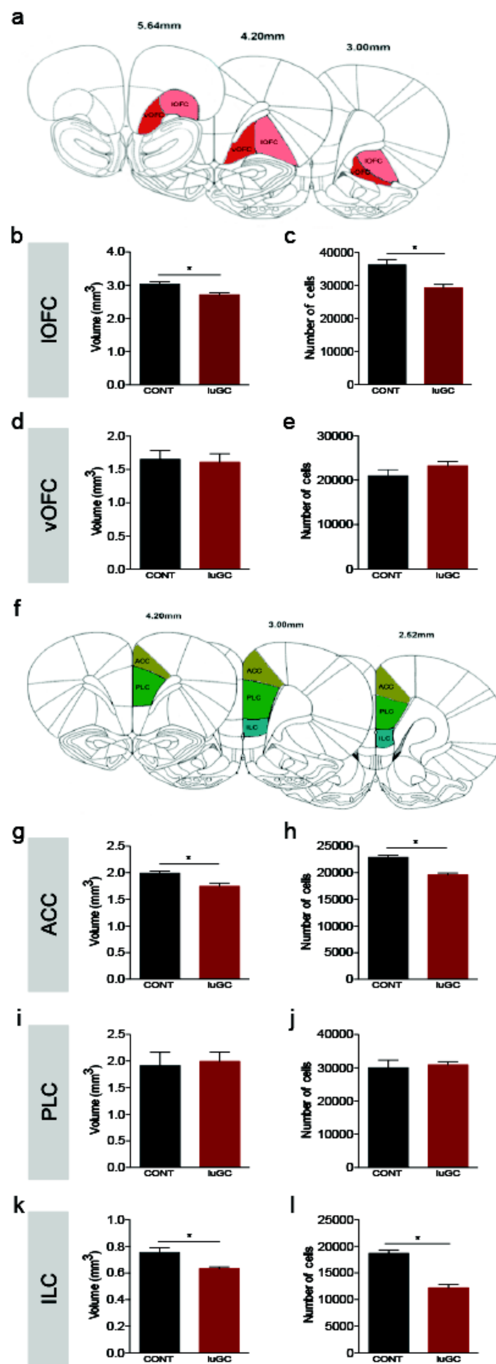
The following section includes six supplementary figures.



Supplementary Figure 1. iuGC treatment does not alter Pavlovian conditioning or enhances habit formation. (a) Pavlovian outcome (represented as average of magazine visits (MV) during the presentation of both CS+ and CS-), which was evaluated after 10 days of Pavlovian conditioning, is not altered in iuGC animals (t_9=4.2, p=0.002) or CONT animals (t_9=3.0, p=0.008). (b) Acquisition of the lever-pressing task is similar in CONT and iuGC animals ($F_{(8,200)}$=23.7, p<0.000); the rate of lever pressing is depicted for each daily session. Outcome devaluation tests performed early and later in training are indicated. Devaluation test performed (c) after the first day of RR-20 (CONT, t_{12}=4.1, p=0.002; iuGC, t_{12}=2.9, p=0.124) and (d) after the last training day (CONT, t_{11}=4.6, p=0.001; iuGC, t_{11}=4.4, p=0.001). Lever pressing normalized to the lever pressing of the previous training day is compared between the valued and the devalued condition for each group. Average numbers ± SEM are plotted. iuGC: *in utero* GC exposed animals; CONT: control animals; CONT val: control valued; CONT deval: control devalued; iuGC val: *in utero* GC exposed valued; iuGC deval: *in utero* GC exposed devalued. n=10/group. * p≤ 0.05.

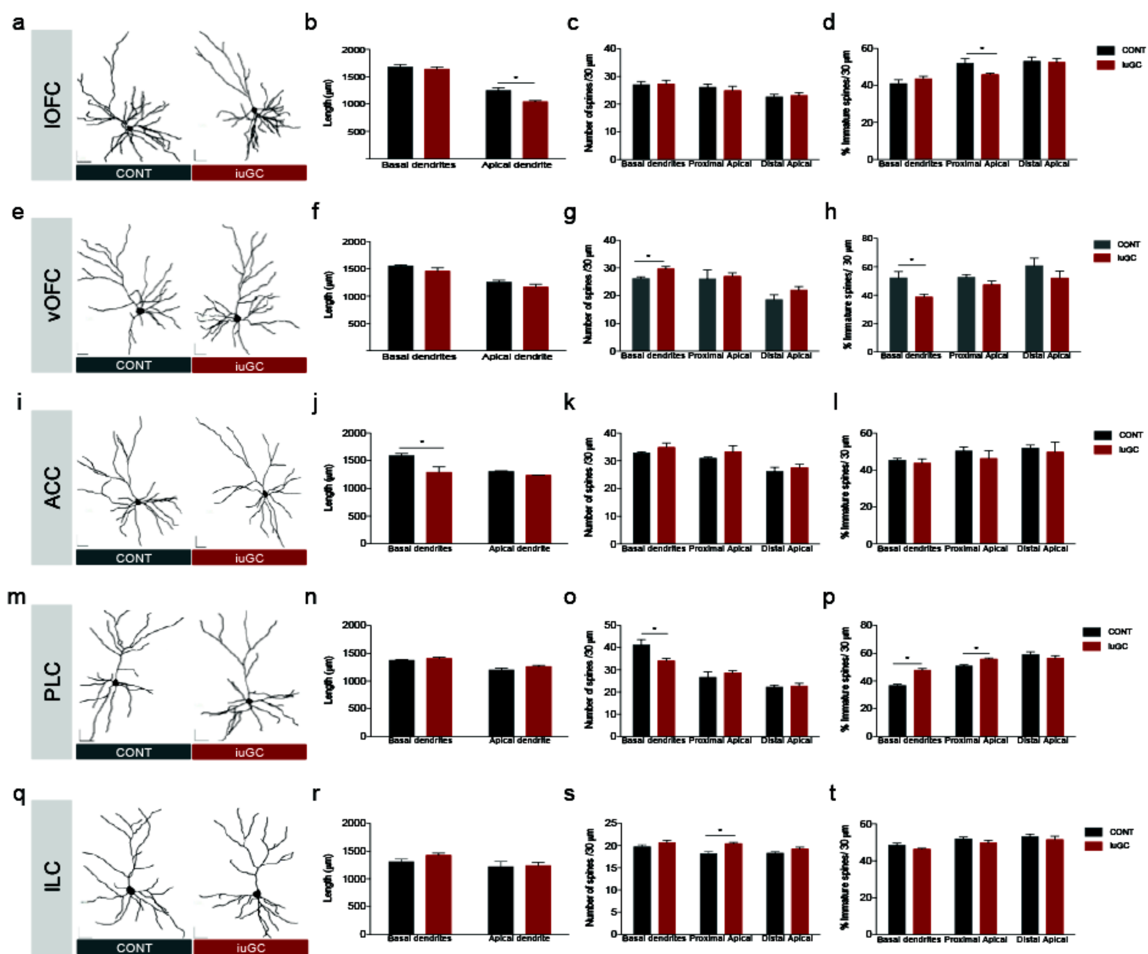


Supplementary Figure 2. Selective PIT performance of animals designated for c-fos analysis shows that iuGC exposure disrupts selective PIT. (a) Pavlovian conditioning of the selective PIT paradigm was similar between CONT animals (performance throughout days of training: $F_{(8,144)}$=18.9, p<0.000; response to CS+ vs. CS-: $F_{(1,144)}$=31.4, p<0.000) and iuGC animals (performance throughout days of training: $F_{(8,128)}$=18.5, p<0.000; response to CS+ vs. CS-: $F_{(1,128)}$=136.5, p<0.000). For each group, mean magazine visits (MV) per min of the CS period presentations and inter-trial interval (ITI, pre-CS) period presentations are plotted. (b) Instrumental conditioning of the selective PIT paradigm revealed no differences between controls and iuGC animals. The number of lever pressing/min performed in each day of training is represented for each group. (c) BPI of CONT and iuGC animals in the selective PIT test session. For each group, the total number of lever presses performed during the BPI is presented. (d) iuGC animals present an impairment in selective PIT performance. Shown is the outcome of the selective PIT paradigm as the total responses on the same lever or the different (dif) lever pressed, according to the CS presented. same – lever pressing on the lever that originates the same reward as the CS presented; dif – lever pressing on the lever that originates a different reward as the CS presented. Graphs represent the total number of lever press during the CS. All graphs are presented as mean ± SEM. iuGC: in utero GC exposed animals; CONT: control animals. n=8-10/group. *p≤0.05, ***p≤0.001.

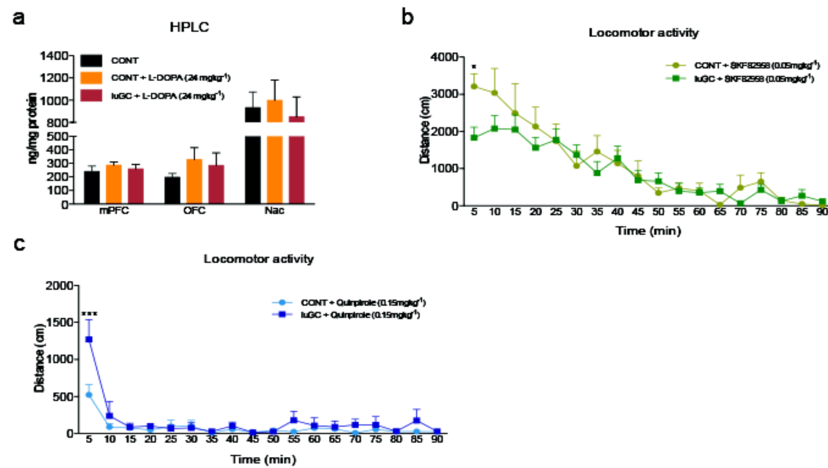


Supplementary Figure 3. Stereological estimations of the mPFC and OFC sub-regions revealed an effect of iuGC exposure. Representative images of coronal brain sections of (a) lOFC and vOFC and (f) ACC, PLC and ILC; numbers represent distance in millimeters to bregma. Average volumes indicate the presence of decrease in (a) volume in lOFC together with a decrease in the (b) number of cells. No differences regarding (c) volume or (d) number of cells were observed in the vOFC. The ACC presents a decrease in the (e) volume, together with a decrease in the (f) total number of cells. No differences regarding (g) volume or (h) number of cells are observed in the PLC. The ILC presents a decrease in the (i) volume, together with a decrease in the (j) number of cells. Average numbers ± SEM are plotted. OFC: orbitofrontal cortex; lOFC: lateral OFC; vOFC: ventral OFC; mPFC: medial prefrontal cortex; ACC: anterior cingulate cortex; PLC: prelimbic cortex; ILC: infralimbic cortex. n=4/group.

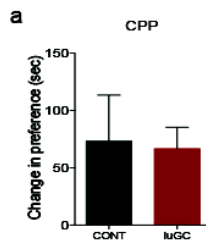
*$p \leq 0.05$.



Supplementary Figure 4. iuGC exposure does not have a profound impact on the neuronal morphology of neurons from layers II/III of the OFC and mPFC. Representative reconstruction of pyramidal neurons of (a) lOFC, (e) vOFC, (i) ACC, (m) PLC and (q) ILC in CONT and iuGC animals. (b) lOFC neurons have a preserved length of the basal dendrites, but showed a decrease in the length of the apical dendrite. (c) The number of total spines within the 30µm segments of the basal dendrites and proximal and distal portions of the apical dendrite analyzed is not altered. (d) On the other hand the percentage of immature spines within the 30µm sections of the proximal parte of the apical dendrite is decreased in iuGC animals. (f) vOFC neurons have a preserved dendritic length. (g) The number of total spines within the 30µm segments of the basal dendrites is increased, but no differences were found in the proximal and distal portions of the apical dendrite. (h) The percentage of immature spines in the analyzed neurons is increased in the basal dendrites but not in the proximal and distal portions of the apical dendrite. (j) ACC neurons have a decrease in the dendritic length of the basal dendrites. The number of (k) total spines or (l) the percentage of immature spines within the 30µm segments of the dendrites reconstructed was not altered. Although the (n) dendritic tree of the PLC neurons seems to be intact, there was a decrease in (o) the number of total spines within the 30µm segments of the basal dendrites and an increase in the (p) percentage of immature spines in the basal dendrites and the proximal portion of the apical dendrite. The ILC also presented a preserved length of the (r) dendrites. Despite the increase in the (s) total number of spines in the proximal portion of the apical dendrite, no changes were observed regarding the percentage of (t) immature spines in ILC neurons. Average numbers ± SEM are plotted. iuGC: *in utero* GC exposed animals; CONT: control animals. OFC: orbitofrontal cortex; lOFC: lateral OFC; vOFC: ventral OFC; mPFC: medial prefrontal cortex; ACC: anterior cingulate cortex; PLC: prelimbic cortex; ILC: infralimbic cortex. n=4/group, at least 10 neurons per animal were analyzed. *$p \leq 0.05$.



Supplementary Figure 5. Effects of the manipulation of the dopaminergic circuitry with the DA precursor L-DOPA or D1 and D2/3 specific receptor agonists. (a) Treatment with 24 $mgkg^{-1}$ of L-DOPA 3 hours prior to sacrifice normalized DA levels in iuGC animals in the NAc ($F_{(2,14)}$ = 0.5, p = 0.613), OFC ($F_{(2,14)}$=1.0, p=0.408) and mPFC ($F_{(2,14)}$=3.3, p=0.070). n=5/group. (b) The locomotor activity of animals treated subcutaneously with SKF82958 (D1 agonist, 0.05 $mgkg^{-1}$) showed a significant effect on iuGC animals' activity in the first 10 min after treatment (post-hoc = 3.1, p=0.05) that normalized for the next 80 min monitored ($F_{(1,10)}$=0.5 , p=0.5). n=6/group (c) The locomotor activity of iuGC animals treated intraperitoneally with quinpirole (D2/3 agonist, 15 $mgkg^{-1}$) was significantly decreased only in the first 10 min after treatment (post-hoc = 6.4, p<0.001), being normal for the remaining of the test ($F_{(1,10)}$=2.3, p=0.158). Average numbers ± SEM are plotted. iuGC: *in utero* GC exposed animals; CONT: control animals. n=6/group. *p≤0.05, ***p≤0.001.



Supplementary Figure 6. Conditioned place preference using dopamine D1-like receptor agonist SKF82958 at 0.05 $mgkg^{-1}$. (a) In the contingent conditioned place preference paradigm (CPP), iuGC and CONT animals spend significantly more time in the SKF82958–associated compartment than in the vehicle-associated compartment. Positive values indicate that animals spent more time on the drug-paired side during the test. Average numbers ± SEM are plotted. iuGC: *in utero* GC exposed animals; CONT: control animals. n=8/group. $^{**}p \leq 0.01$.

Supplementary information 3

The following section includes two supplementary tables.

Supplementary Table 1. Two-way ANOVA results for c-fos activation of iuCG animals versus CONT animals in brain regions analysed. Bonferroni multiple comparison of CONT and iuGC animals that performed Pavlovian and instrumental conditionings but no PIT testing (CONT and iuGC) versus animals that performed selective PIT outcome (CONT test and iuGC test). Results of the percentage of c-fos activation in comparison to CONT animals are depicted. CONT: control animals; iuGC: iuGC animals.

	F	p value	Multiple comparison - Bonferroni		Percentage of activation
			Groups	p value	
lOFC	5.3	0.037*	CONT vs CONT test	0.062	100.7
			CONT vs iuGC	0.156	90.1
			CONT vs iuGC test	0.000***	305.6
			CONT test vs iuGC	0.062	
			CONT test vs iuGC test	1.000	
			iuGC vs iuGC test	0.000***	
vOFC	7.2	0.017**	CONT vs CONT test	0.029*	137.2
			CONT vs iuGC	0.358	89.8
			CONT vs iuGC test	0.000***	390.1
			CONT test vs iuGC	1.000	
			CONT test vs iuGC test	0.000***	
			iuGC vs iuGC test	0.000***	
ACC	40.4	0.000***	CONT vs CONT test	0.066	34.5
			CONT vs iuGC	1.000	11.4
			CONT vs iuGC test	0.000***	100.5
			CONT test vs iuGC	0.013	
			CONT test vs iuGC test	0.000***	
			iuGC vs iuGC test	0.000***	
PLC	3.8	0.071	CONT vs CONT test	0.059	49.4
			CONT vs iuGC	1.000	11.4
			CONT vs iuGC test	0.000***	100.5
			CONT test vs iuGC	1.000	
			CONT test vs iuGC test	0.003**	
			iuGC vs iuGC test	0.000***	
ILC	51.8	0.000***	CONT vs CONT test	0.003**	59.6
			CONT vs iuGC	1.000	31.4
			CONT vs iuGC test	0.000***	231.2
			CONT test vs iuGC	0.090	
			CONT test vs iuGC test	0.000***	
			iuGC vs iuGC test	0.000***	
Nac core	13.5	0.002**	CONT vs CONT test	0.001***	86.3
			CONT vs iuGC	0.300	42.2
			CONT vs iuGC test	0.442	36.8
			CONT test vs iuGC	0.121	
			CONT test vs iuGC test	0.045*	
			iuGC vs iuGC test	1.000	
Nac shell	16.5	0.001***	CONT vs CONT test	0.004**	74.0
			CONT vs iuGC	0.513	34.2
			CONT vs iuGC test	1.000	4.0
			CONT test vs iuGC	0.298	
			CONT test vs iuGC test	0.007**	
			iuGC vs iuGC test	0.765	
BLA	5.8	0.029*	CONT vs CONT test	0.005**	58.4
			CONT vs iuGC	0.231	26.6
			CONT vs iuGC test	0.051	37.9
			CONT test vs iuGC	0.739	
			CONT test vs iuGC test	1.000	
			iuGC vs iuGC test	1.000	
CeA	28.9	0.000***	CONT vs CONT test	0.000***	56.3
			CONT vs iuGC	0.000***	63.1
			CONT vs iuGC test	0.000***	37.5
			CONT test vs iuGC	1.000	
			CONT test vs iuGC test	1.000	
			iuGC vs iuGC test	0.883	
VTA	27.8	0.000***	CONT vs CONT test	0.000***	53.1
			CONT vs iuGC	1.000	13.4
			CONT vs iuGC test	0.161	14.7
			CONT test vs iuGC	0.000***	
			CONT test vs iuGC test	0.000***	
			iuGC vs iuGC test	1.000	

Supplementary Table 2. Mann-Whitney statistical comparison of iuGC versus CONT animals for stereological (volume and number of cells) and morphometric (dendritic length and spine density) parameters. CONT: control animals; iuGC: iuGC animals.

			lOFC	vOFC	ACC	PLC	ILC
Volume		**U**	0.0	7.5	0.0	7.0	0.0
		P value	**0.029***	0.886	**0.029***	0.886	**0.029***
Number of cells		**U**	0.0	3.0	0.0	7.0	0.0
		p value	**0.029***	0.200	**0.029***	0.886	**0.029***
Dendritic length	**Basal dendrites**	**U**	6.0	4.0	0.0	3.0	2.0
		p value	0.686	0.343	**0.029***	0.200	0.229
	Apical dendrite	**U**	0.0	6.0	0.0	2.0	7.0
		p value	**0.029***	0.663	0.057	0.114	0.886
Number of spines	**Basal**	**U**	8.0	0.0	4.0	0.0	6.0
		p value	1.000	**0.029***	0.343	**0.029***	0.663
	Proximal	**U**	6.0	7.0	5.0	4.0	0.0
		p value	0.686	0.885	0.561	0.343	**0.029***
	Distal	**U**	7.0	2.0	5.0	6.0	3.0
		p value	0.885	0.110	0.486	0.686	0.191
Percentage of spines	**Basal**	**U**	5.0	0.0	6.0	0.0	3.0
		p value	0.486	**0.029***	0.686	**0.029***	0.200
	Proximal	**U**	2.0	3.0	6.0	0.0	3.0
		p value	0.114	0.191	0.686	**0.029***	0.200
	Distal	**U**	7.0	4.0	8.0	5.0	5.0
		p value	0.886	0.343	1.000	0.486	0.486

CHAPTER 3

Optogenetic modulation of Nucleus accumbens medium spiny neurons during reward-dependent behaviours

CHAPTER 3.1

Activation of D2 dopamine receptor-expressing neurons in the nucleus accumbens increases motivation

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Activation of D2 dopamine receptor-expressing neurons in the nucleus accumbens increases motivation

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Striatal dopamine receptor D1-expressing neurons have been classically associated with positive reinforcement and reward, whereas D2 neurons are associated with negative reinforcement and aversion. Here we demonstrate that the pattern of activation of D1 and D2 neurons in the nucleus accumbens (NAc) predicts motivational drive, and that optogenetic activation of either neuronal population enhances motivation in mice. Using a different approach in rats, we further show that activating NAc D2 neurons increases cue-induced motivational drive in control animals and in a model that presents anhedonia and motivational deficits; conversely, optogenetic inhibition of D2 neurons decreases motivation. Our results suggest that the classic view of D1–D2 functional antagonism does not hold true for all dimensions of reward-related behaviours, and that D2 neurons may play a more prominent pro-motivation role than originally anticipated.

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Dopaminergic neurotransmission in the nucleus accumbens (NAc) has an essential role in reward behaviours, although the causal biological contribution of this mechanism remains controversial. While some argue that dopamine signals the discrepancy between predicted and experienced reward (reward prediction error)[1–3], others suggest that it mediates motivational drive by the attribution of incentive salience to reward-related stimuli[4].

Up to 95% of NAc neurons are medium spiny neurons (MSN), typically segregated into those expressing D1 dopamine receptors (direct pathway) and those expressing D2 dopamine receptors (indirect pathway)[5–7]. The existence of a functional opposition between D1 and D2 MSN has for long been assumed[8,9], but the establishment of a causal relationship between the activation of each neuronal subtype and its effect on complex motivated behaviours has proved to be challenging. D1-MSN activation is canonically related to positive rewarding events, inducing persistent reinforcement, whereas D2-MSN signalling is thought to mediate aversion (both in dorsal striatum and NAc)[10–12]. Nonetheless, recent studies raised some questions regarding this functional/behavioural bias, especially regarding D2 neurons in the NAc[13–15]. Taking this into consideration, we used an optogenetic approach to address the impact of modulating NAc D1 and D2 neurons in motivation-dependent behaviours.

Results

NAc D1 and D2 activation is correlated with motivation. First, to verify that the NAc was critically recruited in motivation-dependent tasks, we evaluated the neuronal activation pattern of different brain regions after the Pavlovian-to-Instrumental transfer (PIT) and the Progressive-Ratio tasks (PR). PIT evaluates the ability of Pavlovian conditioned cues that predict reward to enhance instrumental response[16,17], i.e., a measure of incentive salience, whereas the PR measures the willingness to work to obtain a reward (breakpoint)[18]. All corticolimbic regions analysed presented a significant increase in c-fos on task execution (Fig. 1a–d and Supplementary Fig. 1a–r; two-way analysis of variance (ANOVA), $P<0.001$, $n=6$). To further understand the contribution of each brain region for the behavioural performance, we performed an exploratory factor analysis through Principal Axis Factoring, which makes no *a priori*

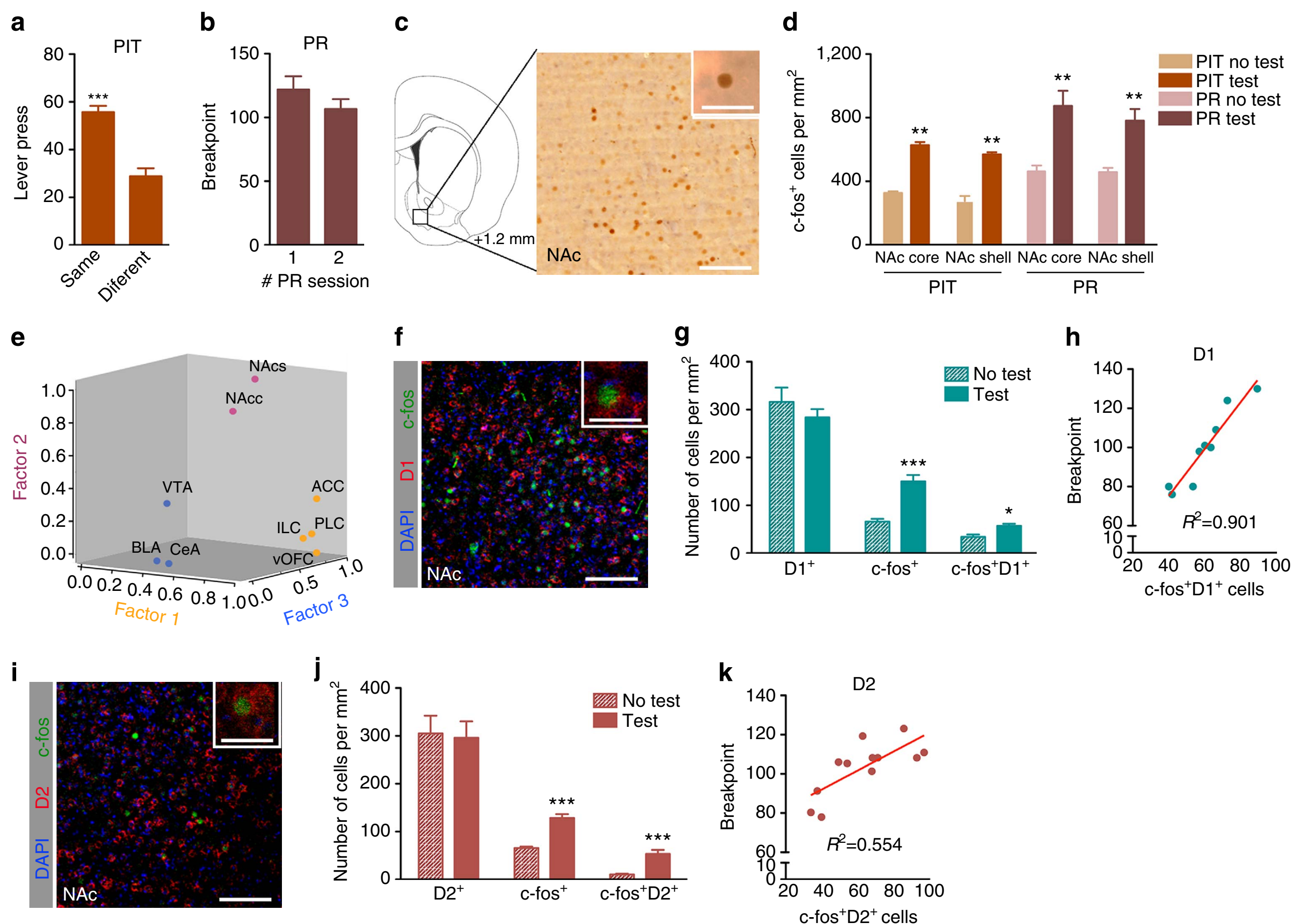

Figure 1 | NAc D1 and D2 neuronal activation predicts performance in motivation-related tasks. (**a**) PIT outcome ($n=10$). (**b**) Breakpoint for two PR sessions ($n=10$). (**c**) Representative immunostaining of c-fos in the NAc; Scale bar: 100 μm; inset: 20 μm. (**d**) Animals performing PIT or PR tests have increased c-fos$^+$ cells in the NAc ($n=6$). (**e**) Principal factor analysis was done to evaluate the contribution of each brain region for the behavioural performance. This analysis shows that the NAc core and shell regions (NAcc and NAcs; factor 2) are grouped distinctively from other limbic regions (BLA, CeA and VTA; factor 3) and from cortical regions that are all grouped together in factor 1 (ACC, PLC, ILC, lOFC and vOFC). Representative images of immunofluorescence for c-fos and dopamine receptor D1 (**f**) or c-fos and dopamine receptor D2 (**i**) in the NAc of animals that performed PR task; Scale bar: 100 μm; inset: 20 μm. Insets represent double positive cells. (**g**) Number of c-fos$^+$/D1$^+$ cells and c-fos$^+$/D2$^+$ (**j**) cells in the NAc ($n=6$). (**h**) Correlation between individual Breakpoint and number of c-fos$^+$/D1$^+$ cells in the NAc ($n=9$). (**k**) Correlation between individual breakpoint and number of c-fos$^+$/D2$^+$ cells in the NAc ($n=12$). Error bars denote s.e.m. *$P\leq0.05$, **$P\leq0.01$, ***$P\leq0.001$.

assumptions about relationships among factors. By defining three factors, this analysis clearly grouped all cortical regions in factor 1 (ACC: anterior cingulate cortex; PLC: prelimbic cortex; ILC: infralimbic cortex; lOFC and vOFC: lateral and ventral orbitofrontal cortex); factor 2 grouped NAc core and shell; and factor 3 included other limbic structures (CeA: central nucleus of the amygdala; BLA: basolateral amygdala; and ventral tegmental area (VTA)) (Fig. 1e). All communalities and factor loadings were satisfactory, revealing no problematical cross loadings. Cronbach's Alpha denotes good, or excellent, reliability scores (Supplementary Table 1). Pearson's correlation coefficients revealed that there is a positive association between the three factors and behavioural test score, although, only NAc (Pearson's correlation, $P=0.002$) and other limbic regions (Pearson's correlation, $P=0.017$) were significant. Using a multiple linear regression model, we verified that 46.9% of behavioural performance could be explained by the three predictors; however, only NAc was significant ($\beta=0.5$, $P=0.042$).

Considering previous results, we then measured the recruitment of NAc D1$^+$ and D2$^+$ neurons during PR performance. Importantly, there was a significant increase in both NAc c-fos$^+$/D1$^+$ and c-fos$^+$/D2$^+$ cells on PR performance ($n=6$; Fig. 1f–g, c-fos$^+$/D1$^+$: unpaired t-test, $P<0.001$; Fig. 1i,j, c-fos$^+$/D2$^+$: unpaired t-test, $P=0.001$). Both were positively correlated with individual breakpoint values (Fig. 1h, c-fos$^+$/D1$^+$: Pearson's correlation, $R^2=0.9$, $P<0.001$, $n=9$; Fig. 1k, c-fos$^+$/D2$^+$: Pearson's correlation, $R^2=0.7$, $P=0.006$, $n=12$).

Absence of D1–D2 antagonism in motivation. Since both D1 and D2 neurons were activated during PR, we decided to causally test the effect of cell type-specific activation on motivated behaviour. For this purpose, we injected a cre-inducible adeno-associated viral construct coding for channelrhodopsin (ChR2) (pAAV-EF1a-DIO-hChR2-enhanced green fluorescent protein (eYFP)) unilaterally in the NAc of D1 and D2-cre transgenic mouse lines and subjected animals to PR task (Fig. 2a–d). No differences in training were observed between groups (Fig. 2e). As anticipated, activation of D1 neurons during cue exposure increased cumulative presses throughout session as well as the breakpoint (Fig. 2f–h; 85% increase; paired t-test, $P=0.011$, $n=5$). Surprisingly, activation of D2 accumbal neurons also enhanced cumulative presses and breakpoint (Fig. 2f–h; 69% increase; paired t-test, $P=0.003$, $n=6$; results from other stimulation parameters in Supplementary Fig. 2).

Exploring the role of D2 neurons in motivation. While the behavioural results obtained with D1 neuronal activation were expected, D2 activation effects in motivation were paradoxical, in the light of previous findings suggesting that D2 neurons are associated with negative rather than positive valence events[10,12,19]. Considering this, we decided to further explore the role of NAc D2 neurons in behaviour. We injected in the NAc of rats a construct carrying ChR2 under the control of the D2 receptor minimal promoter—pAAV-D2R-hChR2(H134R)-eYFP (Fig. 3a,b)—to allow specific optogenetic activation of D2$^+$ neurons. 58% of NAc D2$^+$ neurons were successfully transfected with D2-driven ChR2 or EYFP (D2$^+$/YFP$^+$ cells; Fig. 3c,d); we rarely observed D1$^+$/YFP$^+$ cells (1.5%; Supplementary Fig. 3a–b). By evaluating the optically evoked response using single-cell *in vivo* electrophysiology, we showed that D2-driven opsin was functional at different stimulation frequencies (Fig. 3e–g; two-way ANOVA, $P<0.001$, $n=19$ cells; Supplementary Fig. 3c). Approximately half of the NAc cells (54.3%) increased their firing rate during 40 Hz stimulation (42.9% did not respond and 2.9% decreased activity); and 25.7% of the cells presented altered firing rate during the 60 s after the stimulus (8.6% decreased activity, 17.1% increased activity; Fig. 3h).

To confirm specificity of D2 pathway, we performed electrophysiological recordings in the ventral pallidum (VP) (Fig. 3i), which receives direct projections from both D1 and D2 NAc MSNs[13,20]. NAc D2 neuronal stimulation elicited an overall reduction in the firing rate of the VP (Fig. 3j,k; one-way ANOVA, $P<0.001$, $n=30$ cells), with an average spike latency of 6 ms (Fig. 3p), consistent with the expected monosynaptic (GABAergic) input from the NAc to VP. Because it has been shown that only D1$^+$ (but not D2$^+$) MSNs project directly to the midbrain[13,21,22], we also recorded the VTA during D2 neuronal activation in the NAc. Confirming the selectivity of D2 pathway, we observed a general increase in global VTA activity (Fig. 3m,n; one-way ANOVA, $P<0.001$, $n=25$ cells), with an average spike latency of 160 ms, suggestive of indirect modulation (Fig. 3p). These results were further confirmed by recording experiments of these downstream regions during stimulation of NAc terminals (Supplementary Fig. 4). Moreover, immunofluorescence against YFP showed that VP (but not VTA) is directly innervated by D2-ChR2 terminals (Fig. 3l,o).

We next tested the effects of NAc D2 neuronal activation in the PR task. All groups acquired similar levels of lever pressing during training (Supplementary Fig. 3d–e). D2 optical stimulation (473 nm light; pulses of 12.5 ms at 40 Hz for 1 s) occurring at the same time as the unconditioned stimulus (light above the active lever), induced a significant increase in cumulative presses throughout the session, which translated into a 35% enhancement of the breakpoint (Fig. 4a–c; unpaired t-test, $P<0.001$, $n=13$–16; Supplementary Fig. 3f,g shows PR with different stimulation protocols). This enhancement was not due to changes in the number of pellets earned in the session (Fig. 4d). Importantly, when this stimulation occurred during the inter-trial interval (ITI), it had no effect (Fig. 4e,f; unpaired t-test, $P=0.395$, $n=5$–7). In an additional PR session (with optical stimulation), animals were satiated to induce reward devaluation. Both groups decreased lever pressing, demonstrating sensitivity to the decreased value of the outcome, albeit this was more evident in D2-ChR2 group (Fig. 4g, two-way ANOVA, $P<0.001$). Optical stimulation of NAc D2 neurons during the conditioned stimulus presentation also robustly enhanced PIT performance, despite similar baseline performance (Supplementary Fig. 5a–d; paired t-test, $P=0.01$). No changes were observed in feeding behaviour or locomotion of stimulated animals (Supplementary Fig. 5e–f).

Confirming the behavioural findings, the number of c-fos$^+$/D2$^+$/eYFP$^+$ cells in the NAc was higher in stimulated D2-ChR2 rats when compared with stimulated D2-eYFP control rats on PR task (Fig. 4h,i; unpaired t-test, $P=0.001$, $n=5$ animals).

Our next goal was to understand the impact of inhibiting D2$^+$ accumbal neurons in motivation. To do so, a vector containing halorhodopsin (NpHR) under the control of D2 minimal promoter (pAAV-D2R-NpHR-eEYFP) was injected unilaterally in the NAc (Fig. 5a). Optical activation of NpHR (589 nm laser; 10 s constant light at 5mW) lead to a general decrease in the NAc neuronal firing rate (Fig. 5b,c; one-way ANOVA, $P=0.0015$, $n=20$ cells), with 58.3% of the cells presenting decreased activity and 22.2% increased activity, while 19.4% did not respond (Fig. 5d). At a behavioural level, optical inhibition of NAc D2 neurons during cue exposure decreased cumulative lever presses (Fig. 5e; two-way ANOVA, $P<0.000$) and strongly reduced breakpoint (Fig. 5f,g; 32% decrease, unpaired t-test, $P=0.001$, $n=5$-8). No significant effects in locomotion and feeding behaviour were observed due to D2 optogenetic inhibition (Supplementary Fig. 6).

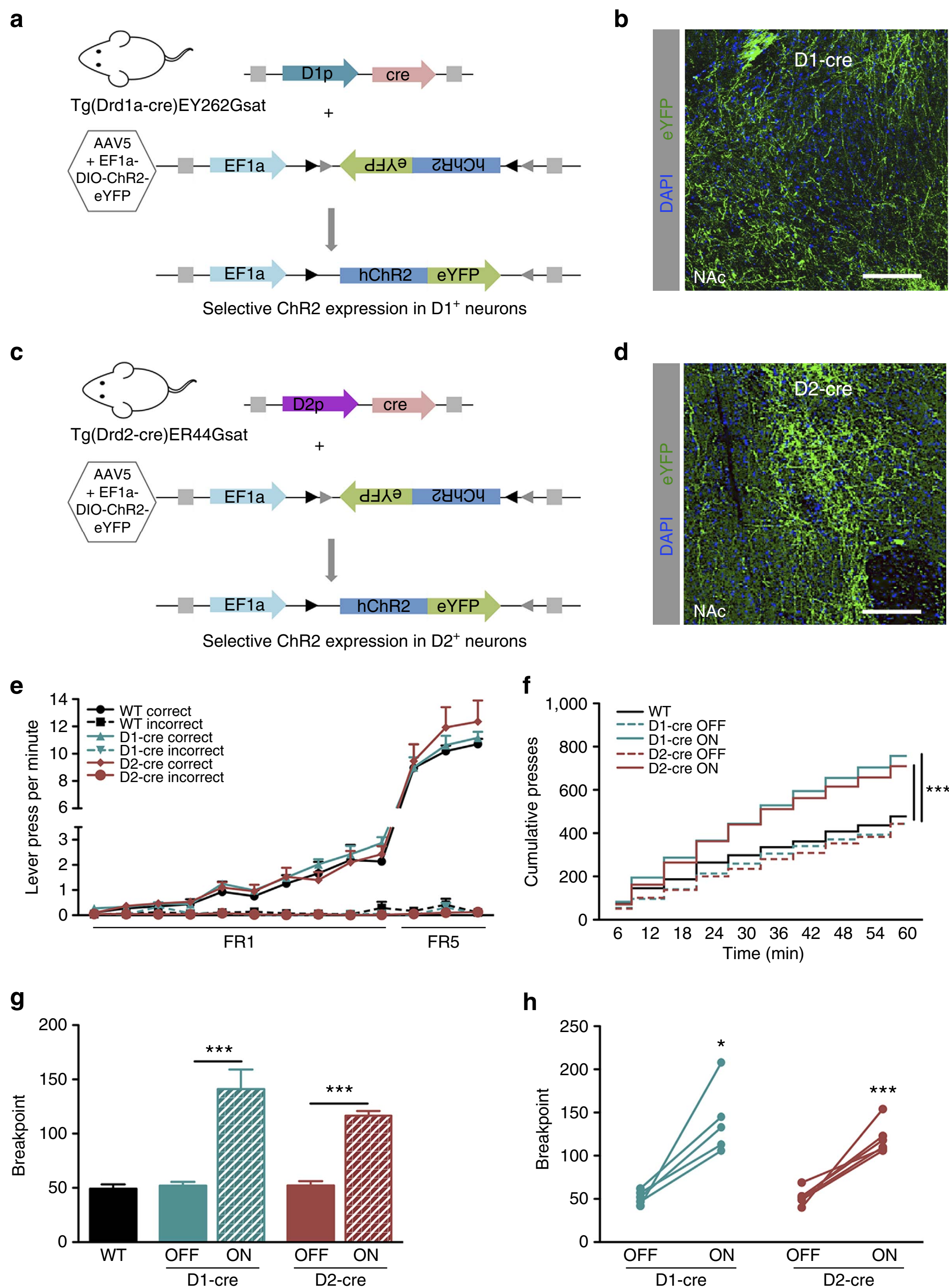

Figure 2 | Optogenetic activation of NAc D1 and D2 neurons enhances motivation in mice. (**a**) Strategy used for optogenetic activation of NAc D1 neurons in mice. A cre-dependent ChR2 construct (AAV5-EF1a-DIO-ChR2-eYFP) was injected unilaterally in the NAc of D1-cre transgenic mice (Tg(Drd1a-cre)EY262Gsat). (**b**) Expression of ChR2-eYFP in D1-cre animals; Scale bar: 50 μm. (**c**) Strategy used for optogenetic activation of NAc D2 neurons in mice. A cre-dependent ChR2 construct (AAV5-EF1a-DIO-ChR2-eYFP) was injected unilaterally in the NAc of D2-cre transgenic mice (Tg(Drd2-cre)ER44Gsat). (**d**) Expression of ChR2-eYFP in D2-cre animals; scale bar: 50 μm. (**e**) No differences were found in the learning curves of PR test between groups ($n_{D1\text{-}cre} = 5$; $n_{D2\text{-}cre} = 6$). (**f**) Optogenetic activation of D1 or D2 neurons (12.5 ms light pulses at 40 Hz, during 1 s of cue exposure) increased cumulative presses throughout session. (**g,h**) Optogenetic activation of NAc D1 or D2 neurons strongly enhanced breakpoint. Error bars denote s.e.m. *$P \leq 0.05$, ***$P \leq 0.001$.

Rescue of deficits in a prenatal glucocorticoid exposure model. We then assessed whether D2 accumbal stimulation was sufficient to normalize motivation in a rat model of *in utero* glucocorticoid exposure (iuGC) that presents hypodopaminergia in the meso-limbic circuit[23,24], NAc D2 dysfunction and a significant impairment in motivation[15]. Control and iuGC animals were unilaterally injected with D2-driven ChR2 construct and subjected to the PR and PIT tasks. In the PR task, training was similar between groups (Fig. 6a,b; $n = 10$). On the test day, iuGC rats presented a 53% decrease in their breakpoint (Fig. 6c; 62.5 ± 6.4 versus 117 ± 8.9; one-way ANOVA, $P = 0.003$), despite consuming the same number of pellets in the session (Fig. 6d).

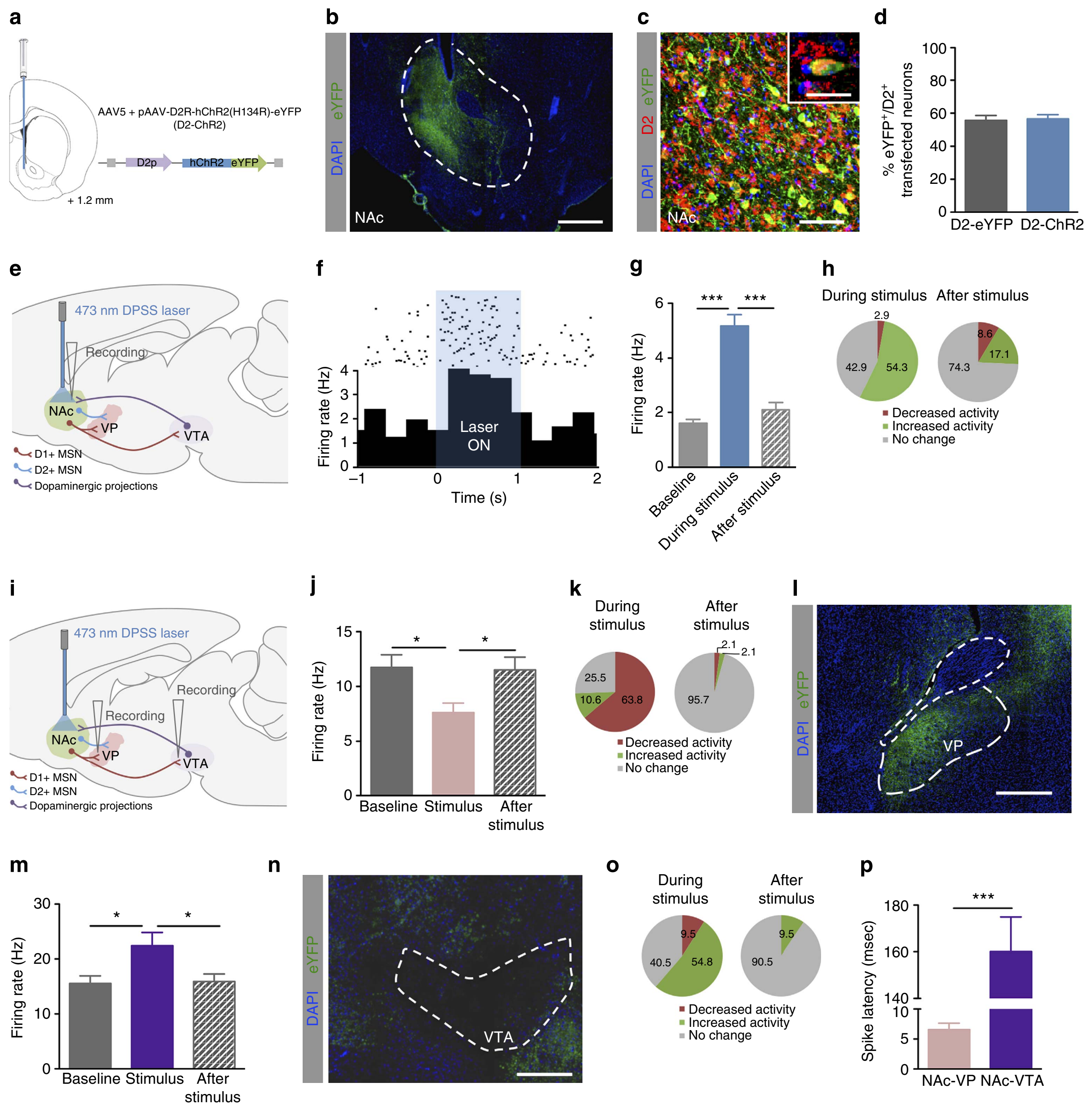

Figure 3 | *In loco* and downstream electrophysiological recordings. (**a**) Rats received unilateral injection of AAV5-D2-hChR2(H134R)-eYFP in the NAc; number represent distance to bregma. (**b**) Immunofluorescence against YFP showing transfection restricted to the NAc; scale bar: 200 μm; ac: anterior commissure. (**c**) Immunofluorescence showing expression of eYFP in $D2^+$ neurons; scale bar: 200 μm; inset: 40 μm. (**d**) Around 58% of $D2^+$ neurons express D2-eYFP or D2-ChR2 ($n=6$). (**e**) Schematic representation of the optogenetic stimulation and *in vivo* single-cell electrophysiological recording experiments. (**f**) Representative time histogram of NAc electrophysiological single units in response to a 40 Hz stimulus. (**g**) Increase in NAc firing rate during optogenetic stimulation (40 Hz, 12.5 ms pulses for 1 s). (**h**) Approximately half of the cells increased firing rate (54%), 42.9% did not respond and 2.5% decreased their activity ($n=19$ cells). (**i**) Schematic representation of the *in vivo* single-cell electrophysiological recording experiments in downstream regions. (**j**) NAc D2 neuronal activation decreases global VP firing rate ($n=30$ cells). (**k**) During stimulation, 63.8% of VP cells decrease their firing rate, consistent with GABAergic inputs from D2 MSNs from the NAc, 25.5% do not respond and 10.6% increased activity. Considering a 60 speriod after the stimulation, most of the cells return to their basal activity levels. (**l**) eYFP expression in the VP showing that D2-ChR2$^+$ terminals arising from the NAc strongly innervate this brain region; scale bar: 500 μm. (**m**) NAc D2 neuronal stimulation increases global VTA firing rate ($n=25$ cells). (**n**) During stimulation, 54.8% of cells increase their firing rate, 40.5% do not respond and 9.5% decreased activity; most of the cells return to their basal activity levels after stimulation. (**o**) eYFP expression in the VTA showing rare D2-ChR2$^+$ terminals; scale bar, 500 μm. (**p**) Spike latency after NAc D2 optogenetic stimulation shows that VP cells fire almost immediately, consistent with a monosynaptic input from the NAc whereas VTA cells present higher latency (indirect/polysynaptic modulation). Error bars denote s.e.m. *$P\leq0.05$, ***$P\leq0.001$.

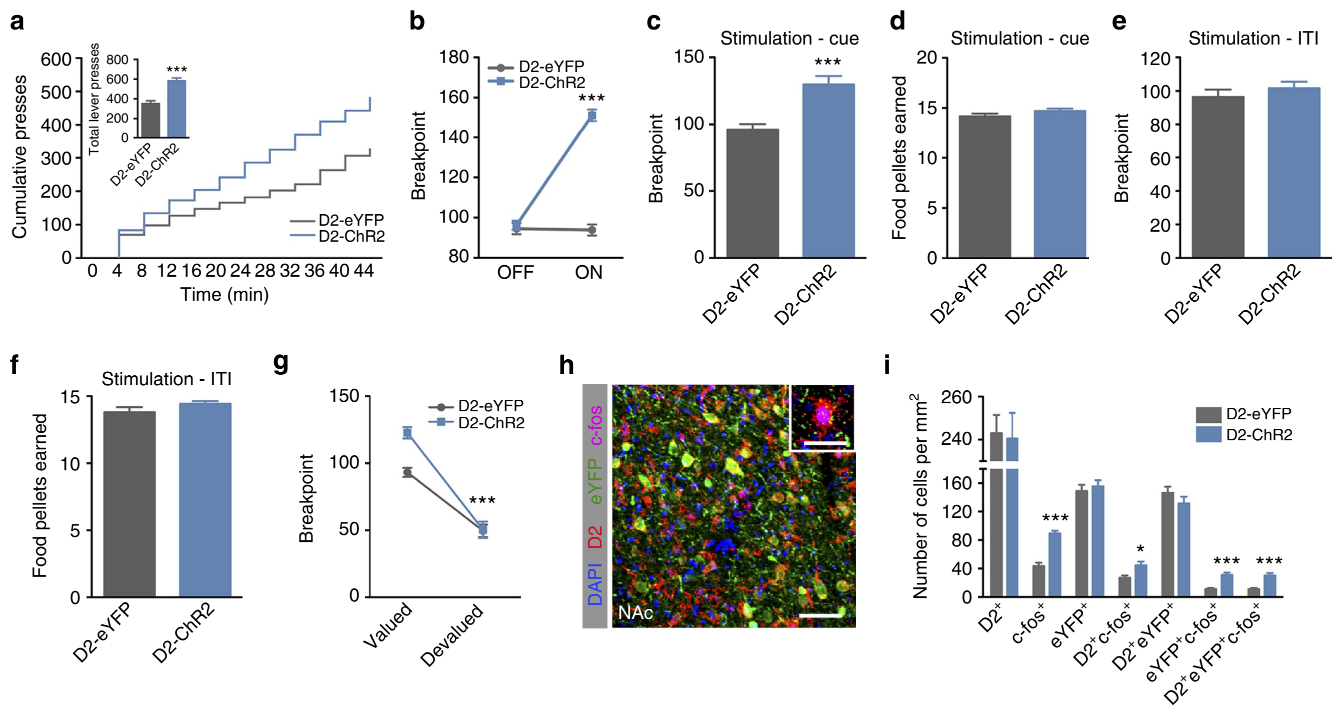

Figure 4 | Optogenetic activation of NAc D2 neurons increases motivation in rats. (**a**) Optogenetic stimulation of D2-ChR2 animals during cue exposure (40 Hz, 12.5 ms pulses for 1 s) increases cumulative presses in the PR test ($n_{D2\text{-}ChR2}=16$; $n_{D2\text{-}eYFP}=13$). (**b**,**c**) D2 activation during cue exposure induces a significantly higher breakpoint; though animals receive the same number of pellets in the session (**d**). (**e**) D2 activation during inter-trial interval (ITI) does not change breakpoint ($n_{D2\text{-}ChR2\text{-}eYFP}=7$; $n_{D2\text{-}eYFP}=5$). (**f**) Total number of food pellets earned in the PR session, in which optical stimulation was given during the ITI. (**g**) Outcome devaluation decreases lever presses in both groups. (**h**) Representative immunofluorescence for c-fos$^+$/D2$^+$/eYFP$^+$ in the NAc; scale bar: 200 μm; inset: 40 μm. (**i**) Quantification of c-fos$^+$/D2$^+$/eYFP$^+$ cells in the NAc of stimulated animals showing the recruitment of D2$^+$/eYFP$^+$ cells ($n=5$ animals). Error bars denote s.e.m. *$P\leq0.05$, ***$P\leq0.001$.

Concordantly, these animals present an overall lower neuronal activation in the NAc when compared with control animals that also performed the test (Fig. 6e,f; one-way ANOVA, $P=0.001$, $n=6$); importantly, there was a marked reduction in the recruitment of D2$^+$ neurons (unpaired t-test, $P=0.001$).

Optogenetic activation of D2 neurons during the PR test session significantly increased the breakpoint of iuGC animals (Fig. 6g; unpaired t-test, $P<0.000$, $n=11–15$), comparable to the one of control rats (iuGC-D2-ChR2 average of 95.85 ± 4.5 versus control-D2-eYFP average of 96.1 ± 4.1; unpaired t-test, $P=0.97$). In agreement, the number of c-fos$^+$/eYFP$^+$ positive neurons was also significantly higher than non-stimulated iuGC animals (Fig. 6j,k; unpaired t-test, $P<0.001$, $n=6$). Importantly, the behavioural rescuing effect was not observed if the stimulation occurred during the ITI (Fig. 6h). We also tested animals' response after reward devaluation in an additional PR session. In this scenario, both control and iuGC groups decreased lever pressing, demonstrating sensitivity to the decreased value of the outcome (Fig. 6i, one-way ANOVA, $P<0.001$, $n=5–7$).

In further support of an important role of D2 neurons in iuGC motivational deficits, we found that in the PIT test, optical stimulation of NAc D2 neurons also normalized the response of iuGC group (Supplementary Fig. 7).

Discussion

Performance in motivation-related tasks has been tightly linked to the NAc: enhancing dopamine transmission in this region increases the willingness of animals to work for food[25–27], whereas blunting dopamine signals attenuates it[26,28,29]. However, the function of D1 and D2 neurons is still debatable. Traditional views propose that tonic dopamine release mainly activates high-affinity D2 receptors, while phasic dopamine events activate low-affinity D1 receptors[30,31], and signal the value of learned reward-predictive cues that drive-motivated behaviour[32,33]. However, recent findings show that phasic dopamine release also drives a rapid activation of D2 MSNs[34], suggesting that some of the behavioural effects of phasic release may be partially mediated by D2 signalling.

Here we report that activation of both D1 and D2 neurons in the NAc is strongly correlated with behavioural performance in motivation-dependent paradigms. In addition, we show that brief optogenetic activation of NAc D1 and D2 neurons during reward-predictive cues strongly augments motivation in mice. Our findings are clearly distinct from the classic view supporting a functional opposition between D1 and D2 MSNs, indicating that both neuronal subtypes can contribute in the same direction in the modulation of motivation. Other optogenetic approaches specifically targeting NAc D1 and D2 neurons showed that D1 neuronal activation resulted in enhancement in conditioned place preference for cocaine, while the activation of D2 neurons attenuated preference, leading to the hypothesis that the D1 pathway has an action in promoting (drug) reward responses, while D2 pathway mediates the opposite[10]. These counterintuitive results may indicate that activation of D1 and D2 neurons in different behavioural contexts or during different stages of the task may lead to distinctive outcomes. In addition, these neurons may differentially modulate the response towards natural rewards versus drugs of abuse[35].

Other D1/D2 optogenetic studies using natural rewards/reinforcers relied on the modulation of dorsomedial striatal neurons. Activation of D1 MSNs resulted in positive responses to

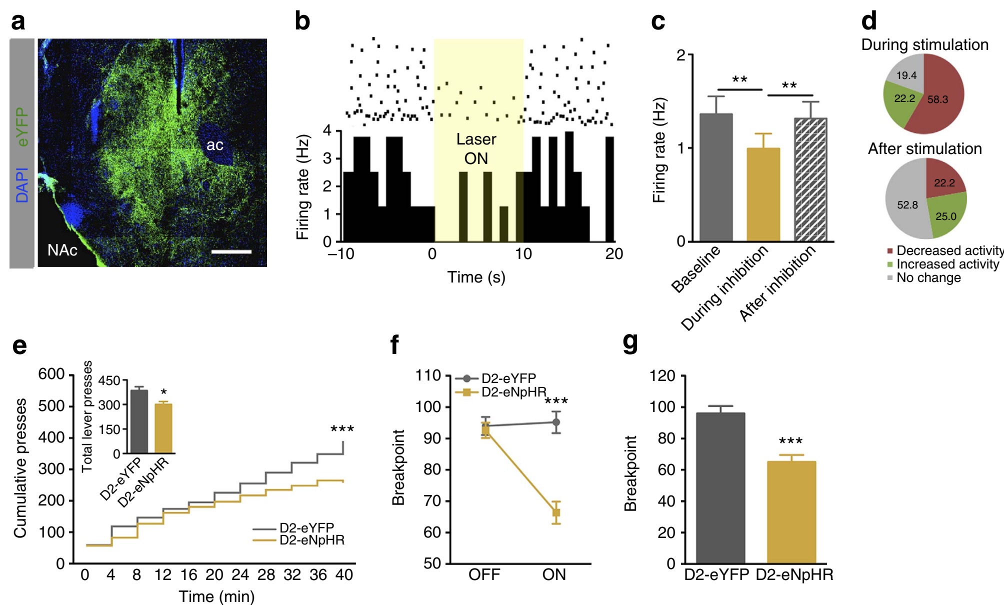

Figure 5 | Inhibition of NAc D2 neurons - electrophysiological and behavioural correlates. (**a**) Representative immunofluorescence against YFP in the NAc of an animal injected with AAV5-D2-eNpHR-eYFP. Scale bar: 400 μm; ac: anterior commissure. (**b-c**) Optogenetic stimulation of D2-eNpHR (10 s constant light at 15 mW) decreases NAc firing rate ($n = 20$ cells). (**d**) During stimulation, 58.3% of cells decrease their firing rate, whereas 22.2% of cells increase their activity and 19.4% do not respond. After the stimulation (period of 60 s), 52.8% of cells return to their basal activity levels, 22.2% of cells present decreased firing rate, and 25% of cells present increased activity. (**e**) Optogenetic inhibition of D2 neurons during cue exposure (15 mW constant light during 10 s) decreases cumulative presses and breakpoint (**f,g**) in the PR test ($n_{D2\text{-}ChR2} = 8$; $n_{D2\text{-}eYFP} = 5$). Error bars denote s.e.m. $^{**}P \leq 0.01$, $^{***}P \leq 0.001$.

rewarding stimuli, contrary to activation of D2 MSNs that induced aversion and suppression of reward/motivated behaviour in both place preference and operant tasks[11]. Our contradictory results regarding D2 neurons are probably the result of anatomical/functional specificities between the ventral and dorsal striatum. Indeed, a recent study showed that, in contrast to dorsal striatum, both NAc MSNs populations may inhibit or disinhibit thalamic activity depending on their projection pattern and not on their genetic (D1 or D2) characteristics[13], emphasizing the need to revisit the current view of dorsal and ventral D1 and D2 MSNs as identical entities.

The unexpected behavioural outcome of D2 neuronal activation was further explored by selectively manipulating D2 neurons using a distinct methodological approach in rats. Akin to results in mice, brief activation (or inhibition) of NAc D2 neurons during reward-predictive cues strongly enhances (or diminishes) motivation. These findings were further extended by showing that activating accumbal D2 neurons in a model of prenatal glucocorticoid exposure that presents D2 dysfunction[15,23] rescues motivation deficits of these animals.

It is important to stress that up to 80% of NAc cholinergic interneurons also express D2 dopamine receptor[36–39], and their selective activation enhances phasic dopamine release in the NAc, which may ultimately synergize to drive-motivated behaviours[40,41]. However, cholinergic interneurons do not seem to be differently recruited between eYFP- and ChR2-stimulated animals (Supplementary Fig. 8), so the observed motivational effect seems to be mostly mediated by the activation of D2 MSNs of the NAc. This can arise through D2 MSNs direct modulation of VP activity, a brain region suggested to be a convergent point for motivational (and hedonic) signals[42]. Likewise, the observed indirect activation of VTA neurons (via VP or not) may also contribute to the observed motivation augmentation[43–45]. Interestingly, VP also reciprocally modulates NAc activity[46]. Thus, a complex cascade of neuromodulatory events can arise due to NAc D2 neuronal activation, highlighting the need to perform additional studies to better comprehend how activation of D2 (and D1) neurons can enhance motivated behaviour.

In conclusion, herein we show that different motivation-dependent tests recruit D1 and D2 neurons in the NAc, and that selective optogenetic activation of both neuronal populations enhances cue-induced motivational drive, suggesting that the classic view of D1–D2 functional antagonism does not hold true for all dimensions/types of reward-related behaviour.

Methods

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

In the case of the model of iuGC, pregnant Wistar han rats (age of 9–11 weeks) were individually housed under the same standard laboratory conditions and food (4RF25, Mucedola SRL) and water were provided *ad libitum*. Subcutaneous injections of dexamethasone (Sigma) at 1 mg kg^{-1} (iuGC animals) or sesame oil (control group) were administered on gestation days 18 and 19. On postnatal day 21, male progeny was weaned and maintained under standard laboratory conditions. Male offspring derived from at least four different litters were used.

Mice. Male and female C57/Bl6 transgenic and non-transgenic mice (age of 2 months at the beginning of the tests) were housed at weaning in groups of five animals per cage. The progeny produced by mating D1-cre (Drd1a-cre, 262, Gensat) or D2-cre (Drd2-cre, ER44, Gensat) heterozygous transgenic male mice with wild-type C57/Bl6 females were genotyped at weaning by PCR. All animals were maintained under standard laboratory conditions: an artificial 12-h light/dark cycle (lights on from 08:00 to 20:00 hours), with an ambient temperature of 21 ± 1 °C and a relative humidity of 50–60%; the mice were given a standard diet (4RF25 during the gestation and postnatal periods, and 4RF21 after weaning, Mucedola SRL) and water *ad libitum*.

All behavioural experiments were performed during the light period of the light/dark cycle. Health monitoring was performed according to FELASA guidelines[47], 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 Veterinária. All

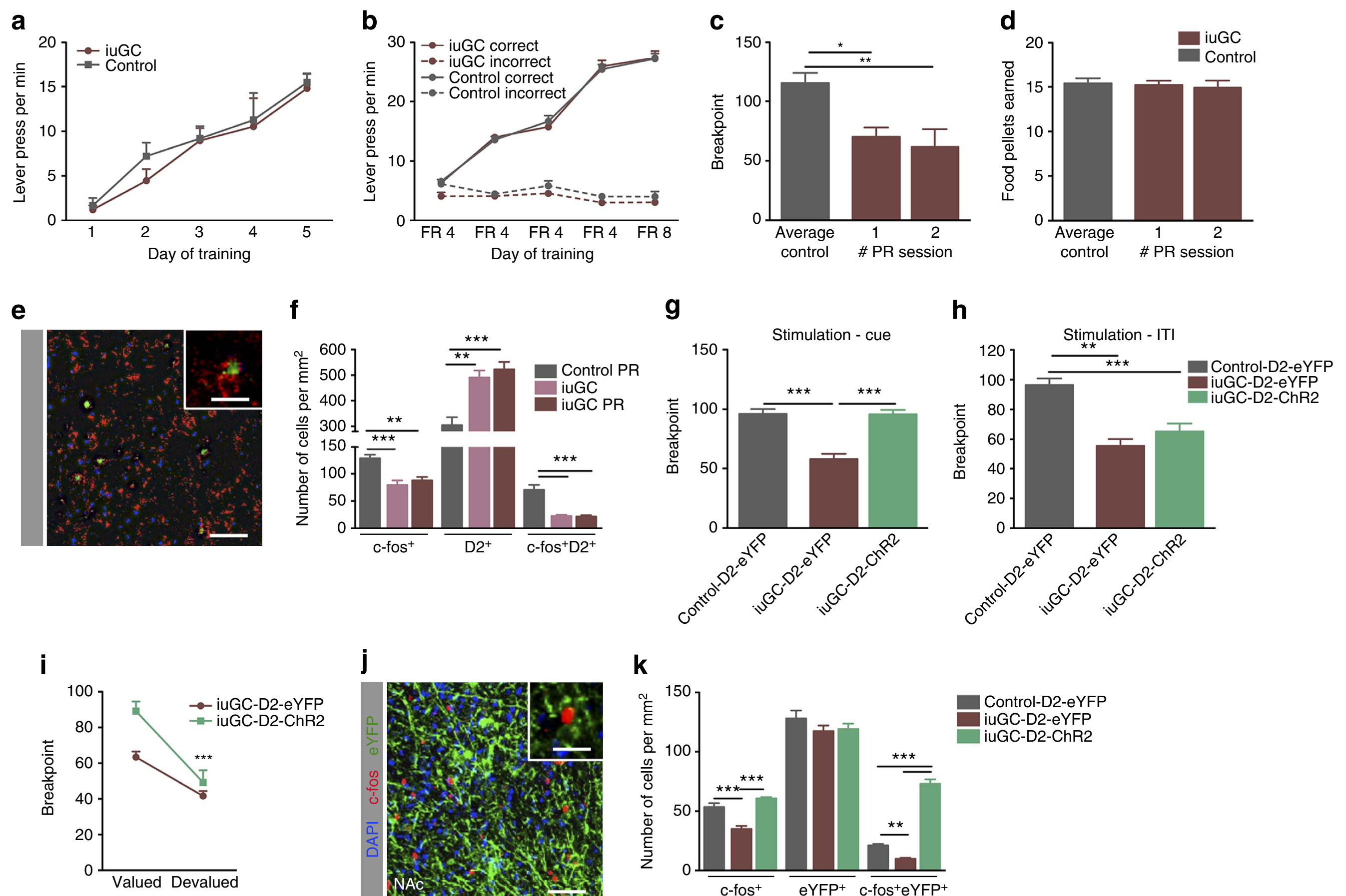

Figure 6 | NAc D2 neuronal activation rescues the behavioural deficits in a model of D2 dysfunction. (**a**) Continuous reinforcement (CRF) training sessions of the PR test shown as average number of lever presses per minute ($n=10$). (**b**) Fixed ratio (FR) training sessions of the PR test are shown as average number of lever presses per minute. (**c**) iuGC rats have a lower breakpoint when compared with control animals; though the total number of food pellets earned in the PR test sessions is similar (**d**). (**e**) Representative image of double immunofluorescence for c-fos and D2 in the NAc; scale bar: 200 µm; inset: 40 µm. (**f**) iuGC animals present a reduction in the recruitment of D2 neurons (c-fos$^+$/D2$^+$ cells) ($n=6$); iuGC animals present reduced total c-fos$^+$ cells and increased D2$^+$ cells in the NAc, regardless of having performed the PR task or not. (**g**) D2 optogenetic stimulation during cue exposure normalizes iuGC breakpoint (iuGC-D2-ChR2 rats) ($n_{control\text{-}D2\text{-}eYFP}=11$; $n_{iuGC\text{-}D2\text{-}ChR2}=15$; $n_{iuGC\text{-}D2\text{-}eYFP}=13$). (**h**) When the optical stimulation was given during inter-trial interval (ITI), no effect in motivation was observed ($n_{control\text{-}D2\text{-}eYFP}=7$; $n_{iuGC\text{-}D2\text{-}ChR2}=7$; $n_{iuGC\text{-}D2\text{-}eYFP}=5$). (**i**) Breakpoint before and after food devaluation is depicted as average of absolute value; both groups decrease lever presses in devalued condition. (**j**) Representative image of immunofluorescence for c-fos and eYFP in the NAc of an animal that performed the PR task. Scale bar: 200 µm; inset: 40 µm. (**k**) Quantification of c-fos$^+$ and eYFP$^+$ cells in the NAc after PR performance with optogenetic stimulation shows that ChR2 neurons are being activated ($n=6$); stimulated iuGC-D2-ChR2 animals present a substantial increase in the number of c-fos$^+$/eYFP$^+$ cells in comparison to stimulated D2-eYFP animals. Error bars denote s.e.m. $^*P\leq0.05$, $^{**}P\leq0.01$, $^{***}P\leq0.001$.

protocols were approved by the Ethics Committee of the Life and Health Sciences Research Institute.

Mouse genotyping. DNA was isolated from tail biopsy using the Citogene DNA isolation kit (Citomed). In a single PCR genotyping tube, the primers Drd1a F1 (5′-GCTATGGAGATGCTCCTGATGGAA-3′) and CreGS R1 (5′-CGGCAAACG GACAGAAGCATT-3′) were used to amplify the D1-cre transgene (340 bp), and the primers Drd2 (32108) F1 (5′-GTGCGTCAGCATTTGGAGCA-3′) and CreGS R1 (5′-CGGCAAACGGACAGAAGCAT-3′) to amplify the D2-cre transgene (700 bp). An internal control gene (lipocalin 2, 500 bp) was used in the PCR (LCN_1 (5′-GTCCTTCTCACTTTGACAGAAGTCAGG-3′) and LCN_2 (5′-CACATCTCATGCTGCTCAGATAGCCAC-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 ImageLab 4.1 (Bio-Rad).

Rat Behaviour and apparatus. Rats were placed and maintained on food restriction (~7 g per day of standard lab chow) to maintain 90% free-feeding weight. 45 mg food pellets (F0021; BioServ), used in the behavioural protocol, were placed in their home cages on the day before the first training session to familiarize the rats with the food pellets. Behavioural sessions were performed in operant chambers (Med Associates). Each chamber contained a central, recessed magazine that provided access to 45 mg food pellets (Bio-Serve) or 100 µl of sucrose solution (20% wt per vol in water) delivered by a pellet dispenser and a liquid dipper, respectively, two retractable levers with cue lights located above them that were located on each side of the magazine. Magazine entries were measured automatically by an infrared beam located at the entry of the magazine. A 1 kHz tone and an amplified white noise, each with a sound of 80 dB, where available as discrete auditory cues. A 2.8 W, 100 mA 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.

Rat PR schedule of reinforcement. All training sessions started with illumination of the house light that remained until the end of the session[18,48]. On the first training session (CRF; continuous reinforcement sessions) one lever was extended. The lever would remain extended throughout the session, and a single lever press would deliver a food pellet (maximum of 50 pellets earned within 30 min). In some cases, food pellets were placed behind the lever to promote lever pressing. After successful completion of the CRF training rats were trained to lever press on the opposite lever using the same training procedure. In the 4 following days the side of the active lever was alternated between sessions. Then, rats were trained to lever press one time for a single food pellet in a fixed ratio (FR) schedule consisting in 50 trials in which both levers are presented, but the active lever is signalled by the illumination of the cue light above it. FR sessions began with extension of both levers (active and inactive) and illumination of the house light and the cue light over the active lever. Completion of the correct number of lever press led to a pellet delivery, retraction of the levers and the cue light turning off for a 20 s ITI. Rats

were trained first with one lever active and then with the opposite lever active in separate sessions (in the same day). In a similar manner, rats were then trained using an FR4 reinforcement schedule for 4 days and a FR8 for 1 day. On the test day, rats were exposed to PR or FR experimental sessions (one session per day) according 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); which was previously shown to elicit stable behaviour[18,48]. FR4 sessions were identical to FR4 sessions described above. Food rewards were earned on an FR4 reinforcement schedule during FR sessions that consisted of 50 trials. PR sessions were identical to FR4 sessions except that 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 elapsed without completion of the response requirement in a trial.

For the sessions with optical stimulation/inhibition, before the PR session began, rats were connected to an opaque optical fibre in the NAc through previously implanted cannula guide. Optical fibres were removed after each session. At the beginning of each trial of the PR session—when the retractable levers are exposed to the animal together with the cue light—animals received an optical stimulation.

Optical stimulation was performed as follows: 473 nm; frequency of 40 Hz; 12.5 ms pulses over 1 s (50% duty cycle); 10 mW at the tip of the implanted fibre. Other alternative stimulation protocols were also used: (1) 473 nm, constant light delivery over 1 s, 10 mW at the tip of the implanted fibre; (2) frequency of 10 Hz, 50 ms pulses over 1 s (50% duty cycle), 10 mW at the tip of the implanted fibre; (3) frequency of 20 Hz, 20ms pulses over 1 s (50% duty cycle), 10 mW at the tip of the implanted fibre.

Optical inhibition was performed as follows: 589 nm; 10 s constant light; 15 mW at the tip of the implanted fibre. Other additional inhibition protocols were also used: (1) 589 nm; 10 s constant light, 5 mW at the tip of the implanted fibre) 589 nm, frequency of 40 Hz, 12.5 ms pulses over 10 s.

Rat PIT test. Pavlovian training comprised nine daily sessions in which each of two auditory conditioned stimulus (CS, tone and white noise) was paired with a different outcome (pellet or sucrose solution). Each of the CS exposure that lasted for 2 min was presented four times per session using a pseudo randomized order, with an ITI of 2 min in average. Data were plotted as the number of magazine visits performed during both CS presentations (16 min in total) and the number of magazine visits performed during the ITI (pre-CS period). Animals were then trained for the instrumental conditioning. Training was performed in two separate sessions per day (one session for each lever) and the order of training was alternated during days (average interval between the two sessions was 3 h). Each session finished after 30 rewards were delivered or 30 min had elapsed. In the first 2 days, lever pressing was in a CRF order. The probability of getting a reward decreased according to the following sequence: days 3–4, random ratio (RR) 5; days 5–6, RR10. The number of lever presses per minute per session was registered and plotted. Twenty-four hours later, subjects were placed in the operant chamber to test for PIT transfer with both levers inserted. After a baseline performance interval (BPI) that lasted for 8 min, four blocks of each auditory CS were presented randomly and lever presses were registered. During each stimulus presentation, lever presses were considered correct if it encoded the same reward as the audible sound. When encoding was different, the actions were considered incorrect. The number of lever presses performed during the test is plotted. Same—lever pressing on the lever that originates the same reward as the CS presented; dif—lever pressing on the lever that originates a different reward as the CS presented.

For the sessions with optical stimulation, before the PIT session began, rats were connected to an opaque optical fibre for optical stimulation in the NAc through previously implanted cannula guide. Optical fibres were removed after each session. At the beginning of each CS presentation, rats received an optical stimulation (473 nm; frequency of 40 Hz; 12.5 ms light pulses over 1 s (50% duty cycle); 10 mW at the tip of the implanted fibre).

Rat locomotor activity. Locomotor behaviour was investigated using the open field test. Briefly, rats were attached to an optical fibre connected to a laser (473 or 589 nm) and immediately placed in the centre of an arena (Med Associates) and their locomotion was monitored online over a period of 40 min. Optogenetic stimulation was given every 150 s with the following conditions: 40 Hz, 12.5 ms light pulses over 1 s; 10 mW at the tip of the implanted fibre. Optogenetic inhibition was given every 150 s with the following conditions: 10 s constant light delivery; 15 mW at the tip of the implanted fibre. Total distance travelled was used as indicator of locomotor activity.

The duration and the number of stimulations given (15 stimulations) of locomotor activity protocol were matched to the number of stimulations given during the PR test session.

Rat food consumption test. Food intake tests were conducted in a familiar chamber containing bedding on the floor in which rats had serial access to pre-weighed quantities of regular chow pellets (20–22 g; 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 20 g of regular chow followed by 20 min of access to 20–22 g of palatable food pellets and chow. Laser stimulation was given once each 60 s period (stimulation: 40 Hz, 12.5 ms pulses over 1 s, 10 mW at the tip of the implanted fibre; inhibition: 10 s constant light, 15 mW). Intake tests were 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 to form a baseline measurement). Chow and palatable food pellets were reweighed at the end of the test to calculate the amount consumed.

Mouse behaviour and apparatus. Mice were housed in groups of three animals per cage and placed and maintained on food restriction to maintain 90–95% free-feeding weight. 20 mg food pellets (F0071; BioServ), used in the behavioural protocol, were placed in their home cages on the day before the first training session to familiarize the mice with the food pellets. Behavioural sessions were performed in operant chambers (Med Associates). Each chamber contained a central, recessed magazine that provided access to 20 mg food pellets, delivered by a pellet dispenser, two ultra-light retractable levers with cue lights located above them that were located on each side of the magazine. Magazine entries were measured automatically by an infrared beam located at the entry of the magazine. A 2.8 W, 100 mA 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.

Mouse PR. Mice were initially trained to press the lever on a FR1 reinforcement schedule whereby a single lever press elicited the delivery of a food pellet to the receptacle. One lever was designated as 'active' (triggering delivery of food reward) and the allocation of right and left levers was counterbalanced between mice. Each trial was separated by a 5 s time-out. Each session lasted 30 min or until animals earned 30 pellets. After 9 days of FR1 training, mice performed 3 sessions of FR5. The mice were then trained in the PR schedule of reinforcement.
The response ratio schedule during PR testing was calculated using the following formula (rounded to the nearest integer): $=[5e^{(R*0.2)}]$-5, where R is equal to the number of food rewards already earned plus 1 (that is, next reinforcer). PR sessions lasted a maximum of 1 h. Failure to press the lever in any 10 min period resulted in termination of the session, which was not observed in any animal.

Light stimulation was given on one single session with the following stimulation protocol: 473 nm; frequency of 40 Hz, 12.5 ms pulses over 1 s (50% duty cycle), 10 mW at the tip of the implanted fibre. Other alternative stimulation protocol was also used: 473 nm; frequency of 20 Hz, 25 ms pulses over 1 s (50% duty cycle),10 mW at the tip of the implanted fibre.

Mouse locomotor activity. Locomotor behaviour was investigated using the open field test. Briefly, mice were attached to an optical fibre connected to a laser (473 nm) and immediately placed in the centre of an arena (Med Associates) and their locomotion was monitored online over a period of 40 min. Optogenetic stimulation was given every 150 s (15 stimulations) with the following conditions: 40 Hz, 12.5 ms light pulses over 1 s (50% duty cycle); 10 mW at the tip of the implanted fibre. Total distance travelled was used as indicator of locomotor activity.

Immunohistochemistry. For c-fos activation analysis, all animals were submitted to either the PIT or PR protocols described above. On the test day half of the animals of each group performed the test, whereas the other half did not perform the test. Rats that performed the test were sacrificed 90 min after initiation of testing and the rats that did not perform the test were sacrificed on the same day. Both groups were anaesthetised with pentobarbital (Eutasil) and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde. Brains were processed and sectioned coronally on a vibratome at a thickness of 50 μm. Briefly, free-floating sections were pretreated with 3% hydrogen peroxide, rinsed in phosphate-buffered saline, blocked with 5% fetal bovine serum for 2 h at room temperature and incubated overnight at room temperature with rabbit anti-c-fos (1:1,000, Ab-5, Merck Millipore) polyclonal antibody. Afterwards, sections were washed and incubated with the appropriate secondary biotinylated antibody for 2 h, processed with an avidin–biotin complex solution and detected with 0.5 mg ml^{-1} 3,3′-diaminobenzidine. Sections were washed and mounted on glass slides, air-dried, counterstained with hematoxylin and coverslipped with Entellan (Merck). Estimation of cell density was obtained by crossing cell number values with the corresponding areas, determined using an Olympus BX51 optical microscope and the StereoInvestigator software (Microbrightfield).

Immunofluorescence. 90 min after initiation of the PR test, rats were deeply anaesthetised with pentobarbital (Eutasil) and were transcardially perfused with 0.9% saline followed by 4% paraformaldehyde. Brains were removed and post-fixed in 4% paraformaldehyde. Coronal vibratome sections (50 μm) were incubated with the primary antibodies 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 mouse anti-GFP (1:200, ab1218, Abcam). Appropriate secondary fluorescent antibodies were used (1:500, Invitrogen). Finally, all sections were stained with 4′,6-diamidino-2-phenylindole (DAPI; 1 mg ml^{-1}). For each animal, positive cells within the brain regions of interest were analysed and cell counts were performed by confocal microscopy (Olympus FluoViewTMFV1000). As a notice, c-fos is mainly nuclear; c-fos^{+}/$D1^{+}$ ($D2^{+}$ or $ChAT^{+}$) cells were considered when D1, D2 or ChAT staining delimited c-fos staining. 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).

Constructs and virus preparation. eYFP, hChR2(H134R)-eYFP and eNpHR3.0-eYFP were cloned under the control of D2 dopamine receptor minimal promoter region (Drd2; ENSRNOG00000008428; Gene ID: 24318), which included 1,540 bp upstream of the first (non-coding) exon (kindly provided by Dr. Karl Deisseroth)[49]. Constructs were packaged in AAV5 serotype by the UNC Gene Therapy Center Vector Core (UNC). Cre-inducible AAV5/EF1a-DIO-hChR2(H134R)-eYFP was obtained directly from the UNC Center. AAV5 vector titres were $3.7–6 \times 10^{12}$ virus molecules per ml as determined by dot blot.

Surgery and cannula implantation. Rats were anaesthetised with 75 mg kg^{-1} ketamine (Imalgene, Merial) plus 0.5 mg kg^{-1} medetomidine (Dorbene, Cymedica). Virus was unilaterally injected into the NAc (coordinates from bregma, according to Paxinos and Watson[50]: + 1.2 mm anteroposterior (AP), + 1.2 mm mediolateral (ML), and -6.5 mm dorsoventral (DV)) (Supplementary Fig. 9). Rats were then implanted with an optic fibre (200 µm core fibre optic; Thorlabs) with 2.5 mm stainless steel ferrule (Thorlabs) using the injection coordinates (with the exception of DV: − 6.4 mm) that were secured to the skull using 2.4 mm screws (Bilaney) and dental cement (C&B kit, Sun Medical). Rats were removed from the stereotaxic frame, sutured and let to recover for two weeks before initiation of the behavioural protocols.

Mice were anaesthetised with 75 mg kg^{-1} ketamine (Imalgene, Merial) plus 1 mg kg^{-1} medetomidine (Dorbene, Cymedica). Virus was unilaterally injected into the NAc (coordinates from bregma, according to Paxinos and Franklin[51]: + 1.4 mm AP, + 0.85 mm ML, and − 4.0 mm DV) (Supplementary Fig. 9). Mice were then implanted with an optic fibre (200 µm core fibre optic; Thorlabs) with 2.5 mm stainless steel ferrule (Thorlabs) using the injection coordinates (with the exception of DV: − 3.9 mm) that were secured to the skull using dental cement (C&B kit, Sun Medical). Mice were removed from the stereotaxic frame, sutured and let to recover for two weeks before initiation of the behavioural protocols.

In vivo single-cell electrophysiology. Four weeks post surgery, rats were anaesthetised with urethane (1.44 g kg^{-1}, Sigma). The total dose was administered in three separate intraperitoneal injections, 15 min apart. Adequate anaesthesia was confirmed by the lack of withdrawal responses to hindlimb pinching. A recording electrode coupled with a fibre optic patch cable (Thorlabs) was placed in the NAc (coordinates from bregma, according to Paxinos and Watson[50]: + 1.2 mm AP, + 1.2 mm ML, and − 6.0 to − 7.0 mm DV), using a stereotaxic frame (David Kopf Instruments) with non-traumatic ear bars (Stoeling). Other recording electrodes with fibre optic attached were placed in the VP (coordinates from bregma, according to Paxinos and Watson[50]: 0 to − 0.12 mm AP, + 2.3 to + 2.5 mm ML, and − 7 to − 7.6 mm DV) and in the VTA (coordinates from bregma, according to Paxinos and Watson[50]: − 5.3 mm AP, + 0.9 mm ML, and − 7.5 to − 8.3 mm DV) (Supplementary Fig. 9). Single neuron activity was recorded extracellularly with a tungsten electrode (tip impedance 5–10 Mat 1 kHz) and data sampling was performed using a CED Micro1401 interface and Spike 2 software (Cambridge Electronic Design). The DPSS 473 nm laser system or DPSS 589nm laser system (CNI), controlled by a stimulator (Master-8, AMPI) were used for intracranial light delivery. Fibre optic output was pre-calibrated to 10-15 mW from the fibre tip before implantation. Optical stimulation was performed as follows: 473 nm; frequency of 20 Hz, 30 Hz, 40 Hz or 50 Hz; 12.5 ms pulses over 1 s, 10 mW. Optical inhibition was performed as follows: 589 nm; 10 s constant light, 15 mW.

Firing rate histograms were calculated for the baseline (60 s before stimulation), stimulation period and after stimulation period (60 s after the end of stimulation). The cells were considered as responsive or not responsive to the stimulation on the basis of their firing rate change with respect to the baseline period. Neurons showing a firing rate increase or decrease by more than 20% from the mean frequency of the baseline period were considered as responsive[52]. Spike latency was determined by measuring the time between half-peak amplitude for the falling and rising edges of the unfiltered extra-cellular spike.

Statistical analysis. Normality tests were performed for all data analysed. Statistical analysis between two groups was made using Student's *t*-test. Two-way ANOVA was used when appropriate. Bonferroni's *post hoc* multiple comparisons was used for group differences determination.

To capture the factorial structure of brain region measures, an Exploratory Factor Analysis was performed through Principal Axis Factoring forced to three dimensions with oblimin rotation. The internal reliability of the three dimensions was measured with Cronbach's alpha. The three dimensions were then computed using the regression method. Pearson correlations were calculated to obtain the strength of the relation between the test score and the three brain regions scores. Next, a multiple linear regression was performed to identify significant predictors of test score (using the three factors as predictors).

Results are presented as mean ± s.e.m. All statistical analysis was performed using IBM SPSS Statistics (v.22) and results were considered significant for $P \leq 0.05$.

Data availability. The data that support the findings of this study are available from the corresponding author on request.

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Author contributions

A.J.R., C.S.-C. and N.S. developed the concept and designed experiments. C.S.-C. performed and analysed most of the experiments. B.C. and S.B. assisted in the behavioural procedures and all animal experimentation. A.D.-P. performed and assisted in the analysis of electrophysiological recordings. L.P. assisted in the acquisition of the confocal images. P.C. assisted in the statistical analysis. C.S.-C. and A.J.R wrote the paper. All authors discussed and revised the manuscript.

Additional information

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

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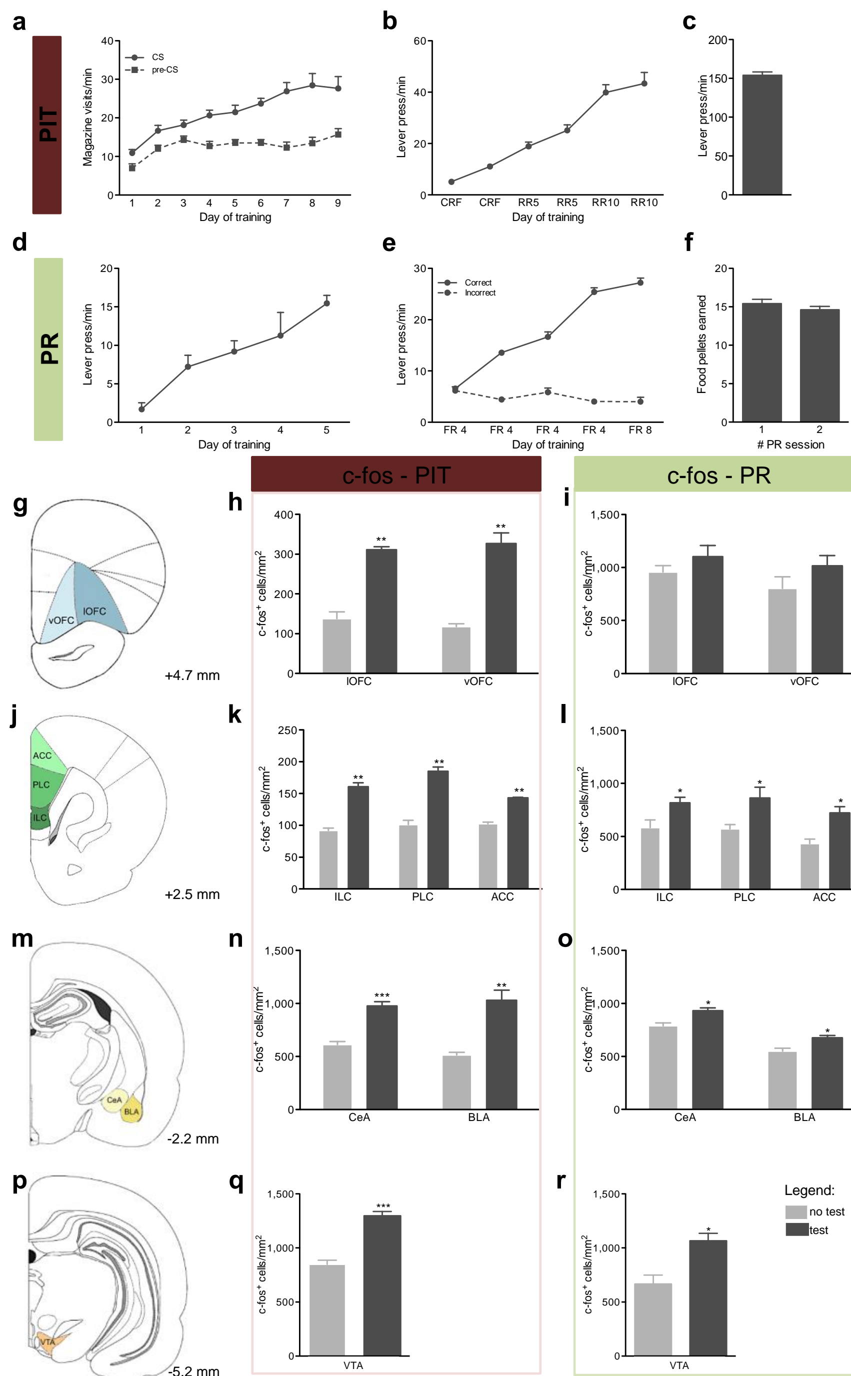
Supplementary Information

Activation of D2 dopamine receptor-expressing neurons in the nucleus accumbens increases motivation

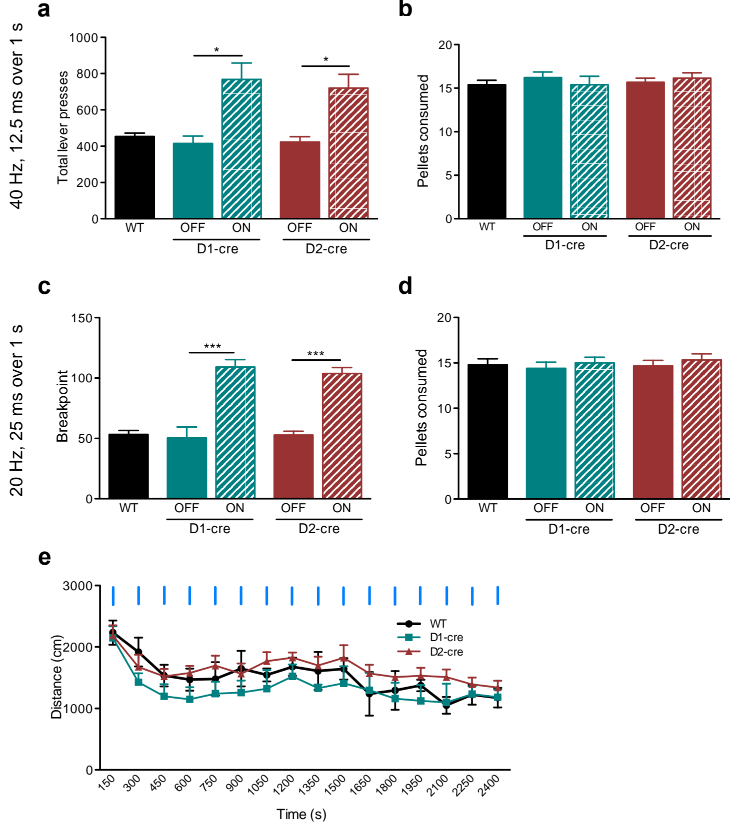
Nature communications (2016) 7, 11829

Supplementary Figure 1



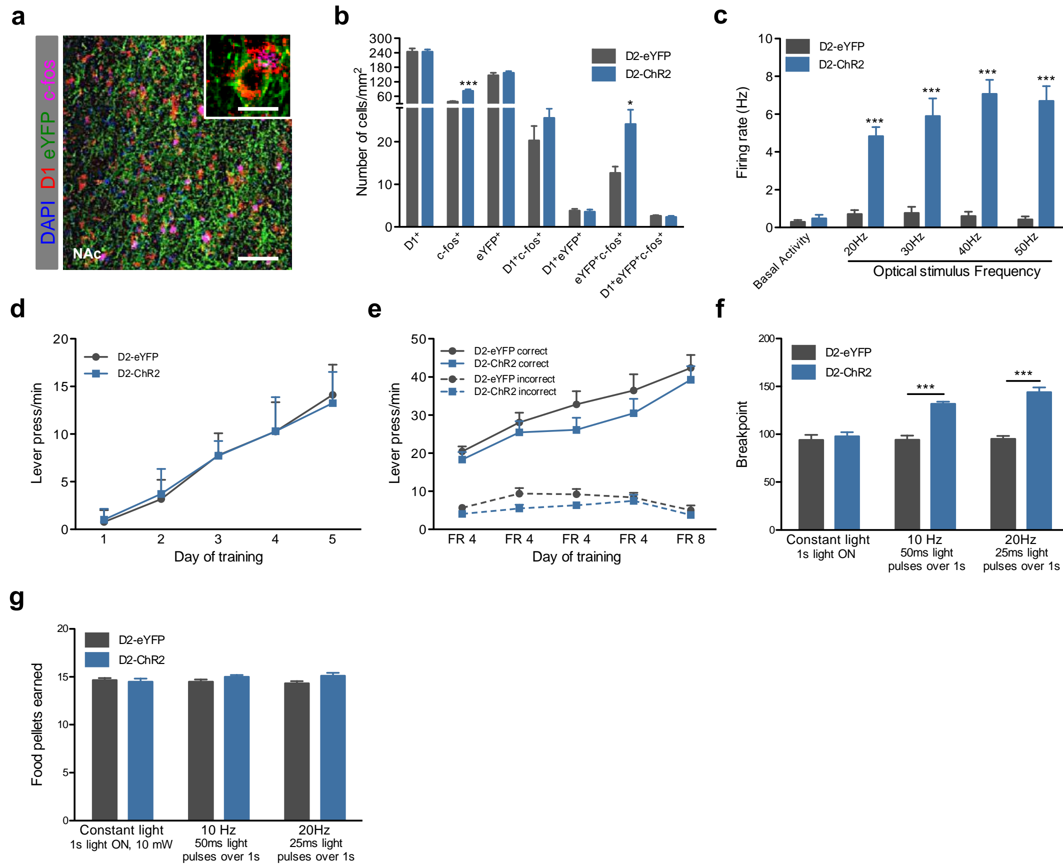
Supplementary Figure 1. Performance in two distinct motivation-dependent tasks – PIT and PR tests – and c-fos immunostaining. (**a**) Pavlovian conditioning shown as mean magazine visits per minute. (**b**) Instrumental conditioning depicted as the number of lever presses per minute performed in each training day. (**c**) Baseline performance, shown as total number of lever presses. (**d**) Continuous reinforcement (CRF) training sessions of the PR test, presented as average number of lever presses per minute. (**e**) Fixed ratio (FR) training sessions of the PR test, depicted as average number of lever presses per minute. (**f**) Average number of food pellets earned for each PR session. n=10. (**g-r**) PIT and PR induced a general activation of all cortical and limbic regions analyzed: lOFC and vOFC (**h, i**); ACC, PLC and ILC (**k, l**); BLA and CeA (**n, o**); and VTA (**q, r**) (n=6). Representative images of coronal brain sections are shown – lOFC and vOFC (**g**); ACC, PLC and ILC (**j**); BLA and CeA (**m**); and VTA (**p**); numbers represent distance in millimeters to bregma. Error bars denote s.e.m. *p≤0.05, **p≤0.01, ***p≤0.001.

Supplementary Figure 2



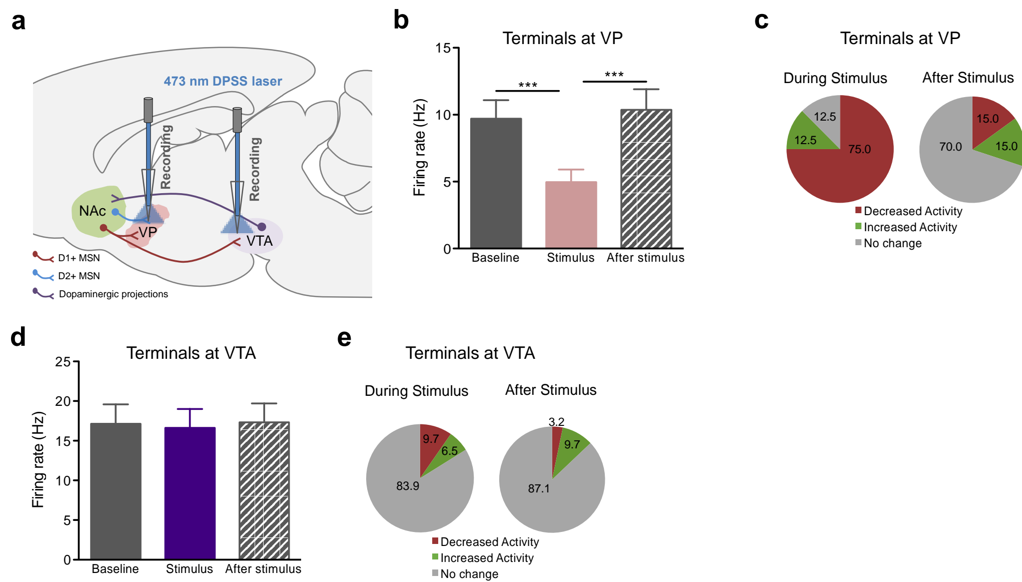
Supplementary Figure 2. Optogenetic activation of NAc D1 and D2 neurons in mice. (**a, c**) Different protocols of optogenetic stimulation of accumbal D1 and D2 neurons increased mice breakpoint ($n_{D1\text{-}cre}$=5; $n_{D2\text{-}cre}$=6). (**b, d**) No differences in the total number of pellets earned during session were found. (**e**) No major effects in locomotion of D1-cre- or D2-cre-stimulated versus non-stimulated rats. Error bars denote s.e.m. *p≤0.05, ***p≤0.001.

Supplementary Figure 3



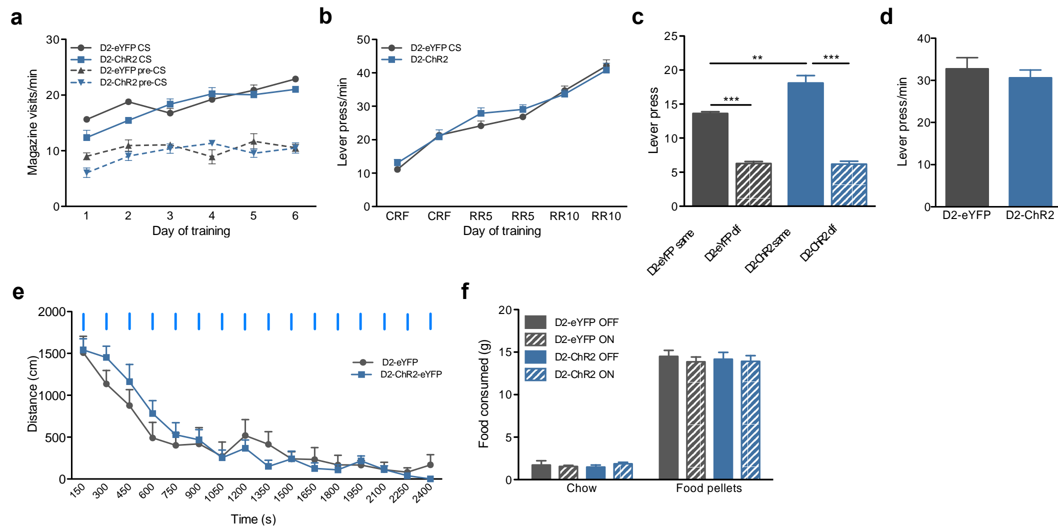
Supplementary Figure 3. Optogenetic activation of NAc D2 neurons. (**a**) Representative immunofluorescence of the NAc using antibodies against dopamine receptor D1, YFP and c-fos in an animal injected with AAV5-D2-hChR2(H134R)-eYFP that performed the PR test. Inset shows a D1$^+$/YFP$^+$ cell (bottom) and a D1$^+$/YFP$^+$/c-fos$^+$ cell (top). Scale bar: 200 μm; inset: 40 μm. (**b**) Cell counting revealed that only few D1$^+$ cells co-express YFP (average of 3.6 YFP$^+$ cells out of 244.4 D1$^+$ cells, which correspond to 1.5% of D1$^+$ neurons). No statistical difference in the activation of D1$^+$ neurons was found between stimulated D2-eYFP and D2-ChR2 animals (20.3 vs 25.62 D1$^+$/c-fos$^+$ cells; p=0.224). (**c**) D2-ChR2 opsin is functional at different stimulation frequencies (12.5 ms pulses for 1s; n_{rats}=5, $n_{neurons}$>15). (**d**) CRF training sessions of the PR schedule, shown as average number of lever presses per minute ($n_{D2\text{-}ChR2\text{-}eYFP}$=16; $n_{D2\text{-}eYFP}$=13). (**e**) FR training sessions of the PR test, presented as average number of lever presses per minute ($n_{D2\text{-}ChR2\text{-}eYFP}$=16; $n_{D2\text{-}eYFP}$=13). (**f**) Different protocols of optogenetic stimulation of accumbal D2 neurons reliably increased breakpoint with the exception of constant stimulation ($n_{D2\text{-}ChR2\text{-}eYFP}$=8; $n_{D2\text{-}eYFP}$=6). (**g**) Total number of food pellets earned in the PR session with different stimulation parameters. Error bars denote s.e.m. *p≤0.05, ***p≤0.001.

Supplementary Figure 4



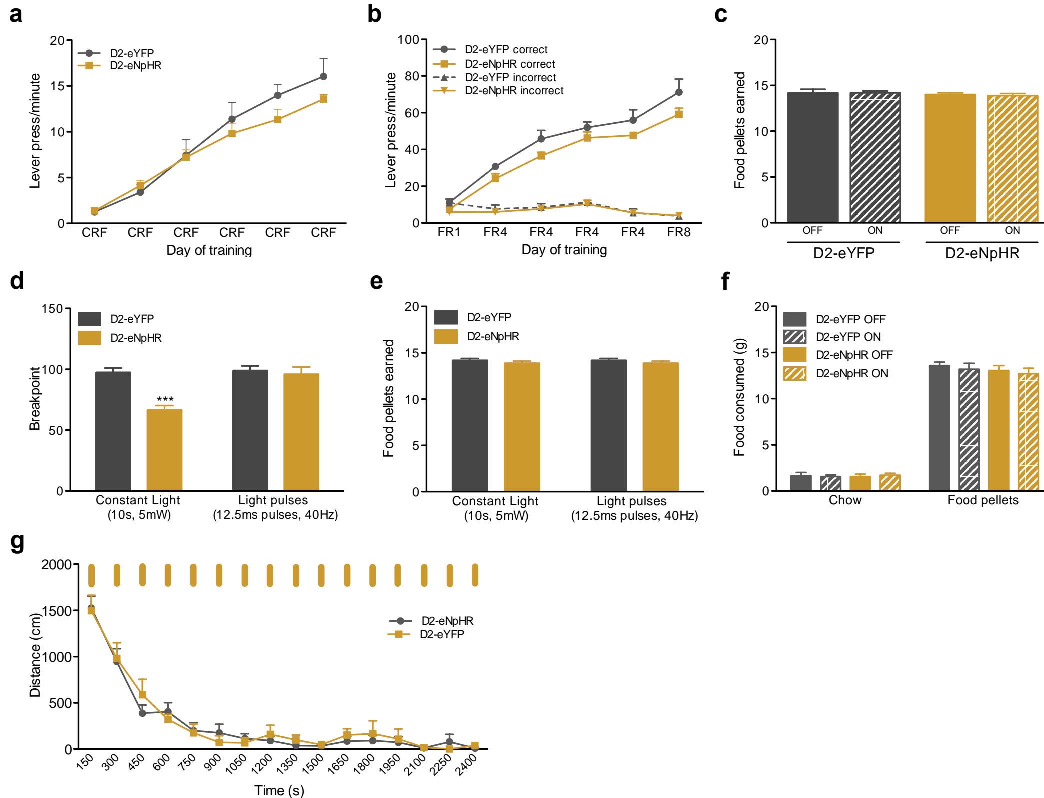
Supplementary Figure 4. Optogenetic modulation of NAc D2 terminals in the ventral pallidum (VP) and ventral tegmental area (VTA). (**a**) Schematic representation of the *in vivo* single-cell electrophysiological recording experiments with optogenetic stimulation performed in NAc terminals at VP (and VTA). (**b-c**) Stimulation of NAc terminals at the VP decreases global VP firing rate (n=30 cells), similarly to stimulation of cell bodies (depicted in Fig. 3). (**d-e**) When stimulation was performed in the VTA, no major effect in firing rate was found (n=31 cells), consistent with the absence of direct inputs to this brain region. Error bars denote s.e.m. ***p≤0.001.

Supplementary Figure 5



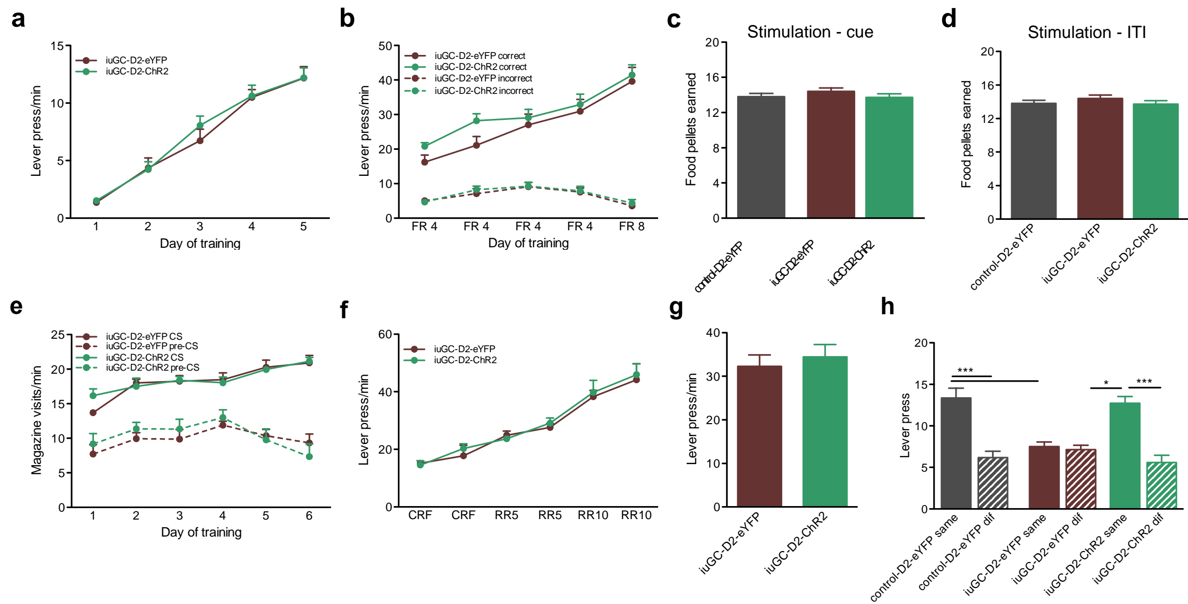
Supplementary Figure 5. Effects of NAc optogenetic activation of D2 neurons in Pavlovian-to-Instrumental Transfer (PIT) and other behaviors. (**a**) Pavlovian conditioning of the PIT test, shown as mean magazine visits per minute ($n_{D2\text{-}ChR2}$=7; $n_{D2\text{-}eYFP}$=5). (**b**) Instrumental conditioning of the PIT paradigm, depicted as number of lever presses per minute. (**c**) PIT is enhanced after NAc D2 activation (40 Hz, 12.5 ms pulses for 1 s) during conditioned stimulus (CS) presentation in D2-ChR2 animals. *Same: lever associated with CS; dif: other lever.* (**d**) Total number of lever presses in the baseline period of the PIT test. (**e**) No differences were observed in locomotor activity of D2-ChR2 stimulated versus D2-YFP-stimulated rats (40 Hz, 12.5 ms pulses during 1 s; 15 stimulations). (**f**) No effects in free feeding behavior – regular chow or sugar pellets in stimulated animals. Error bars denote s.e.m. **p≤0.01, ***p≤0.001.

Supplementary Figure 6



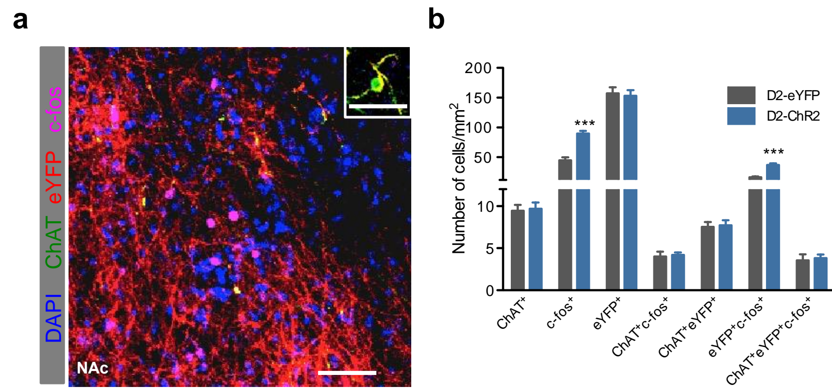
Supplementary Figure 6. Inhibition of accumbal D2 neurons - electrophysiological and behavioral correlates. (**a**) Continuous reinforcement (CRF) training sessions of the PR test is presented as average number of lever presses per minute ($n_{D2\text{-}eYFP}$=5; $n_{D2\text{-}eNpRH}$=8). (**b**) Fixed ratio (FR) training sessions of the PR test are shown as average number of lever presses per minute. (**c**) Average number of food pellets earned in the PR session with the following optical stimulation: 10 s constant light at 15 mW during cue exposure. (**d**) Breakpoint of rats that received different D2-eNpHR optical stimulation parameters Constant light at 5 mW stimulation protocol also decreased breakpoint, but not pulsed stimulation. (**e**) Average number of food pellets earned in the PR session with different optical stimulation parameters. (**f**) No major effects of D2 neuronal inhibition in free food consumption or locomotion (**g**). Error bars denote s.e.m. ***p≤0.001.

Supplementary Figure 7



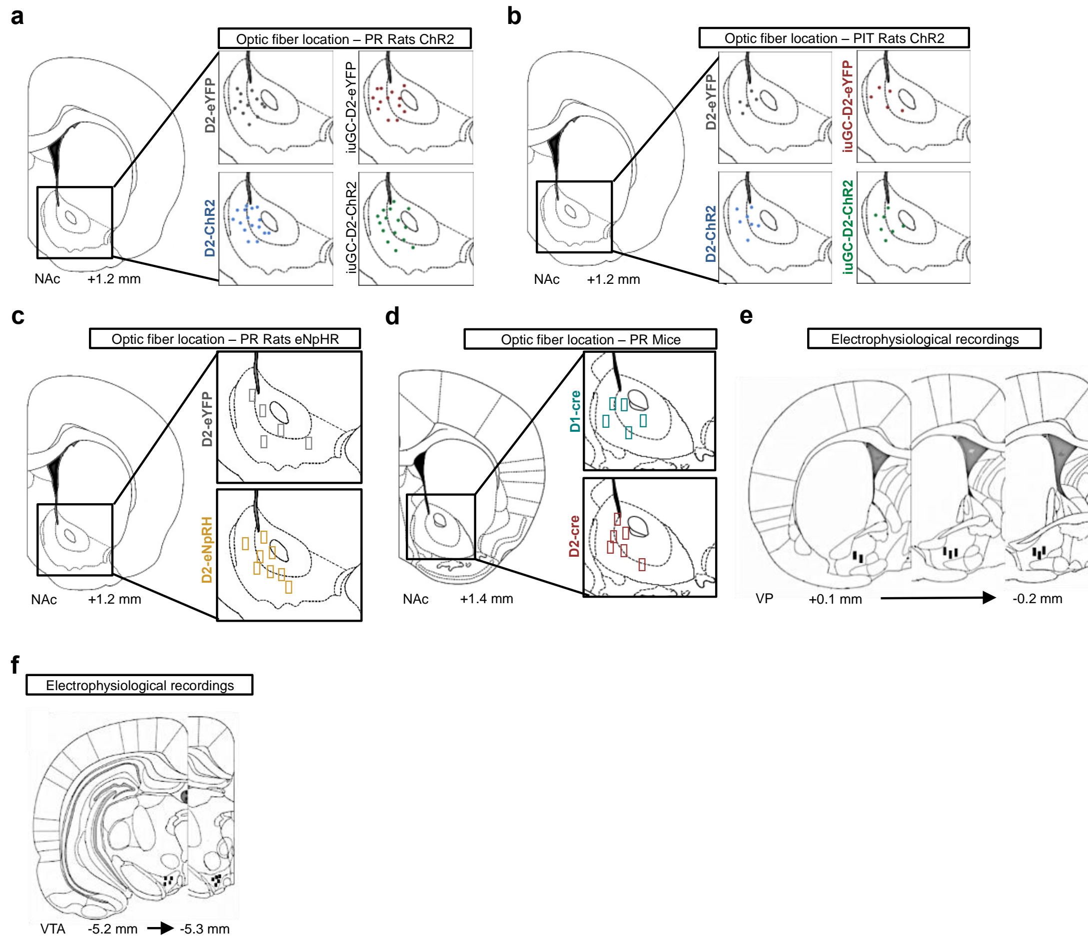
Supplementary Figure 7. Effects of NAc optogenetic activation of D2 neurons in behavior of control and iuGC animals. (**a**) Continuous reinforcement (CRF) and (**b**) fixed ratio (FR) training sessions of the PR test of iuGC-D2-ChR2 and iuGC-D2-eYFP groups. Data is presented as average number of lever presses per minute ($n_{iuGC\text{-}D2\text{-}ChR2}$=15; $n_{iuGC\text{-}D2\text{-}eYFP}$=13). (**c**) Average number of food pellets earned in the PR session of iuGC-D2-ChR2 and iuGC-D2-eYFP groups with stimulation during cue exposure. (**d**) Average number of food pellets earned in the PR session with stimulation during ITI ($n_{control\text{-}D2\text{-}eYFP}$=5; $n_{iuGC\text{-}D2\text{-}ChR2}$=7; $n_{iuGC\text{-}D2\text{-}eYFP}$=5). (**e**) Pavlovian conditioning of the PIT paradigm, shown as mean magazine visits per minute. (**f**) Instrumental conditioning of the PIT paradigm, depicted as the number of lever presses per minute. (**g**) Baseline performance of the PIT test session. (**h**) Stimulation of D2 neurons rescued PIT impairment in iuGC animals. Error bars denote s.e.m. *p≤0.05, ***p≤0.001.

Supplementary Figure 8



Supplementary Figure 8. Quantification of $ChAT^{+}$/c-fos^{+}/$eYFP^{+}$ neurons after D2 optogenetic stimulation during PR test. (**a**) Representative triple immunofluorescence using antibodies against YFP, ChAT and c-fos in the NAc of an animal injected with AAV5-D2-ChR2-eYFP. Inset shows a rare YFP^{+}/$ChAT^{+}$/c-fos^{-} cell; scale bar: 200 µm; inset: 40 µm. (**b**) There are very few $ChAT^{+}$ cells per mm^2 of NAc (average of 9.5 cells/mm^2). There is no significant increase in the number of YFP^{+}/$ChAT^{+}$/c-fos^{+} in stimulated D2-ChR2 animals in comparison to stimulated D2-YFP animals. Error bars denote s.e.m. ***$p \leq 0.001$.

Supplementary Figure 9



Supplementary Figure 9. Confirmation of optic fiber and electrode localization in different experiments. (**a**) Schematic representation of optic fiber placement in the NAc of rats that performed the progressive ratio (PR) paradigm with D2-ChR2 optogenetic activation. (**b**) Schematic representation of optic fiber placement in the NAc of rats that performed the Pavlovian-to-Instrumental Transfer (PIT) test with D2-ChR2 optogenetic activation. (**c**) Schematic representation of optic fiber placement in the NAc of rats that performed the PR paradigm with D2-eNpHR vector. (**d**) Schematic representation of optic fiber placement in the NAc of D1-cre and D2-cre mice + DIO-ChR2 that performed the PR test. (**e**) Schematic representation of recording electrode placement in the VP and VTA (**f**). Numbers represent distance in millimeters to bregma.

Supplementary Table 1

Supplementary Table 1. Factorial structure of brain regions analyzed by Exploratory Factor Analysis (EFA) through Principal Axis Factoring forced to three dimensions. Internal reliability assessed with Cronbach's alpha.

	Communalities		Factors (F)		
	Initial	**Extraction**	**F1[a.]: Cortical regions**	**F2[b.]: NAc**	**F3[c]: Other Limbic regions**
ILC	0.951	0.974	0.998		
lOFC	0.874	0.883	0.973		
PLC	0.958	0.946	0.933		
vOFC	0.822	0.799	0.849		
ACC	0.932	0.905	0.727		
NAcc	0.881	0.891		0.980	
NAcs	0.856	0.931		0.829	
CeA	0.800	0.822			0.952
VTA	0.802	0.839		0.315	0.694
BLA	0.650	0.556	-0.305		0.558
	Cronbach's Alpha		0.838	0.933	0.838

ACC: anterior cingulate cortex; BLA: basolateral amygdala; CeA: central amygdala; ILC: infralimbic cortex; lOFC: lateral orbitofrontal cortex; NAcc: nucleus accumbens core; NAcs: nucleus accumbens shell; PLC: prelimbic cortex; vOFc: ventral orbitofrontal cortex; VTA: ventral tegmental area.

[a.] F1, Factor 1

[b.] F2, Factor 2

[c.] F3, Factor 3

CHAPTER 3.2

Nucleus accumbens D2-dependent increase in motivation: impact of selective manipulation of local microcircuits

Manuscript in preparation

Nucleus accumbens D2-dependent increase in motivation: impact of selective manipulation of local microcircuits

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Abstract

The nucleus accumbens (NAc) is a key element of the reward system, playing a central role in reinforcement and motivation. Around 95% of the NAc neurons are GABAergic medium spiny neurons (MSNs), mainly divided into those expressing dopamine receptor D1 (D1R) and those expressing dopamine receptor D2 (D2R). The remaining 5% are comprised by different subtypes of interneurons.

Previous work from our group showed that NAc D2R-expressing neuronal optogenetic activation/inhibition enhances/decreases motivation in a progressive ratio task. In order to understand what type of neurons were involved in this behavioural effect, we combined optogenetic activation of NAc D2R-expressing neurons with *in loco* pharmacological delivery of specific neurotransmitter antagonists.

Our results suggest that the increase in motivation is dependent on cholinergic-controlled release of dopamine in the NAc, which requires both D1R and D2R, and is also dependent on glutamatergic signalling.

Introduction

The reward circuit has been simplistically described as dopaminergic projections arising from the ventral tegmental area (VTA) to the nucleus accumbens (NAc)[1–3]. Evidence in animal models and humans showed that the motivational aspects of reward processing are greatly mediated by this neural circuit[4–6].

The most abundant NAc neuronal population are the medium spiny neurons (MSNs; 95%), that are typically divided into those that express the dopamine receptor D1 (D1R, D1-MSNs), and those that express the dopamine receptor D2 (D2R, D2-MSNs)[7–9]. In addition to dopaminergic inputs from the VTA, these MSNs receive dense monosynaptic glutamatergic innervation from the medial prefrontal cortex (mPFC), hippocampus and amygdala[10–12], and project directly to the VTA through the direct pathway (mediated exclusively by D1-MSNs), or indirectly via the ventral pallidum (VP; mediated by both D1- and D2-MSNs)[7,13–16]. Additionally, MSNs are known to synapse with each other[17], maintaining GABAergic accumbal activity under a very balanced control.

The remaining 5% of NAc neurons are local interneurons. These include large tonically active cholinergic interneurons (CIN) that receive glutamatergic inputs from the cortex and thalamus, GABAergic inputs from MSNs, and dopaminergic inputs from the substantia nigra (SN)/VTA[18,19], and that synapse primarily onto MSNs[18–20]. Another subclass of striatal interneurons are the fast spiking (FS) GABAergic interneurons that express parvalbumin. FS interneurons receive inhibitory inputs from other interneurons and from the globus pallidus (GP), as well as excitatory glutamatergic inputs from the thalamus and cortex. Their primary inhibitory synapses occur onto MSNs[21–23], providing a strong feed-forward inhibition that shapes the firing activity of MSNs[24–26]. A final and distinct group of interneurons are the low threshold spiking (LTS) interneurons, which express somatostatin (SOM), neuropeptide Y (NPY), and nitric oxide synthase (NOS)[27–30]. LTS neurons are implicated in long-term plasticity, as they receive glutamatergic inputs from the thalamus and cortex, and synapse directly onto MSNs[28,31].

Other types of interneurons have been identified, namely tyrosine hydroxylase (TH) interneurons[32,33] and calretinin (CR) interneurons[34–36], but their exact function in striatal signalling remains largely unexplored.

Importantly, both cholinergic and GABAergic interneurons play a crucial role in NAc activity and respond to salient stimuli[21,37], modulating reward-dependent behaviours. For example, recent studies showed that selective activation of cholinergic interneurons increases dopamine release in

the NAc[38,39], which may synergize to promote motivated behaviours. Additionally, CINs of primates were shown to acquire a stereotypical, synchronous pause in their activity in response to cues that predict saliency to rewards in operant tasks[19,21,40]. Interestingly, these responses were shown to be highly dependent on inputs from dopaminergic projections to the striatum, as the pause response disappears if dopaminergic transmission is interrupted[41–43]. In addition, GABAergic interneurons exert powerful monosynaptic inhibition of the MSNs through multiple perisomatic synapses[44,45]. Remarkably, the electrophysiological activity of this class of interneurons is largely uncoordinated during cue and reward phases of conditioning, and they only fire during choice excursion[46], suggesting that inhibition from GABAergic interneurons contributes to reward retrieval.

In the past years, compelling data supported a role for D1-MSNs in positive reinforcement[47–49], while D2-MSNs have mostly been associated with aversion[48,49]. Nonetheless, recent anatomical, electrophysiological and behavioural data emerged in opposition to this dichotomous hypothesis. For example, whereas the division of direct and indirect neurons based on the respective expression of D1R and D2R in dorsal striatum appears to be accurate, this does not hold true for the ventral striatum (NAc) since the indirect pathway contains a mixture of D1-MSNs and D2-MSNs [16,50]. This implies that both NAc D1- and D2-MSNs can inhibit or disinhibit thalamic activity, which can have clear repercussions in behaviour. Indeed, a previous study from our group showed that activation of NAc D1- and D2-MSNs is positively correlated with motivation and that optogenetic activation of either neuronal population is sufficient to increase motivation in different reward-related tasks (Chapter 3.1). In spite of this, it is important to refer that other neuronal populations might also be directly recruited by this optogenetic manipulation since some classes of interneurons also express D2R[51–54]. Moreover, activation of D2-MSNs can also affect D1-MSN activity and other striatal interneurons[55,56]. So, in order to better understand the impact of D2-MSN optogenetic activation in different NAc neuronal populations and their contribution for motivation enhancement, we combined optogenetic activation of D2R-expressing neurons with pharmacological delivery of specific antagonists locally in the NAc. With this approach we show that D2R-expressing neuronal activation effects in motivation appear to be dependent on cholinergic-mediated dopamine release from the VTA, which required both D1R and D2R. In addition, we show that the behavioural effects are dependent on NAc glutamatergic signalling through NMDA and AMPA receptors.

Material and methods

Animals

Male Wistar Han rats (age of 2-3 months old at the beginning of the experiments) were used. Animals were maintained under standard laboratory conditions with a 12 h light/dark cycle (lights on from 8am to 8pm) and room temperature of 21±1°C, with relative humidity of 50-60%; rats were individually housed after optical fibre implantation; standard diet (4RF21, Mucedola SRL) and water were given *ad libitum*, until the beginning of the behavioural experiments, in which animals switched to food restriction to maintain 85% of their initial body weight.

Behavioural manipulations occurred during the light period of the light/dark cycle. Health monitoring was performed according to FELASA guidelines[57], 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 animals experimenters were certified by the Portuguese regulatory entity – Direção Geral de Veterinária. All protocols were approved by the Ethics Committee of the Life and Health Sciences Research Institute (ICVS).

Behavior

Subjects and apparatus

Rats were habituated to 45 mg food pellets (F0021; BioServ), which were used as reward during the behavioural protocol, one day prior to training initiation. Behavioural sessions were performed in operant chambers (Med Associates) that contained a central, recessed magazine to provide access to 45 mg food pellets (Bio-Serve), two retractable levers with cue lights located above them that were positioned on each side of the magazine. Chamber illumination was obtained through a 2.8 W, 100 mA light positioned at the top-center of the wall opposite to the magazine. The chambers were controlled by a computer equipped with Med-PC software (Med Associates).

Progressive ratio (PR) schedule of reinforcement

The behavioural protocol for rats was the same as previously described (Chapter 3.1). In short, animals were trained to lever press to obtain food pellets in a continuous reinforcement (CRF) schedule for 6 consecutive days. Animals were then trained in a fixed-ratio (FR) 1 for one

day, an FR 4 for 4 days and an FR 8 for 1 day. On the test day, rats performed one session of progressive-ratio (PR). PR sessions ended after 15 min elapsed without completion of the response requirement in a trial. The breakpoint and number of pellets consumed during test sessions were analyzed.

For the sessions with optical stimulation, before the PR session began, rats were connected to an opaque optical fiber through previously implanted guided cannulas. Optical fibers were removed after each session. At the beginning of each trial of the PR session – when the retractable levers are exposed to the animal together with the cue light that indicates the active lever– animals received an optical stimulation.

Optical stimulation was performed as follows: 473 nm; frequency of 40 Hz; 40 light pulses of 12.5 ms each (50% duty cycle); 10 mW at the tip of the implanted fiber.

Constructs and virus preparation

eYFP and hChR2(H134R)-eYFP were cloned under the control of the D2R minimal promoter region as described before[58]. Constructs were packaged in AAV5 serotype by the UNC Gene Therapy Center Vector Core (UNC). AAV5 vector titters were 3.7-6x10^{12} viral molecules/ml as determined by dot blot.

Surgery and cannula implantation

Rats were anesthetized with 75 mg kg^{-1} ketamine (Imalgene, Merial) plus 0.5 mg kg^{-1} medetomidine (Dorbene, Cymedica). Virus was unilaterally injected into the NAc (coordinates from bregma, according to Paxinos and Watson[59]: +1.2mm anteroposterior, +1.2 mm mediolateral, and -6.5 mm dorsoventral). Rats that performed the PR with only optical stimulation were implanted with an optic fibre (200μm diameter) attached to a 2.5 mm ferrule (Thorlabs), and rats that performed the PR test with both optical stimulation and local administration of antagonists were implanted with opto-fluid cannulas (Doric Lenses) using the injection coordinates (except for the dorsoventral: -6.4 mm) that were secured to the skull using 2.4 mm screws (Bilaney) and dental cement (C&B kit, Sun Medical). Rats were removed from the stereotaxic frame, sutured and let to recover for two weeks before initiation of the behavioural trainings.

Immunofluorescence (IF)

90 minutes after initiation of the PR test, rats were deeply anesthetized with pentobarbital

(Eutasil) and were transcardially perfused with 0.9% saline followed by 4% paraformaldehyde. Brains were removed and post-fixed in 4% paraformaldehyde. Coronal vibratome sections (50 μm) were incubated with the primary antibody rabbit anti-c-fos (1:500, ABE457, Millipore) and goat anti-GFP (1:500, ab6673, Abcam). Appropriate secondary fluorescent antibodies were used (1:1000, Invitrogen). Finally, all sections were stained with 4′,6-diamidino-2-phenylindole (DAPI; 1 mg ml^{-1}). Images were collected and analysed by confocal microscopy (Olympus FluoViewTMFV1000). Cell counts were normalized to the brain region area.

Drugs

All drugs were delivered 10 minutes before animals were placed in the operant chambers to perform the PR test, through an opto-fluid system chronically implanted in the NAc. Injections were performed using a 5 μL gastight seringe (Hamilton), attached to the implanted injection cannula of the rats through 22-gauge tubing, at a constant rate of 1 μL per minute.

The drugs used in experimental procedures were: R(+)-SCH-23390 hydrochloride (D1R antagonist, 0.25 μg in 0.5 μL of saline, Sigma); (S)-(-)-Sulpiride (D2R antagonist, 0.2 μg in 1 μL, Sigma); Scopolamine Hydrobromide (muscarinic receptor antagonist, 25 μg in 1 μL, Sigma); Mecamylamine hydrochloride (nicotinic receptor antagonist, 22.5 μg in 1 μL, Sigma); Dihydro-β-erythroidine hydrobromide (DhβE, nicotinic β2-containing receptor antagonist, 0.7 μg in 1 μL, Tocris); D(−)-2-Amino-5-phosphonopentanoic acid (AP5, NMDA receptor antagonist, 0.1 μg in 1 μL, Sigma); 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, AMPA receptor antagonist, 0.5 μg in 1 μL, Sigma); CGP-55845 hydrochloride ($GABA_B$ receptor antagonist, 44 ng in 0.5 μL, Sigma); 1(S),9(R)-(-)-Bicuculine methobromide ($GABA_A$ receptor antagonist, 75 ng in 0.5 μL, Sigma).

Statistical analysis

Normality tests were performed for all data analysed. Statistical analysis between two groups was made using Student's *t*-test. One-way or two-way analysis of variance (ANOVA) was used when appropriate. Bonferroni's *post hoc* multiple comparisons were used for group differences determination.

Results are presented as mean ± s.e.m. All statistical analysis was performed using GraphPad Prism (v5.0) and IBM SPSS Statistics (v.22) and results were considered significant for $p \leq 0.05$.

Results

Optogenetic stimulation of D2R-expressing neurons in the NAc increases motivation

We injected in the NAc of rats a construct carrying the expression of channelrohodopsin (ChR2) and enhanced yellow fluorescent protein (eYFP) under the control of the D2R minimal promoter region – pAAV-D2R-hChR2(H134R)-eEYFP – or the control virus – pAAV-D2R-eYFP (Fig. 1a). Next, animals were submitted to a progressive ratio (PR) test to evaluate their willingness to work for food, a direct measure of individual motivation[60]. During training of both CRF and FR schedules all groups acquired similar levels of lever pressing (Fig. 1b,c). Optical stimulation of D2R-expressing neurons in the NAc (473 nm light; 40 light pulses of 12.5 ms at 40 Hz), occurring at the same time as the unconditioned stimulus (light above the active lever) was presented (Fig. 1d), induced a significant increase in the breakpoint of D2-ChR2 rats in comparison with D2-eYFP stimulated rats (Fig. 1e; $t_{15}=7.7$, $p<0.001$, 35% increase), in agreement with our previous findings (Chapter 3.1). Importantly, this increase in motivation was not the reflex of differences in the number of food pellets earned during the session (Fig. 1f).

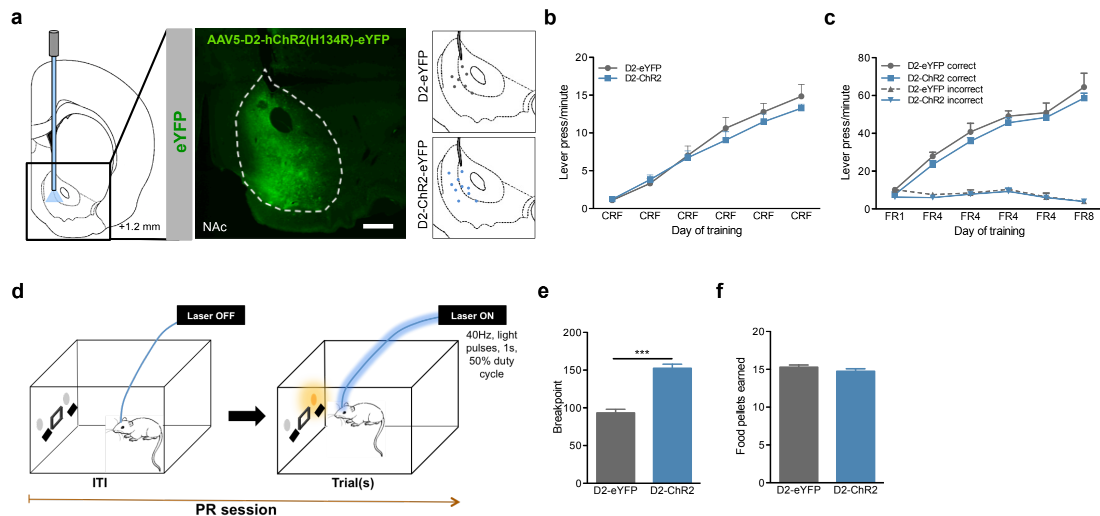

Figure 1. Optical stimulation of D2R-expressing neurons in the NAc increases motivation. (**a**) We unilaterally injected AAV5-D2-ChR2(H134R)-eYFP (D2-ChR2 group) or AAV5-D2-eYFP (D2-eYFP group) in the NAc of rats. Expression of eYFP was confirmed by YFP immunostaining; scale bar = 500 μm; cannula placement was confirmed for all animals. Four weeks after virus injection, animals were tested in the PR test. (**b**) CRF training sessions of the PR test, presented as average number of lever presses per minute. (**c**) FR training sessions of the PR test, depicted as average number of lever presses per minute. (**d**) Schematic representation of the PR test session – rats received optical stimulation (473 nm, 40 Hz, 40 light pulses of 12.5 ms, 10 mW at the tip of the patch cable) at the beginning of each trial (when cue light above the active lever was turned ON). ITI: inter-trial interval. (**e**) Optogenetic activation of accumbal D2R-expressing neurons during cue exposure strongly enhanced breakpoint (35% increase). (**f**) Average number of food pellets earned during the PR session is similar between groups. ($n_{D2\text{-}eYFP}$=7; $n_{D2\text{-}ChR2}$=8). Error bars denote s.e.m. ***p≤0.001.

Optogenetic stimulation of D2R-expressing neurons in the NAc: effects in other brain regions

In order to confirm the impact of optogenetic activation of NAc D2R-expressing neurons in the recruitment of brain regions of the reward circuit, we examined the pattern of expression of c-fos, an immediate early gene, after the PR test with D2 accumbal optogenetic stimulation (Fig. 2a). As expected, stimulated D2-ChR2 rats showed a significant increase in c-fos staining in the NAc, comparing with stimulated control D2-eYFP rats (Fig. 2b; t_8=4.2, p=0.0031).

We also evaluated the number of c-fos$^+$ cells in downstream regions: the VP, which is directly innervated by NAc D2-MSNs; the VTA, which is innervated solely by NAc D1-MSNs[16], but receives VP-projections[61], and the SN, which is mainly innervated by dorsal striatum MSNs[62]. A trend for increased number of c-fos$^+$ cells in the VP was found (Fig. 2c; t_8=1.9, p=0.0917). A significant

increase in VTA c-fos^{+} cells was observed (Fig. 2d; t_8=3.7, p=0.006), differently to the SN that presented no significant changes (Fig. 2e; t_8=1.1, p=0.2977).

We also analyzed c-fos expression in different medial prefrontal cortex (mPFC) sub-regions, because there is a dual processing system in which the NAc receives primary inputs from different cortical regions including orbital prefrontal cortex, insular cortex, and cingulate cortex[63,64], but also modulates these regions indirectly through a VP-thalamic output[64]. Interestingly, all sub-regions of the mPFC analyzed showed an increase in the number of c-fos^{+} cells: anterior cingulate cortex (ACC t_8=4.0, p=0.004), prelimbic cortex (PLC, t_8=2.7, p=0.028) and infralimbic cortex (ILC, t_8=2.7, p=0.029) (Fig. 2f).

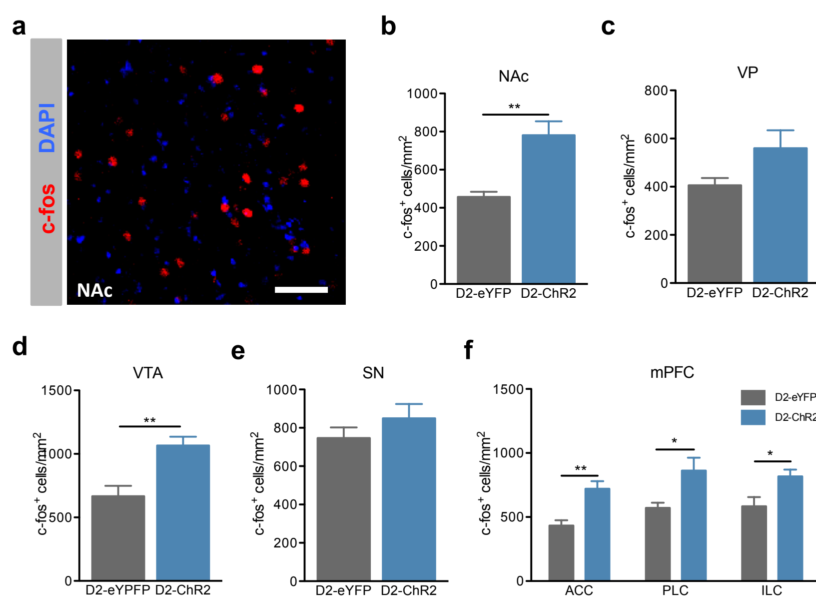

Figure 2. c-fos expression in different brain regions upon optogenetic stimulation of NAc D2R-expressing neurons. (**a**) Representative example of immunostaining for c-fos in the NAc of a D2-ChR2 rat; scale bar = 50 μm. The number of c-fos positive cells per square millimeter is represented for the (**b**) NAc, (**c**) VP, (**d**) VTA, (**e**) SN, and (**f**) different sub-regions of the mPFC. Upon NAc D2 optical stimulation, there is an increase in c-fos^{+} cells in the NAc, VTA and mPFC sub-regions. A tendency for increased activation of VP was also found. No changes in SN c-fos^{+} cells were observed between control and D2-ChR2 animals. (n=5/group). Error bars denote s.e.m. *p≤0.05, **p≤0.01.

NAc: nucleus accumbens; VP; ventral pallidum; VTA: ventral tegmental area; SN: substantia nigra; mPFC: medial prefrontal cortex; ACC: anterior cingulate cortex; PLC: prelimbic cortex; ILC: infralimbic cortex.

D2-induced increase in breakpoint is dependent on $GABA_B$ receptor activation

MSNs have a GABAergic nature and synapse with each other in the NAc[17]. In addition, local interneurons provide an additional source of GABA that also controls MSN activity[21,44,65].

To further understand the impact of GABAergic transmission in the control of D2-mediated enhancement of motivation, we used hybrid fibre optic cannulas, which allow dual delivery of drugs and light in the same place. Immediately before behavioural testing and optogenetic activation of D2R-expressing neurons, we injected in the NAc either a $GABA_A$ receptor antagonist (bicuculline, 75 ng) or a $GABA_B$ receptor antagonist (CGP 55845 hydrochloride, CGP-55845, 44ng). $GABA_A$ antagonist did not alter breakpoint of control animals, though there was a trend for increased number of lever presses (D2-eYFP vs D2-eYFP+Bicuculline, $t_{12}=2.0$, $p=0.07$, 15% increase); it also failed in altering the breakpoint of D2-stimulated animals (D2-ChR2 vs D2-ChR2+Bicuculline, $t_{14}=1.3$, $p=0.2188$) (Fig. 3a).

On the other hand, administration of the $GABA_B$ antagonist induced a significant increase in the breakpoint of control rats (Fig. 3c; D2-eYFP vs D2-eYFP+CGP-55845, $t_{12}=2.8$, $p=0.019$, 19% increase). Interestingly, administration of CGP-55845 also led to an additional increase in the breakpoint of D2-stimulated animals (Fig. 3c; $t_{12}=5.9$, $p<0.001$, 16% increase). No differences between groups in the number of pellets earned during the PR session were found (Fig. 3b,d).

These results suggest that GABA signalling arising from MSNs or local interneurons act as a break that controls the levels of motivational drive in a $GABA_B$ dependent manner. Moreover, the positive effects of D2 activation in motivation appears not to be dependent on local GABA release by D2R-expressing neurons.

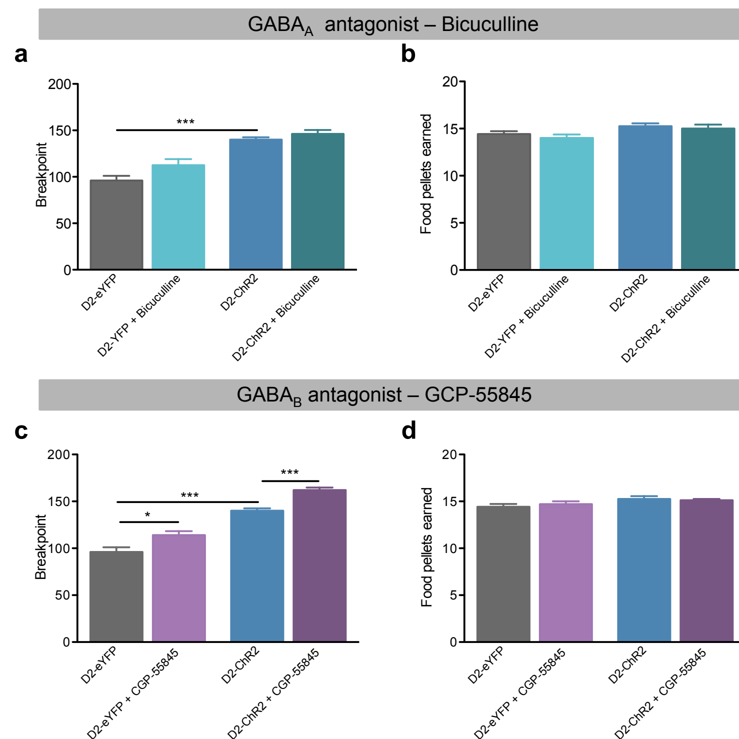

Figure 3. Effects of GABA receptor antagonists in the breakpoint of D2-stimulated animals. Ten minutes before the performance of PR test, rats received local injection of either bicuculline (75 ng) or GCP-55845 (44 ng) in the NAc. (**a**) Optogenetic activation of D2R-expressing neurons strongly enhanced breakpoint; bicuculline had no effect in control or D2-stimulated animals. (**b**) Average number of food pellets earned for the PR session is similar between groups. ($n_{D2\text{-}eYFP}$=7; $n_{D2\text{-}eYFP+Bicuculline}$=7; $n_{D2\text{-}ChR2}$=8; $n_{D2\text{-}ChR2+Bicuculline}$=8). (**c**) CGP-55845 treatment increased the breakpoint of control group (D2-eYFP) and D2-stimulated animals. (**d**) Average number of food pellets earned for the PR session is similar between groups. ($n_{D2\text{-}eYFP}$=7; $n_{D2\text{-}eYFP+CGP\text{-}55845}$=7; $n_{D2\text{-}ChR2}$=8; $n_{D2\text{-}ChR2+CGP\text{-}55845}$=8). Error bars denote s.e.m. * $p \leq 0.05$, ***$p \leq 0.001$.

Cholinergic modulation of D2-induced increase in motivation

In addition to GABAergic modulation, MSNs activity is tightly controlled by cholinergic interneurons, which are able to modulate dopamine release from VTA terminals in the NAc[38,66,67], promoting behavioural conditioning[68].

Using a similar approach as above, we injected in the NAc a combination of muscarinic (mAChR) and nicotinic acetylcholine (ACh) receptor (nAChR) antagonists prior to PR paradigm (scopolamine, sco, 25μg; mecamylamine, mec, 22.5 μg, respectively). Blockade of cholinergic signalling had no major effect in the motivation of control animals; however, it abolished the motivation enhancement effect induced by optogenetic activation of the D2R-expressing neuronal population (Fig. 4a; D2-ChR2 vs D2-ChR2 scop+mec, $F_{(7,56)}$=15.6, $p<0.001$, 26% decrease).

Further studies using either one of the antagonists revealed that this blockage of D2-driven improvement in motivation was mediated by nAChR but not mAChR (Fig. 4a; D2-ChR2 vs D2-ChR2+mec, $t_{16}=8.7$, $p<0.001$, 34.3% decrease; D2-ChR2 vs D2-ChR2+sco, $t_{16}=2.0$, $p=0.0611$, 11% decrease). No differences between groups in the number of pellets earned during the session were found (Fig. 4b).

In the NAc, MSNs only express mAChR (M1 and M4)[69] and not nAChR[70–72], which are usually expressed in VTA dopaminergic terminals[70,72–75] and GABAergic interneurons[44,76]. In fact, tonic striatal ACh is able to promote dopamine release through specific beta2-subunit-containing (β2*)-nAChR receptors in the VTA terminals[77–79]. Thus, we injected DhβE (specific antagonist of β2*-nAChR, 0.7 μg) in the NAc of rats before performing the PR test. We observed that stimulated D2-ChR2+DhβE rats had a significantly lower breakpoint when compared with stimulated D2-ChR2 rats (Fig. 4c; $t_{14}=5.7$, $p<0.001$, 20.8% decrease), similar to control group. No effect of DhβE administration in control animals was found in the breakpoint (Fig. 4c; $t_{12}=1.9$, $p=0.0846$) or in the number of pellets earned (Fig. 4d) during the session.

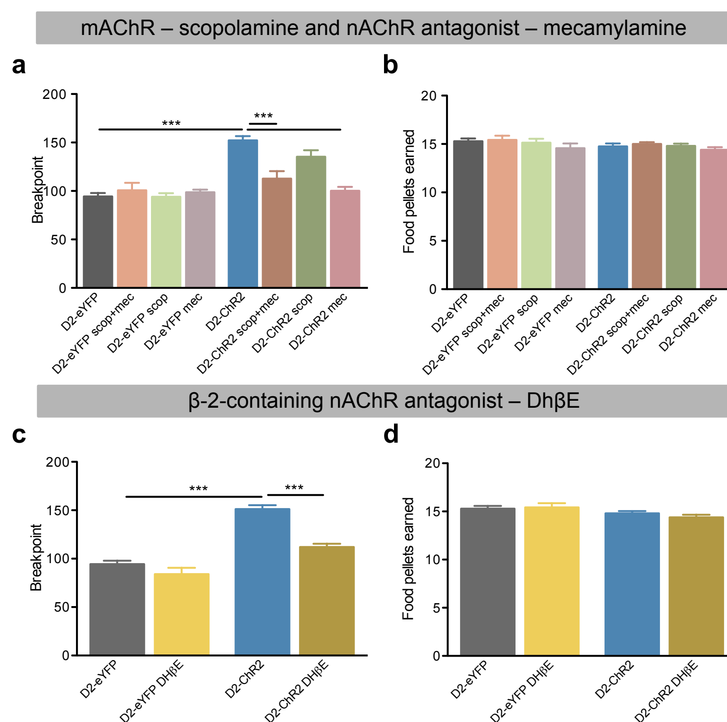

Figure 4. The increase in breakpoint caused by NAc D2 stimulation is dependent on local cholinergic activity. (**a**) Ten minutes before the performance of PR test, rats received local injection of scopolamine (sco; 25 μg), mecalamyne (mec; 22.5 μg) or sco+mec in the NAc; optogenetic activation of D2R-expressing neurons strongly enhanced breakpoint; this effect is lost with administration of a combination of muscarinic and nicotinic antagonists. This cholinergic dependence is mediated solely by nicotinic receptors since mec normalized breakpoint, but not sco.

(**b**) Average number of food pellets earned for the PR session is similar between groups. ($n_{D2\text{-}eYFP}$=7; $n_{D2\text{-}eYFP\ sco+mec}$=7; $n_{D2\text{-}eYFP\ sco}$=7; $n_{D2\text{-}eYFP\ mec}$=7; $n_{D2\text{-}ChR2}$=8; $n_{D2\text{-}ChR2\ sco+mec}$=8; $n_{D2\text{-}ChR2\ sco}$=8; $n_{D2\text{-}ChR2\ mec}$=8). (**c**) The increase in the breakpoint induced by optogenetic activation of NAc D2R-expressing neurons is absent with DHβE local administration; DhβE is a specific antagonist for β2*-nAChR located in VTA dopaminergic terminals. (**d**) Average number of food pellets earned for the PR session is similar between groups. $n_{D2\text{-}eYFP}$=7; $n_{D2\text{-}eYFP\ DH\beta E}$=7; $n_{D2\text{-}ChR2}$=8; $n_{D2\text{-}ChR2\ DH\beta E}$=8. Error bars denote s.e.m. ***p≤0.001.

Enhancement of motivation by D2 optogenetic activation requires dopamine signaling through D1R and D2R

The previous results suggested that D2 optogenetic activation modulates ACh release that in turn acts in the VTA dopaminergic terminals, eventually leading to dopamine release. Thus, we next tried to clarify the role of D1R and D2R in this process. To do so, we injected in the NAc R(+)-SCH-23390 hydrochloride (SCH-23390 0.5 μg; D1R antagonist), or sulpiride (0.2 μg; D2R antagonist) before performance of PR test. After, animals performed the PR test with optogenetic stimulation of D2R-expressing neurons (Fig. 5a).

Interestingly, D1R or D2R antagonist administration induced a significant reduction in the breakpoint of control animals (D2-eYFP) (Fig. 5a, SCH-23390: t_{12}=2.2, p=0.049, 20.5% decrease; Fig. 5c, sulpiride: t_{12}=3.2, p=0.008, 23.7% decrease). Additionally, pharmacological inhibition of D1R or D2R abolished the increase in motivation induced by NAc D2 optogenetic activation (Fig. 5a; D2-ChR2 vs D2-ChR2+SCH-23390: t_{18}=8.9, p<0.001, 34% decrease; Fig. 5c, D2-eYFP vs D2-eYFP+sulpiride: t_{12}=3.2, p=0.008, 46.7% decrease), suggesting that the behavioural improvement is dependent on both types of dopamine receptors signalling.

None of the pharmacological treatments changed the number of pellets earned in the PR session (Fig. 5b, d).

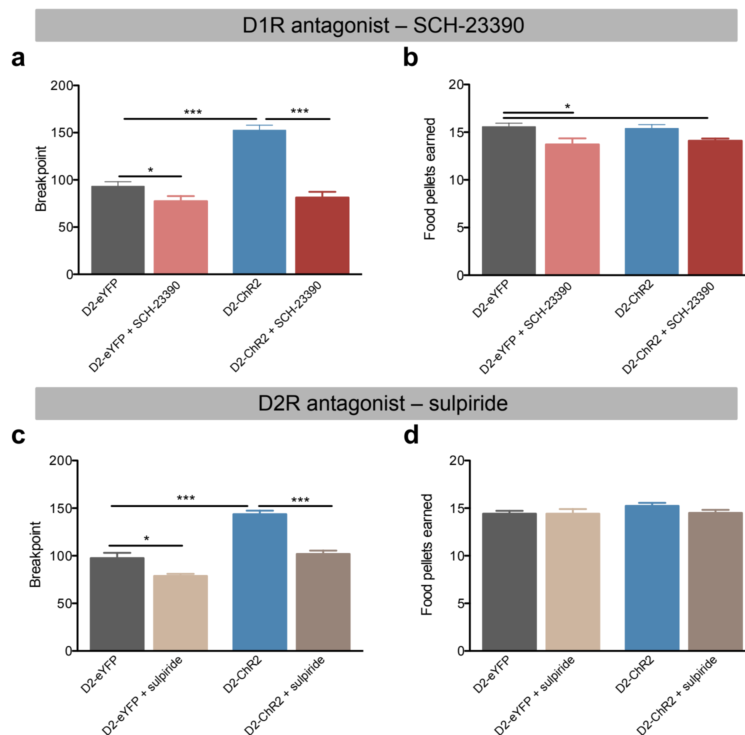

Figure 5. The increase in breakpoint caused by NAc D2 stimulation is dependent on both D1R and D2R. Ten minutes before PR testing, rats received local injection of either SCH-23390 (0.25 µg) or sulpiride (0.2 µg) in the NAc. (**a**) Optogenetic activation of D2R-expressing neurons strongly enhanced breakpoint that is lost with local administration of D1R antagonist. (**b**) Average number of food pellets earned for the PR session is similar between groups. $n_{D2\text{-}eYFP}=7$; $n_{D2\text{-}eYFP+SCH\text{-}23390}=7$; $n_{D2\text{-}ChR2}=8$; $n_{D2\text{-}ChR2+SCH\text{-}23390}=8$. (**c**) Optogenetic activation of D2R-expressing neurons significantly increases breakpoint that is lost with local administration of D2R antagonist. (**d**) Average number of food pellets earned for the PR session is similar between groups. $n_{D2\text{-}eYFP}=7$; $n_{D2\text{-}eYFP+sulpiride}=7$; $n_{D2\text{-}ChR2}=8$; $n_{D2\text{-}ChR2+sulpiride}=8$. Error bars denote s.e.m. * $p \leq 0.05$, *** $p \leq 0.001$.

The increase in breakpoint caused by D2 stimulation is dependent on glutamatergic activity

The NAc receives strong glutamatergic projections from the mPFC, amygdala and hippocampus[7,13–15], and the release of glutamate within this brain region plays a fundamental role in reward processing[80–83]. In addition, our results showed a significant activation of all mPFC regions upon D2 accumbal optogenetic manipulation.

Therefore, we administered a specific NMDA receptor antagonist (AP5, 0.1 µg) or a specific AMPA receptor antagonist (CNQX, 0.5 µg) in the NAc prior to behavioural testing with optogenetic manipulation (Fig. 6). No effect was observed in control animals' breakpoint after administration of CNQX (Fig. 6a; D2-eYFP vs D2-eYFP+CNQX, $t_{12}=0.03$, $p=0.97$) or AP5 (Fig. 6c; D2-eYFP vs D2-eYFP+AP5, $t_{12}=0.8$, $p=0.43$). However, inhibition of NAc AMPA receptors abolished the positive

effects of D2 neuronal activation in the breakpoint (Fig. 6a; D2-ChR2 vs D2-ChR2+CNQX, t_{14}=2.7, p=0.0194, 15% decrease). Accordingly, inhibition of NMDA receptors also reverted the effects of D2 neuronal activation in motivation (Fig. 6c; D2-ChR2 vs D2-ChR2+AP5, t_{14}=5.8, p<0.001, 19.4% decrease).

No differences in the number of pellets earned during the PR session were observed with either treatment (Fig. 6b,d).

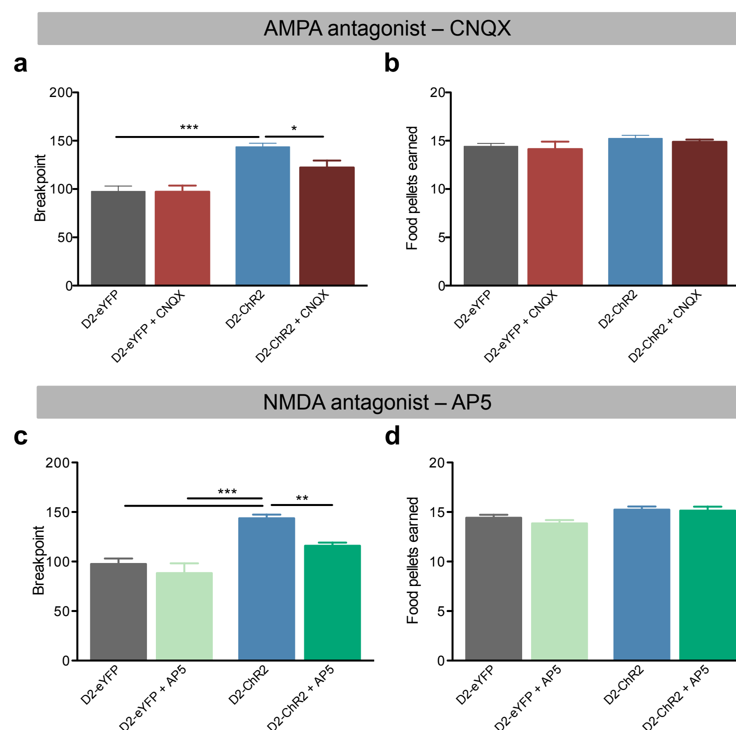

Figure 6. Effects of glutamate receptors antagonists in the breakpoint of D2-stimulated animals. Ten minutes before the performance of PR test, rats received local injection of either AMPA (CNQX; 0.5 µg) or NMDA receptors antagonists (AP5; 0.1 µg) in the NAc. (**a**) Administration of AMPA receptor antagonist normalizes breakpoint of D2-stimulated animals. (**b**) Average number of food pellets earned for the PR session is similar between groups. ($n_{D2\text{-}eYFP}$=7; $n_{D2\text{-}eYFP+CNQX}$=7; $n_{D2\text{-}ChR2}$=8; $n_{D2\text{-}ChR2+CNQX}$=8). (**c**) Optogenetic activation of D2R-expressing neurons strongly enhanced breakpoint; this effect is lost with AP5 treatment. (**d**) Average number of food pellets earned for the PR session is similar between groups. ($n_{D2\text{-}eYFP}$=7; $n_{D2\text{-}eYFP+AP5}$=7; $n_{D2\text{-}ChR2}$=8; $n_{D2\text{-}ChR2+AP5}$=8). Error bars denote s.e.m. ** p≤0.01, ***p≤0.001.

Discussion

Local microcircuits play an important role in striatal function. Here, we show that activation of D2R-expressing neurons during cue exposure increases willingness to work in a PR test, and that a concerted action of different neurotransmitter systems is required for this behavioural effect.

We first evaluated the impact of GABAergic transmission since GABAergic MSNs highly synapse with each other in the NAc[17], providing a weak lateral inhibitory network (feedback inhibition)[65,84–86]. This MSN-MSN reciprocal regulation mainly occurs in a $GABA_A$ receptor mediated manner[86,87]. Our results suggest that the D2-driven enhancement in motivation is not dependent on GABAergic signalling, since neither $GABA_A$ nor $GABA_B$ antagonists normalized this behavioural effect. However, we do observe an additional increase in the breakpoint of both control and D2-stimulated animals upon $GABA_B$ antagonist administration in the NAc, suggesting increased motivational drive.

Paradoxically, though MSNs express $GABA_B$ receptors, application of exogenous $GABA_B$ agonists does not lead to any MSN electrophysiological effect[88]; nonetheless, it significantly supresses glutamatergic inputs onto MSNs via a pre-synaptic mechanism[88,89]. Thus, it is plausible that the blockade of presynaptic $GABA_B$ receptors could enhance corticothalamic glutamatergic release, enhancing motivational drive. Indeed, apart from classic studies showing that NAc cue-evoked firing is abolished by VTA inactivation[90], there is also evidence that cue-evoked excitations of NAc core neurons depend on mPFC glutamatergic projections, and contribute to the behavioural response to reward-predictive cues[91]. Importantly, we do observe an increased activation of mPFC-NAc projecting regions in D2-stimulated animals. Moreover, NAc administration of AMPA and NMDA antagonists prior to optogenetic stimulation abolishes the positive impact of D2 neuronal activation. This effect is likely to be mediated by blockage of glutamatergic activation of MSNs, which express different classes of glutamate receptors[92,93], but not by acting on dopaminergic VTA terminals and corticothalamic glutamatergic terminals that express mGluRs[94].

Yet, it is important to refer that although sparse, GABAergic interneurons (which do not express D2R[95]) display highly branched dendritic and extensive axonal arborisations[34,96,97] and are capable of exerting a powerful control over striatal excitability (feed-forward inhibition)[65,86]. They also express $GABA_B$ receptors[88], so the blockage of this specific feed-forward inhibition might also contribute for the observed increase in motivational drive.

In addition to local GABA control, the striatum also contains CINs, which have both excitatory and inhibitory effects in striatal MSNs[98–100]. In primates, CINs exhibit multiphasic responses to motivationally salient stimuli that mirror those of midbrain dopamine neurons, being important for reward-related learning[18,101,102]. CINs are able to co-release glutamate and ACh[103], and influence striatal activity[68]. *In vivo* selective activation of CINs is sufficient to elicit dopamine release in the NAc directly and independently of the soma, by its binding to β2*-nAChRs in VTA terminals[39,104,105]. It has been suggested that these nAChR act as dynamic detectors of ACh concentrations, enhancing the contrast between tonic and burst dopaminergic firing[77,78,106,107]. Here, we show that β2*-nAChR antagonist administration blocks D2-dependent increase in motivated behaviour, suggesting that ACh-mediated dopamine release from VTA terminals is crucial for the observed behavioural effect. Moreover, we also observe increased VTA neuronal activation in D2-stimulated animals, whereas no effect was observed in the SN, suggesting that this region is being recruited differently during the task in stimulated animals. In fact, considering previous data from our group that show a significant decrease in electrophysiological activity in the VP (Chapter 3.1), we believe that this increase is probably occurring through VP modulation.

In this direction, we next showed that NAc local administration of either D1R or D2R antagonists decreases animals' motivation, in accordance with our previous results showing that the degree of activation of both D1R and D2R accumbal neurons was positively correlated with motivational drive (Chapter 3.1). Importantly, both treatments also abolished D2-driven positive effects in motivation, indicating a synergistic effect of both neuronal populations in motivation[47,49,108–110]. However, one has to bear in mind that D2R antagonists can also act in D2 autoreceptors in midbrain terminals, disinhibiting presynaptic control of dopamine release[111–113].

Interestingly, D2 optogenetic activation during cue exposure also indirectly recruited D1-MSNs, as assessed by an increase in the number of $D1^+$/c-fos$^+$ cells in the NAc upon D2 stimulation (Chapter 3.1). Considering the proposed role for D1R-expressing neurons in reinforcement[114–116], this activation probably contributes for the behavioural output. One possibility is that the cholinergic-mediated increase in dopamine release induced by D2 optical stimulation increases D1R activation, resulting in more AMPA and NMDA receptors at the membrane surface[117,118], making D1-MSNs more responsive to glutamatergic signalling. Accordingly, we show that administration of both AMPA and NMDA receptor antagonists normalized the breakpoint of D2-stimulated animals (but had no effect in control animals). However, the picture gets more

complicated because besides MSNs, these receptors are also expressed in CINs. In fact, it has been suggested that glutamatergic thalamostriatal inputs synchronize CIN activity (via NMDA/AMPA receptors) to coordinate dopamine release through β2*-nAChR receptors in VTA terminals[105,119].

Despite the prominent role of striatal dopamine on reward and motivation[2,4,120–122], this and other studies show that different striatal microcircuits play a significant role in the modulation of motivation[39,68,76,104,123]. We show that accumbal D2 neuronal optogenetic activation enhances motivation, and appears to require dopamine release from VTA terminals in a cholinergic-mediated manner, that acts in both D1R and D2R, and is dependent on glutamatergic inputs acting through striatal NMDA and AMPA receptors.

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CHAPTER 3.3

Nucleus accumbens D2R-expressing neurons bi-directionally modulate Pavlovian conditioning

Manuscript in preparation

Nucleus accumbens D2R-expressing neurons bi-directionally modulate Pavlovian conditioning

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Abstract

The nucleus accumbens (NAc) is a key region for reward processing. Two distinct GABAergic neuronal populations, constituted by medium spiny neurons (MSNs) that express either dopamine receptor D1 (D1R, D1-MSNs), or dopamine receptor D2 (D2R, D2-MSNs), process dopaminergic signals arising from the ventral tegmental area (VTA) to the NAc. While D1-MSNs have been canonically associated with reinforcement and positive stimuli, D2-MSNs are associated with aversion and negative stimuli. Nonetheless, recent data has shown that D2-MSNs also play a relevant role in reward-related behaviours.

Here, we used optogenetic tools to specifically stimulate/inhibit NAc D2R-expressing neurons and assess its effects in behavioural conditioning in two distinct paradigms. Interestingly, the modulatory effects of accumbal activation of D2R-expressing neurons on Pavlovian conditioning vary according to the duration of optical stimulation: short optical stimulation is reinforcing whereas long optical stimulation is aversive. We further show that these distinct protocols of optogenetic activation lead to different electrophysiological outcomes in downstream regions, namely the ventral pallidum (VP) and VTA.

These results highlight the importance of testing different stimulation protocols in optogenetic experiments, and suggest that the same neurons can bi-directionally mediate reward behaviours depending on the type of input they receive.

Introduction

Reinforcement learning in response to positive or negative stimuli is greatly regulated by neural circuits including the basal ganglia that receive dopaminergic inputs from the midbrain[1–6]. Specifically, the nucleus accumbens (NAc) is known to play a critical role in reward behaviours, by the decoding of dopaminergic signals arising mainly from the ventral tegmental area (VTA)[7]. Interestingly, electrophysiological recordings have shown that the striatum actively responds to cues that predict reward[8,9], and optical stimulation of VTA-to-NAc projections is reinforcing[10]. These projecting signals (mostly dopaminergic in nature) act through two distinct neuronal populations of GABAergic medium spiny neurons (MSNs), which together make up to 95% of all NAc neurons. Each of these MSN populations projects to specific output brain regions and expresses distinct types of dopamine receptors at the cell surface[11–14]. Dopamine receptor D1 (D1R)-expressing MSNs (D1-MSNs) directly project to the output nuclei of the basal ganglia, substantia nigra (SN) and VTA (direct pathway), but can also indirectly project to output nuclei of the basal ganglia through the ventral pallidum (VP) (indirect pathway)[15]. Dopamine receptor D2 (D2R)-expressing MSNs (D2-MSNs) project indirectly to the output nuclei of the basal ganglia through the ventral pallidum (VP) [14,15].

Akin to dorsal striatum, it is widely accepted that NAc MSNs exert antagonistic influences on behaviour[16–18]. For example, in dorsomedial striatum, stimulating D1-MSNs induces persistent reinforcement, whereas stimulating D2-MSNs induces transient punishment[16]. In the NAc, optical activation of D1-MSNs enhances cocaine-mediated behavioural conditioning, whereas optical stimulation of D2-MSNs supresses cocaine reward[19]. However, previous data from our team showed that activation of either D1- or D2-MSNs enhances cue-dependent motivational drive (Chapter 3.1), suggesting that both neuronal populations can act in the same direction to promote reward behaviours. In addition, others have shown that stimulation of both D1- and D2-MSNs in the dorsolateral striatum is reinforcing, although these two MSN populations reinforce distinct action strategies[20].

Considering the controversial role of D2-MSNs in rewarded behaviours, in this study we specifically activated/inhibited NAc D2R-expressing neurons using optogenetic tools, and assessed animals' behaviour in two distinct conditioning paradigms. Interestingly, we show that there is a dual effect of D2R-expressing neuronal accumbal activation in Pavlovian conditioning, depending on the optogenetic stimulation protocol applied.

Materials and Methods

Animals

Male Wistar Han rats (age of 2 months old at the beginning of the experiments) were used. Animals were maintained under standard animal house conditions with an artificial 12 h light/dark cycle (lights on from 8am to 8pm), room temperature of 21-22°C and relative humidity of 60%; after cannula implantation, rats were individually housed and standard diet (4RF21, Mucedola SRL) and water were given *ad libitum*. All behavioural experiments were performed during the light period of the light/dark cycle.

Health monitoring was performed according to FELASA guidelines[21], confirming the Specified Pathogen Free health status of sentinel animals. All procedures were conducted in accordance with European Regulations (European Union Directive 2010/63/EU). Animal facilities and the people directly involved in animal experimentation were certified by the Portuguese regulatory entity – Direção Geral de Veterinária. All protocols were approved by the Ethics Committee of the Life and Health Sciences Research Institute.

Constructs and virus preparation

eYFP, hChR2(H134R)-eYFP and eNpHR3.0-eYFP were cloned under the control of D2R minimal promoter region (D2R; ENSRNOG00000008428; Gene ID: 24318), which included 1540 bp upstream of the first (non-coding) exon (kindly provided by Dr. Karl Deisseroth)[22]. Constructs were packaged in AAV5 serotype by the UNC Gene Therapy Center Vector Core (UNC). Virus titters were 3.7-6x10^{12} virus molecules/ml as determined by dot blot.

Surgery and cannula implantation

Rats were anesthetized with 75 mg kg^{-1} ketamine (Imalgene, Merial) plus 0.5 mg kg^{-1} medetomidine (Dorbene, Cymedica). Virus was unilaterally injected into the NAc (coordinates from bregma, according to Paxinos and Watson[23]: +1.2mm anteroposterior (AP), +1.2 mm mediolateral (ML), and 6.5 mm dorsoventral (DV)). Rats were then implanted with an optic fibre (200μm core fibre optic; Thorlabs) with 2.5 mm stainless steel ferrule (Thorlabs) using the injection coordinates (with the exception of DV: 6.4 mm) that were secured to the skull using 2.4 mm screws (Bilaney) and dental cement (C&B kit, Sun Medical). Rats were removed from the stereotaxic frame and sutured. Anaesthesia was reverted by administration of atipamezole (1 mg kg^{-1}). After surgery

animals were given anti-inflammatory (Carprofeno, 5 mg kg^{-1}) for one day, analgesic (butorphanol, 5 mg kg^{-1}) for 3 days, and were let to fully recover for four weeks before initiation of behaviour.

Optogenetic stimulation

For all optogenetic stimulation experiments using ChR2, 10 mW of blue light (measured at the tip of the fiberoptic) was generated by a 473 nm DPSS laser (CNI Laser) and unilaterally delivered to rats through one fiberoptic patch cord (0.22 NA, 200 μm diameter; Thorlabs) that was attached to the implanted fiberoptic cannula, using a connecting ceramic sleeve. For all optogenetic inhibition experiments using eNpHR, 15 mW (measured at the tip of the fiberoptic) of yellow light was generated by a 589 nm DPSS laser (CNI Laser) and unilaterally delivered.

Laser output was controlled using a pulse generator (Master-8; AMPI) to deliver light.

Conditioned Place Preference – CPP

The CPP apparatus consisted of two compartments with different patterns on floors and walls, 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 center 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 chamber during each preference test was monitored using a computerized photo-beam system (Med Associates). The apparatus was designed so that rats did not have preference for a particular chamber. The CPP test consisted of three phases over 4 days. On day 1, individual rats were placed in the center chamber and allowed to freely explore the entire apparatus for 15 min (pre-test). Rats were conditioned for two consecutive days: in the first conditioning session (day 2), rats were confined to one of the side chambers for 30 min and paired with optical stimulation; in the second session (day 3), rats were confined to the other side chamber for 30 min with a different stimulation parameter. Conditioning days were counterbalanced across rats. On day 4 (similar to day 1) rats were placed in the center chamber and allowed to freely explore the entire apparatus for 15 min (post-test). Both post-test and pre-test were conducted identically. Results are expressed as the difference of time spent in the stimulus-paired to no-stimulus-paired side.

Optical stimulation was given as follows: (**1**) 473 nm; frequency of 40 Hz; 40 light pulses of 12.5 ms duration, every minute; (**2**) 473 nm; frequency of 40 Hz; 12.5 ms light pulses, over 10 s, every minute; (**3**) 473 nm; frequency of 40 Hz; 12.5 ms light pulses, over 60 s, with 60 s of

interval (without illumination).

Optical inhibition was performed as follows: 589 nm; 10 s constant light; every minute.

Real-Time Place Preference – RTPP

RTPP test was performed in a custom-made black plastic arena (60 x 60 x 40 cm) consisting of two indistinguishable chambers, for 15 min. One chamber was paired with light stimulation. 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 after manually assessed. Results are presented as total time spent in each chamber.

Optical stimulation was given as follows: 473 nm; frequency of 40 Hz; 12.5 ms light pulses; during the entire period that the animal stayed in the stimulus side.

Optical inhibition was performed as follows: 589 nm; constant light; during the entire period that the animal stayed in the stimulus side.

In vivo single cell electrophysiology

After 4 weeks post-surgery, rats were anesthetized with urethane (1.44 g Kg^{-1}, Sigma). The total dose was administered in 3 separate intra peritoneal injections, 15 min apart. Adequate anesthesia was confirmed by the lack of withdrawal responses to hind limb pinching. A recording electrode coupled with a fiber optic patch cable (Thorlabs) was placed in the NAc (coordinates from bregma, according to Paxinos and Watson[23]: +1.2 mm AP, +1.2 mm ML, and 6.0 to 7.0 mm DV), using a stereotaxic frame (David Kopf Instruments) with non-traumatic ear bars (Stoeling). Other recording electrodes were placed in the VP (coordinates from bregma, according to Paxinos and Watson[23]: 0 to -0.12 mm AP, +2.3 to +2.5 mm ML, and 7 to 7.6 mm DV) and in the VTA (coordinates from bregma, according to Paxinos and Watson[23]: -5.3 mm AP, +0.9 mm ML, and 7.5 to 8.3 mm DV). Single neuron activity was recorded extracellularly with a tungsten electrode (tip impedance 5-10 Mat 1 kHz) and data sampling was performed using a CED Micro1401 interface and Spike 2 software (Cambridge Electronic Design). The DPSS 473 nm laser system or DPSS 589nm laser system (CNI), controlled by a stimulator (Master-8, AMPI) were used for intracranial light delivery. Fiber optic output was pre-calibrated to 10-15 mW from the fiber tip before implantation. Optical stimulations were performed as follows:

- 473 nm; frequency of 40 Hz; 40 light pulses of 12.5 ms duration, 10 mW at the tip of the

fibre.

- 473 nm; frequency of 40 Hz; 2400 light pulses of 12.5 ms, 10 mW at the tip of the fibre.

Firing rate histograms were calculated for the baseline (60 s prior to stimulation), stimulation period and after stimulation period (60 s after the end of stimulation). The cells were considered as responsive or not responsive to the stimulation on the basis of their firing rate change with respect to the baseline period. Neurons showing a firing rate increase or decrease by more than 20% from the mean frequency of the baseline period were considered as responsive[24]. Spike latency was determined by measuring the time between half-peak amplitude for the falling and rising edges of the unfiltered extra-cellular spike.

Immunofluorescence (IF)

Rats were deeply anesthetized with pentobarbital (Eutasil) and then transcardially perfused with 0.9% saline followed by 4% paraformaldehyde. Brains were removed and post-fixed in 4% paraformaldehyde. Coronal vibratome sections (50 μm) were incubated with the primary antibodies mouse anti-D2R (1:500, B-10, Santa Cruz Biotechnology) and goat anti-GFP (1:500, ab6673, Abcam); mouse anti-D1R (1:100, NB110-60017, Novus) and mouse anti-GFP (1:200, Ab1218, Abcam); or goat anti-ChAT (1:750, AB144P, Millipore) and mouse anti-GFP (1:200, Ab1218, Abcam). Appropriate secondary fluorescent antibodies were used (1:500, Invitrogen). Finally, all sections were stained with 4′,6-diamidino-2-phenylindole (DAPI; 1 mg ml^{-1}). For each animal, 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 crossing cell number values with the corresponding areas, determined using an Olympus BX51 optical microscope and the StereoInvestigator software (Microbrightfield).

Statistical analysis

Normality tests were performed for all data analysed. Statistical analysis between two groups was made using Student's *t*-test. One-way or two-way analysis of variance (ANOVA) was used when appropriate. Bonferroni's *post hoc* multiple comparisons was used for group differences determination.

Results are presented as mean ± sem. All statistical analysis was performed using IBM SPSS Statistics (v.22) and results were considered significant for $p \leq 0.05$.

Results

Opposing effects of different optogenetic stimulation parameters of D2R-expressing neurons in Pavlovian conditioning

To specifically manipulate D2R-expressing neurons, we injected in the NAc viral vectors carrying channelrhodopsin (ChR2, D2-ChR2), halorhodopsin (eNpHR, D2-eNpHR), or the enhanced green fluorescent protein (eYFP, D2-eYFP) genes under the control of the D2R minimal promoter region[22]. Four weeks after viral injection, and after behavioural performance, the expression of the constructs was determined by analysis of eYFP signal in the NAc (Fig. 1a). The location of optical fibres was also confirmed for all the animals used in this study (Fig. 1a). Afterwards, we confirmed specificity of the viral infection by performing immunofluorescence against D2R and YFP. As depicted in Figure 1b-d, D2-driven constructs were specific to $D2^+$ neurons (around 60% of $D2^+$ neurons also expressed YFP), reflected in a high number of $D2^+$/$eYFP^+$ cells (Fig 1d); a very small number of $D1^+$ neurons expressed these constructs (around 10 cells/mm^2, corresponding to 4% of $D1^+$ cells also expressed YFP; Fig. 1d). Importantly, 30% of cholinergic neurons (around 3-4 cells/mm^2), which also express D2R[25], were transfected (Fig. 1d).

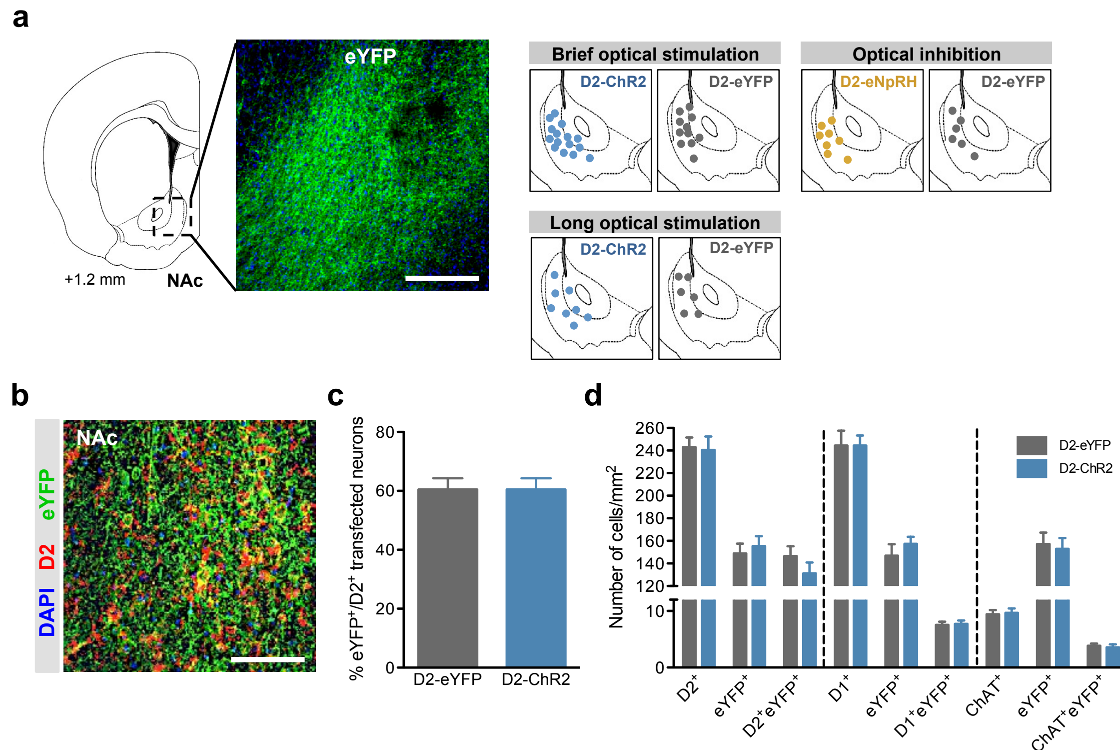

Figure 1. Selectivity of the optogenetic manipulation of D2R-expressing neurons in the NAc. (**a**) Animals received unilateral injection of AAV5-D2-hChR2(H134R)-eYFP (D2-ChR2), AAV5-D2-eNpHR3.0-eYFP (D2-eNpHR) or AAV5-D2-NpHR-eYFP (D2-eYFP) in the NAc. Expression of eYFP in the NAc of a D2-ChR2 animal is shown; the location of cannula implantation was confirmed for all groups. Numbers represent distance in millimetres to bregma; scale bar =100 μm. (**b**) Expression of D2R and eYFP in the NAc of a D2-ChR2 rat, showing co-expression; scale bar = 200 μm. (**c**) Around 60% of D2R-expressing neurons of the Nac neurons express either D2-eYFP or D2-ChR2 (n=5 animals per group). (**d**) Quantification of (1) D2$^+$, eYFP$^+$, D2$^+$/eYFP$^+$ (2) D1$^+$, eYFP$^+$ D1$^+$/eYFP$^+$ and (3) ChAT$^+$, eYFP$^+$ and ChAT$^+$/eYFP$^+$ cells in the NAc of both D2-eYFP and D2-ChR2 animals. These countings show the selectivity of the virus for D2$^+$ neurons. Only 1.5% of D1$^+$ neurons express the D2R-driven construct. Cholinergic interneurons are also transfected. n=5 animals per group. Error bars denote s.e.m.

Rats were then subjected to an unbiased CPP test, a non-contingent (experimenter-induced) conditioning paradigm (Fig. 2a). The protocol consists of 2 days of conditioning: in one of the days, animals receive optogenetic stimulation in one of the chambers and on the other day, animals are placed in the other chamber and no stimulus is given. The order of the conditioning days was counterbalanced and randomized between animals. On test day, animals are allowed to freely explore both chambers.

Brief optogenetic stimulation (473 nm, 40 Hz, 40 light pulses of 12.5 ms pulses, every minute) of D2R-expressing neurons induces a clear preference for the stimulus-associated chamber (Fig. 2b; $F_{(1,24)}$=8.9, p=0.0065), as shown by an increased percentage of time spent in

the stimulus box of D2-ChR2 rats when compared to control D2-eYFP rats. D2-ChR2 rats presented a significant increase in the change in preference in comparison with D2-eYFP rats (Fig. 2c; t_{30}=3.8, p<0.001). As expected, control group (D2-eYFP) did not show any preference (Fig. 2b,c).

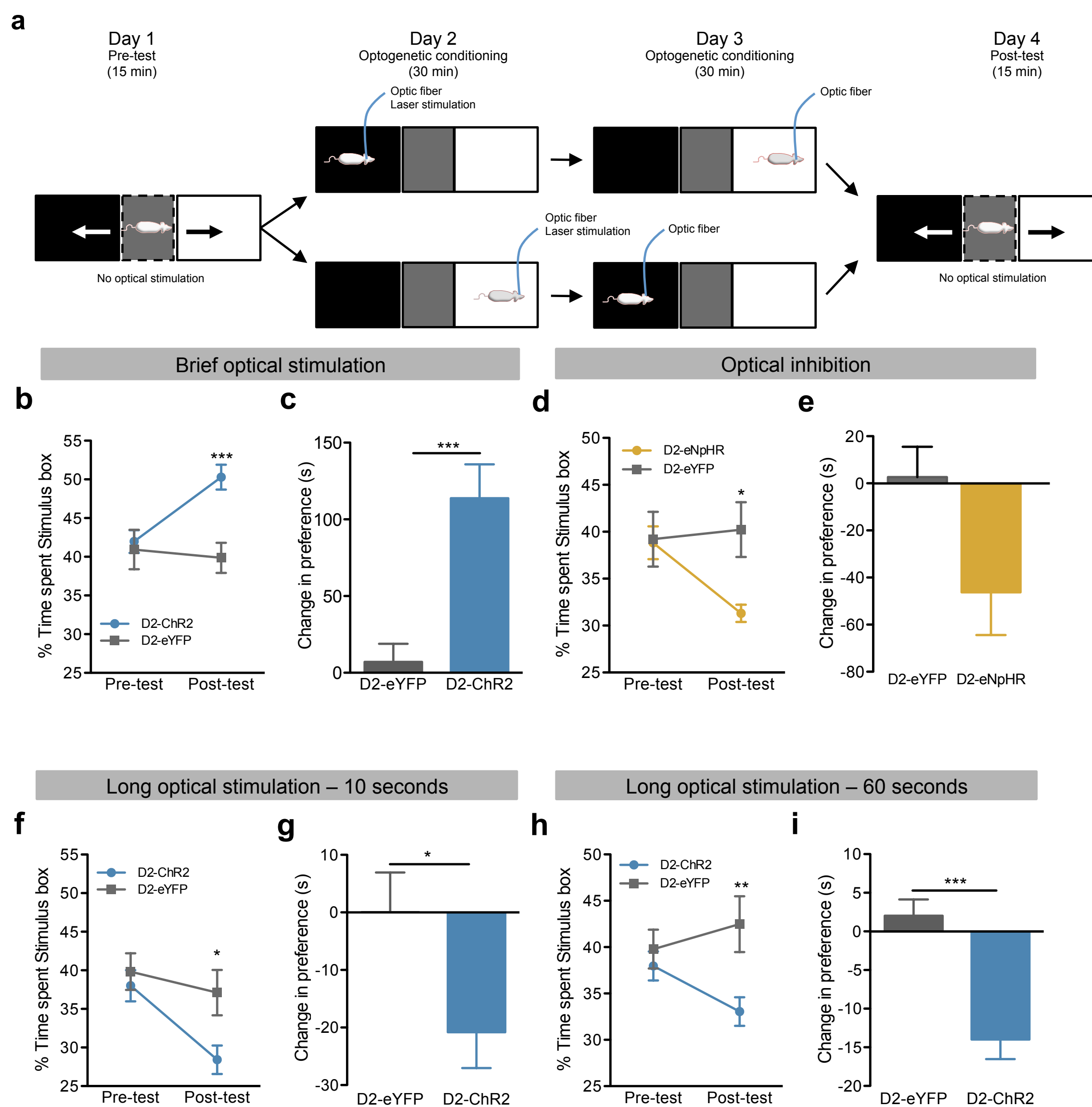

Figure 2. Modulation of NAc D2R-expressing neurons in the CPP test. (**a**) Schematic representation of the CPP protocol: animals were submitted to a pre-test session (15 minutes long) in which they freely explored the 3 boxes. After, they were subjected to two conditioning days: on one of the conditioning days, rats were placed in one of the boxes and given optical stimulation (stimulus box), while on the other conditioning day, rats were confined to the other box with no stimulation given (no-stimulus box). The assigned chamber for each condition and order of conditioning were counterbalanced. On day 4, animals were allowed to freely explore both boxes. (**b**) Brief optogenetic stimulation of NAc D2 neurons (473 mn, 40 Hz, 40 light pulses of 12.5 ms, 10 mW at the tip of the patch cable, every minute) induces conditioning. D2-ChR2 animals show a significantly higher percentage of time spent in the stimulus box than D2-eYFP in the post-test session ($n_{D2\text{-}eYFP}$=11, $n_{D2\text{-}ChR2}$=15). (**c**) Change in preference shown as the difference

in time spent in the stimulus box and the time spent in the no-stimulus box. D2-ChR2 preferred the stimulus box. (**d**) Long optogenetic stimulation of D2R-expressing neurons (473 mn, 40 Hz, 400 light pulses of 12.5 ms, 10 mW at the tip of the patch cable, every minute) induce aversion, shown by a significantly lower percentage of time spent in the stimulus box ($n_{D2\text{-}eYFP}$=6, $n_{D2\text{-}ChR2}$=10) (**e**) Change in preference for D2-ChR2 and D2-eYFP rats submitted to the CPP protocol with long optogenetic stimulation of D2R-expressing neurons. (**e**) A second protocol of long optogenetic stimulation of D2 neurons for 60 seconds (473 mn, 40 Hz, 2400 light pulses of 12.5 ms, 10 mW at the tip of the patch cable, with 60 s of interval) also induces aversion, shown by a significantly lower percentage of time spent in the stimulus box ($n_{D2\text{-}eYFP}$=6, $n_{D2\text{-}ChR2}$=8). (**f**) Change in preference for D2-ChR2 and D2-eYFP rats submitted to the CPP protocol with long optogenetic stimulation of D2-MSNs. (**g**) Optogenetic inhibition of D2R-expressing neurons (589 mn, 10 second constant light, 15 mW at the tip of the patch cable, every minute) induces aversion, since D2-NpHR animals present lower percentage of time spent in the stimulus box ($n_{D2\text{-}eYFP}$=6, $n_{D2\text{-}eNpHR}$=8). (**h**) Change in preference for D2-eNpHR and D2-eYFP rats, shown as the difference in time spent in the stimulus box and the time spent in the no-stimulus box. Error bars denote s.e.m. *p≤0.05, **p≤0.01, ***p≤0.001.

We next tested what was the behavioural effect of inhibiting accumbal D2R-expressing neurons. Interestingly, optical inhibition of this neuronal population (continuous 589 nm light for 10 seconds every minute) induced aversion (Fig. 2d; $F_{(1,12)}$=2.2, p<0.05). Despite these results, D2-eNpHR rats did not present a significant difference in the change in preference when compared with D2-eYFP rats, though there is a trend (Fig. 2e; t_{12}=2.1, p=0.0625).

D2 optical stimulation is aversive in a RTPP protocol

We also performed the RTPP test to evaluate the effects of activation of NAc D2R-expressing neurons (Fig. 3a). RTPP also measures the reinforcing properties of particular stimulus as the CPP test, but is dependent on individual's choice (contingent). In this test, animals are free to explore the two chambers, in which one is paired with the delivery of optical stimulus and the other is not. Every time the animal crossed to the stimulus side of the box (stimulus box), it received optical stimulation (473 nm, 40 Hz, 12.5 ms light pulses, 10 mW) that ceased only when the animal crossed back to the no stimulus side (no-stimulus box).

Interestingly, D2 optical stimulation was aversive to D2-ChR2 rats (Fig. 3b; $F_{(3,24)}$=21.2, p<0.001), since these animals spent a significantly lower percentage of time in the stimulus box when compared with the no-stimulus box (Fig. 3b; t_{14}=6.7, p<0.001; Fig. 3c; t_{12}=3.7, p=0.003). In addition, D2-ChR2 presented a significantly lower number of entries in the stimulus box (Fig. 3d; t_{12}=2.4, p=0.0355), but the time spent per entry did not differ (Fig. 3e).

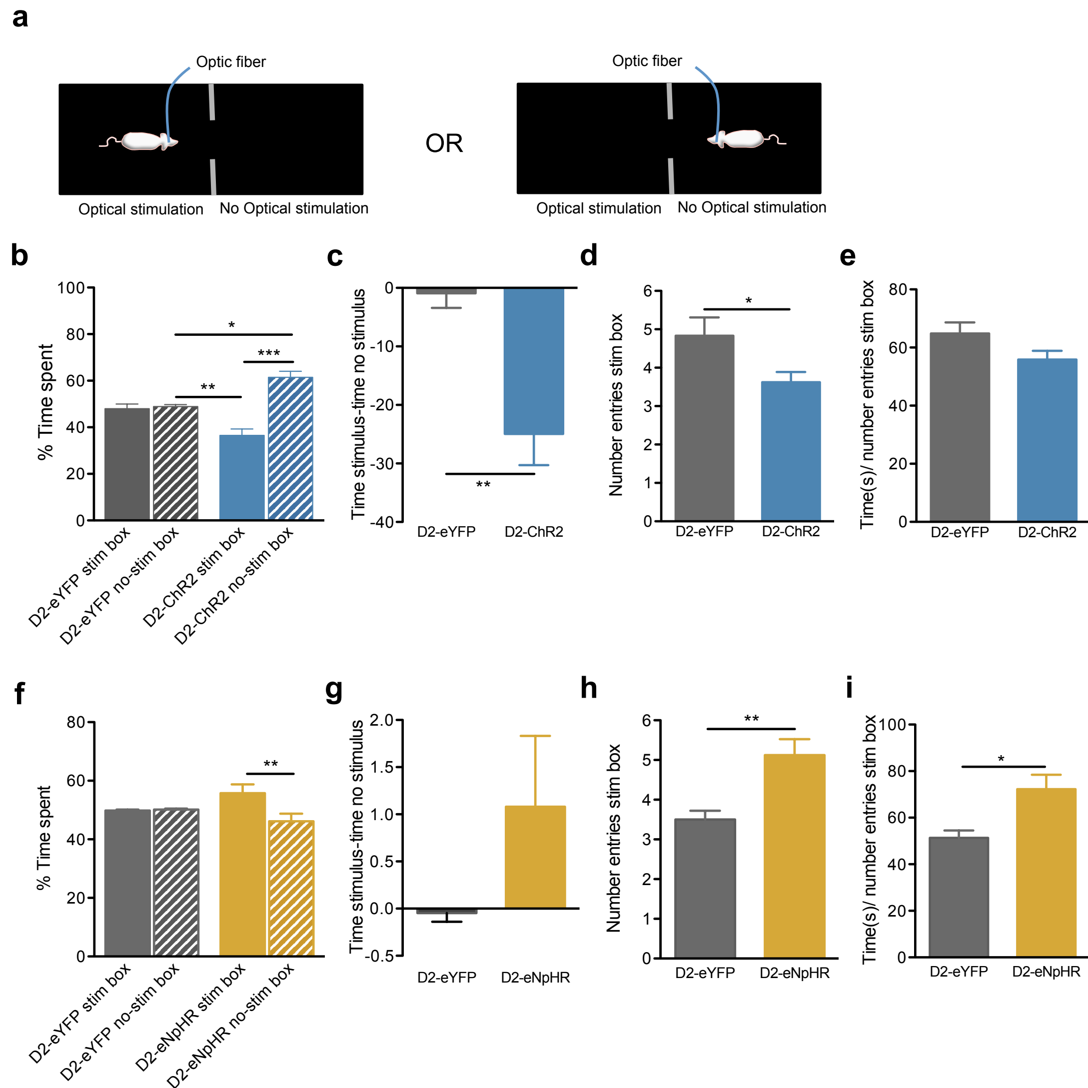

Figure 3. Modulation of NAc D2R-expressing neurons' activity during the RTPP. (**a**) Schematic representation of the RTPP protocol: animals were placed in the box, allowed to freely explore between the two compartments. When animals crossed to the stimulus side (stimulus box) they received optical stimulation (473 nm, 40 Hz, 12.5 ms light pulses, 10 mW at the tip of the patch cable) that only ceased when they crossed back to the no-stimulus side (no-stimulus box). (**b**) D2-ChR2 rats spend a significantly lower percentage of time in the stimulus box. (**c**) Significant difference between time spent in the stimulus box and the time spent in no-stimulus box for D2-ChR2 and D2-eYFP rats. Differences in (**d**) the time spent per entry and (**e**) the number of entries in the stimulus box. (**f**) D2-eNpHR spent a significantly higher percentage of time in the side where they receive optogenetic inhibition (589 nm, constant light, 15 mW at the tip of the patch cable). (**g**) The difference between time spent in the stimulus box and the time spent in no-stimulus box for D2-eNpHR and D2-eYFP rats was not significantly different, though there was a trend for reduced time in the stimulus box. Differences in (**h**) the number of entries in the stimulus box and in (**i**) the time spent per entries in the stimulus box. ($n_{D2\text{-}eYFP}$=6, $n_{D2\text{-}ChR2}$=8, $n_{D2\text{-}eNpHR}$=8). Error bars denote s.e.m. *p≤0.05, **p≤0.01 ***p≤0.001.

We next performed a similar experiment but with D2 optogenetic inhibition (589 nm, 15 mW). Interestingly, D2-eNpHR spent significantly more time in the stimulus box than in the no-stimulus box (Fig. 3f; t_{13}=2.4, p=0.0327), although it did not differ from D2-eYFP rats (Fig. 3g; t_{12}=1.4, p=0.1978). In addition, D2-eNpHR rats entered more in the stimulus box (Fig. 3h; t_{12}=3.2, p=0.0071), and spent more time in the stimulus box per entry (Fig. 3i; t_{12}=2.7, p=0.0193).

Effect of D2 prolonged stimulation on CPP

To confirm the effect of prolonged stimulation of D2R-expresing neurons in conditioning processes we performed a CPP protocol, similar to the one described above, but with an optogenetic conditioning using prolonged D2 stimulation (473nm; 40 Hz, 400 light pulses of 12.5 ms, every minute). Interestingly, this protocol induced aversion to the stimulus-associated box (Fig. 2f; $F_{(1,14)}$=4.7, p=0.0472), shown by a significantly decreased percentage of time spent in the stimulus box when compared with D2-eYFP rats (Fig. 2g; t_{14}=3.34, p<0.001). As expected, control D2-eYFP rats did not show any preference (Fig. 2f,g). Increasing the optogenetic stimulus to 1 minute (473nm; 40 Hz, 2400 loght pulses of 12.5 ms light pulses, with 60 seconds of interval) also caused a significant decrease in the percentage of time spent in the stimulus box (Fig. 2h; $F_{(1,14)}$=0.4, p<0.01) and a significant decrease in preference in comparison with D2-eYFP rats (Fig. 2i; t_{12}=4.3, p=0.0007).

Electrophysiological effects of different optogenetic stimulation parameters

To further understand the enigmatic behavioral data, we performed single-cell *in vivo* electrophysiology of NAc neurons during D2 neuronal stimulation (Fig. 4a). Short optical stimulation of accumbal D2R-expressing neurons (473 nm; 40 Hz, 40 light pulses of 12.5 ms) resulted in a significant increase in the firing rate of accumbal neurons (Fig. 4b,c; $F_{(2,72)}$=21.2, p<0.001). 62.9% of NAc neurons increased firing, 51.4% did not respond and 5.7% decreased activity. 34.3% of the cells presented altered firing rate during the 60 seconds after the stimulus (11.4% decreased activity, 22.9% increased activity) (Fig. 4d).

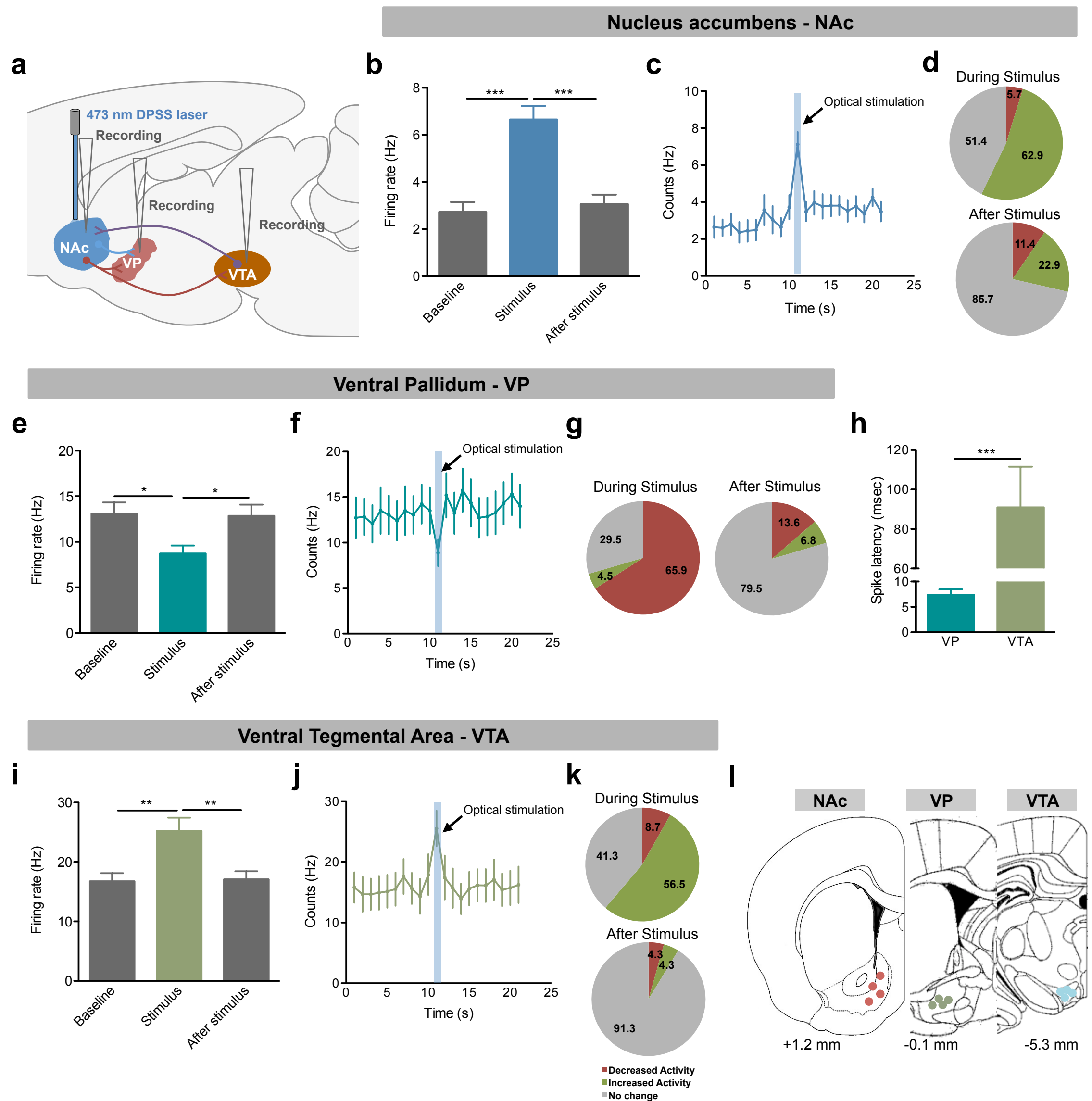

Figure 4. Electrophysiological recordings in the NAc and downstream targets during brief optical stimulation of NAc D2R-expressing neurons. (**a**) Schematic representation of the *in vivo* single-cell electrophysiological recording experiments with optogenetic stimulation in the NAc and downstream regions (VP and VTA). (**b**) Significant increase in NAc firing rate during optogenetic stimulation (40 Hz, 40 light pulses of 12.5 ms). (**c**) Time histogram of NAc electrophysiological activity in response to the optical stimulation. (**d**) More than half of the cells increased firing rate (62.9%), while 11.4% decreased their activity and 51.4% did not respond to D2 neuronal stimulation. (**e**) D2 accumbal optical stimulation significantly decreases VP firing rate. (**f**) Time histogram of VP electrophysiological activity in response to optical stimulation of D2R-expressing neurons within the NAc. (**g**) Short NAc D2 neuronal activation decreases the activity of 65.9% of VP cells, increases the activity of 4.5% of cells and does not change the activity of 29.5% of cells. (**h**) D2 accumbal optical stimulation significantly increases VTA firing rate. (**i**) Time histogram of VTA electrophysiological activity in response to optical stimulation of D2R-expressing neurons within the NAc. (**j**) Brief NAc activation of D2R-expressing neurons increases the activity of 56.5% of VTA cells, decreases the activity of 8.7% of cells and does not change the activity of 41.3% of cells. (**k**) Spike latency of VP and VTA after brief accumbal optical stimulation of D2R-expressing neurons. (**l**) Schematic representation of recording electrode

placement in the NAc, VP and VTA; numbers represent distance in millimetres to bregma. n=25 cells per brain region (4 rats). Error bars denote s.e.m. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

We also performed electrophysiological recordings in the VP (Fig. 4e,f), which receives direct projections from NAc D2-MSNs (but also from D1-MSNs)[13,20]. Importantly, brief optical stimulation of D2R-expressing neurons in the NAc significantly decreased the firing rate of the VP (Fig. 4e,f; $F_{(2,72)}=4.9$, $p=0.01$). Stimulation elicited a decrease in the firing rate of 66.9% of VP cells; 4.5% presented increased activity and 29.5% did not respond (Fig. 4g). After the stimulus, the firing rate was similar to baseline (Fig. 4e,f), with only 13.6% of cells presenting significant decrease in activity (Fig. 4g); 6.8% had increased firing and 79.5% were similar to baseline. Spike latency measured in the VP after D2 optical accumbal stimulation was of 6.6 milliseconds in average (Fig. 4h), consistent with the monosynaptic inputs from the NAc to VP.

In addition, we also recorded the electrophysiological activity of VTA neurons during activation of D2R-expressing neurons in the NAc, which does not receive direct D2-MSN NAc projections, but receive indirect projections through the VP[13,21,22]. We observed a general increase in VTA activity (Fig. 4i,j; $F_{(2,72)}=8.1$, $p=0.0007$), with 56.5% of cells showing increased firing (Fig. 4k). After stimulation the firing rate was similar to baseline activity (Fig 4i-j), and only 4.3% of cells maintained increased firing rate; 4.3% had decreased firing rate and 91.3% had a firing rate similar to baseline (Fig. 4k) .The spike latency was 100 milliseconds in average (Fig. 4h), suggesting the existence of polysynaptic connections.

The electrode placement was confirmed for all animals used in the electrophysiological recordings (Fig. 4l).

We next evaluated the electrophysiological response of the NAc and downstream regions with the longer stimulation period (Fig. 5a). We recorded a 60 s baseline of activity, then stimulated for 60 seconds (473 nm; 2400 light pulses of 12.5 ms) and recorded 60 seconds after. D2R-expressing neuronal stimulation resulted in a significant increase in the neuronal firing of NAc neurons (Fig. 5b,c; $F_{(2,72)}=4.7$, $p=0.01$). More than half of accumbal cells (64.7%) increased their firing rate during the stimulation, 14.7% did not respond and 20.6% decreased activity; after the 60 s of stimulus, 38.2% of the cells still maintained altered firing rate: 8.8% present decreased activity and 29.4% show increased firing rates) (Fig. 5d).

Recordings of the VP neurons showed significant changes in firing rate (Fig. 5e,f; $F_{(2,72)}=4.4$, $p=0.006$): stimulation of accumbal D2R-expressing neurons elicited a significant decrease in the

firing rate of the VP in the first second after the initiation of stimulation (t_{24}=3.6, p=0.0015), but the activity significantly decreased in the rest of the stimulation period in comparison with the baseline (t_{24}=3.5, p=0.002). During the first second of stimulation, 58.5% of cells decreased their activity, but in the following period, only 7.5% presented decreased activity and 47.2% of cells increased their firing rate (Fig. 5g). Spike latency was of 7.4 milliseconds in average (Fig. 5k), consistent with the monosynaptic inputs from the NAc to VP.

Electrophysiological recordings of the VTA showed exactly the opposite of the VP (Fig. 5h,i; $F_{(2,72)}$=4.7, p=0.009). In the first second of optical stimulation, we observed a significant increase in firing rate (t_{24}=2.3, p=0.0288), followed by a significant decrease during the rest of the period (t_{24}=2.7, p=0.0129). In the first second of stimulation 44.4% of cells increased activity, while in the following period, 55.6% showed a decrease in firing (Fig. 5j). Spike latency was 91.0 milliseconds in average (Fig. 5k), suggestive of a polysynaptic connection.

The electrode placement was confirmed for all animals used in the electrophysiological recordings (Fig. 5l).

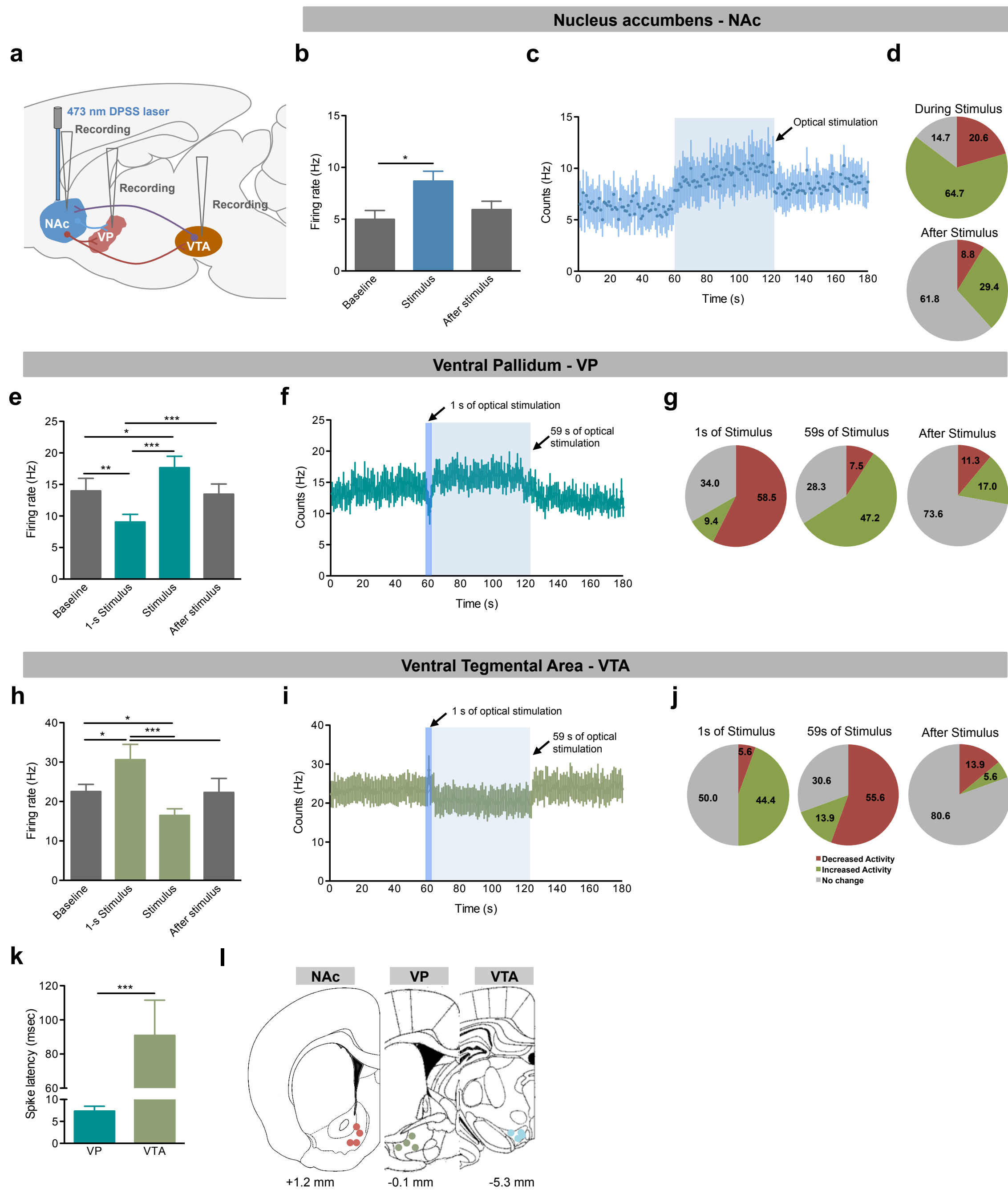

Figure 5. Electrophysiological recordings in the NAc and downstream targets during long optical stimulation of NAc D2R-expressing neurons. (**a**) Schematic representation of the *in vivo* single-cell electrophysiological recording experiments with longer optogenetic stimulation in the NAc and downstream regions (VP and VTA). (**b**) Significant increase in NAc firing rate during optogenetic stimulation (40 Hz, 2400 light pulses of 12.5 ms). (**c**) Time histogram of NAc electrophysiological activity in response to long optical stimulation. (**d**) 64.7% of NAc cells increased firing rate during long otpogenetic stimulation of accumbal D2R-expressing neurons (40 Hz, 2400 light pulses of 12.5) while 20.6% decreased their activity and 14.7% did not respond. (**e**) Accumbal D2R-expressing neuronal optical stimulation significantly decreases VP firing rate after the first s of stimulus, which is reverted in the following 59 s of stimulation. (**f**) Time histogram of VP electrophysiological activity in response to prolonged D2R-expressing neurons optical stimulation within the NAc. (**g**) Prolonged NAc stimulation of D2R-expressing neurons

decreases the activity of 58.5% of VP cells, increases the activity of 9.4% of cells and does not change the activity of 34% of cells in the first second of optical stimulation; however during the following 59 s of stimulation 47.2% of VP cells increases the activity, 7.5% of cells decrease activity and 28.3% of cells do not change the activity. (**h**) Prolonged accumbal optical stimulation of D2R-expressing neurons significantly increases VTA firing rate in the first second of stimulation, which is reverted in the following 59 s of stimulation. (**i**) Time histogram of VTA electrophysiological activity in response to prolonged optical stimulation of D2R-rxpressing neurons within the NAc. (**j**) Prolonged NAc D2R-expressing neuronal activation increases the activity of 44.4% of VTA cells, decreases the activity of 1.6% of cells and does not change the activity of 50% of cells during the fist second of stimulation; in the following 59 s of stimulation 55.6% of cells decrease activity, 13.9% increase and 30.6% do not change activity. (**k**) Spike latency of VP and VTA after prolonged accumbal optical stimulation of D2R-expressing neurons. (**l**) Schematic representation of recording electrode placement in the NAc, VP and VTA; numbers represent distance in millimeters to bregma. n=25 cells per brain region (4 rats). Error bars denote s.e.m. *p≤0.05, **p≤0.01, ***p≤0.001.

Discussion

The present data shows that D2R-expressing neurons of the NAc may play a dual role in Pavlovian conditioning depending on the optical stimulation protocol used to activate these neurons.

In general terms, the CPP paradigm is used to evaluate the rewarding and/or aversive effects of natural of pharmacological stimuli, including drugs of abuse[26,27]. Recently, this test has also been used to evaluate the reinforcing properties of optogenetic activation/inhibition of specific neuronal sub-populations (e.g. dopaminergic neurons of the VTA)[1,28,29]. CPP involves classic Pavlovian conditioning, i.e., the reinforcing/aversive properties of an unconditioned stimulus paired with a particular context (conditioned stimulus) biases animals' choice, inducing preference or aversion. Variations of the classic CPP have been proposed, namely the RTPP, in which animals can choose between a laser-associated chamber and a neutral one, in a contingent manner.

Several studies have shown that optogenetic stimulation of VTA dopaminergic neurons that causes an increased release in dopamine in the NAc is sufficient to trigger place conditioning[28], whereas inhibition of the same neurons causes place aversion[1]. Interestingly, pharmacological studies reveal a similar effect, since it was shown that given a choice between a compartment where animals received previously microinjections of dopamine agonists into the NAc and a compartment paired with vehicle, animals spend more time in the drug-paired compartment[30,31]. Thus, dopamine transmission in the NAc mediates the assessment of the motivational value of reward-related cues[32], such that these cues can subsequently trigger Pavlovian conditioning.

In the NAc, dopamine acts mainly in D1- and D2-MSNs though some interneurons also express dopamine receptors. Interestingly, intra accumbal administration of both dopamine D1R and D2R agonists in the NAc caused place conditioning[33]; however, other studies have proven otherwise, since animals do not self-administer D1R or D2R agonists[34].

More recently, optogenetic studies have been used to specifically stimulate D1- or D2-MSNs and understand their role in behavior. However, since optogenetic activation of D1R- or D2R-expressing neurons does not mimic the D1R or D2R intracellular signaling, optogenetic conditions may not reflect pharmacological manipulation.

Importantly, optogenetic activation (using 10 Hz blue light pulses over 3 minutes) of NAc D1-MSNs is sufficient to increase cocaine sensitization and cocaine place preference[29], while inhibition of the same neurons suppresses cocaine sensitization[35]. On the other hand, the same optical

stimulation of NAc D2-MSNs attenuated cocaine conditioned place preference[29] and supressed cocaine self-administration[11]. Interestingly, the same study showed that neither preference nor aversion for the blue light chamber could be observed in the absence of cocaine[29]. Remarkably, here we show that brief optical stimulation (40 Hz, 40 light pulses of 12.5 ms) of D2R-expressing neurons induces conditioning in the CPP, i.e., is reinforcing. Conversely, in the RTPP, we observed that accumbal optical stimulation of D2R-expressing neurons causes aversion. These apparently paradoxical findings could be explained by differences in the optical stimulus duration. In the CPP animals receive a brief activation stimulus (consisting of 40 light pulses), whereas in the RTPP animals were subjected to an average of 60 seconds of stimulus (average of time spent in laser-associated chamber). Both protocols induced a similar increase in NAc neuronal activity, but clearly different electrophysiological responses in downstream regions. Brief activation of D2R-expressing neurons supressed VP activity, similarly to previous studies showing that electrical stimulation of the NAc results in decreased pallidal activity[36]. VTA activity was increased in an indirect manner. Yet, longer stimulus originated clearly different neuronal responses. The first second of stimulus caused a significantly decrease in VP activity that shifted to an increase as the stimulus continued. In the same direction, while VTA activity increased in the first second of NAc stimulation, it decreased throughout the rest of the stimulation period. This decrease in VTA activity could explain the induced aversion observed with this protocol as previously described[37,38]. Importantly, to our knowledge, this is the first study describing opposing electrophysiological and behavioural effects of accumbal D2R-expressing neuronal manipulation. Nonetheless, at least one study has shown that immediate or prolonged stimulation of GABAergic populations of the laterodorsal tegmental area induced different behavioural results. Immediate activation produced fear-like responses, whereas prolonged stimulation caused anxiety-like behaviours[39]. Nevertheless, no electrophysiological correlates were used to explain these differences.

Our results expose one caveat with optogenetic experiments that needs to be taken into consideration. First, it is important to use stimulation protocols that generate a neuronal response as close as the physiological one, which is remarkably difficult, as not always this is extensively characterized. Second, it is very important to test different stimulation/inhibition protocols, and their impact in neuronal activity, not only in the stimulated region, but also in downstream targets and ultimately in behaviour. Thus, while some results found in the literature may seem at first sight contradictory, they may reflect different experimental conditions. For example, Lobo and colleagues reported that optogenetic stimulation of D2R-expressing neurons in the NAc caused aversion in a

cocaine-dependent CPP[29], contrary to our results that show that the stimulus is reinforcing. Apart from the fact that they used cocaine in their studies, their stimulus consisted in 3 minutes of light pulses[29], whereas we applied light pulses over 1 second (40 light pulses of 12.5 ms duration) every minute. As our results suggest, this can lead to differential responses in the VP and VTA, as well as in other non-analysed brain regions, which are crucial for the modulation of behavioural conditioning[2,18,40,41].

In conclusion, here we show that short optogenetic stimulation of D2R-expressing neurons in the NAc is reinforcing, while longer activation periods of the same neuronal population is aversive.

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CHAPTER 4

General discussion and conclusions

General discussion

In the present work we took advantage of different approaches to better dissect the role of the limbic circuit in reward (BOX 1) and motivation (BOX 2), taking a particular focus on the Nucleus accumbens (NAc), and its specific neuronal populations, namely dopamine receptor D1 (D1R)-expressing medium spiny neurons (MSNs, D1-MSNs) and dopamine receptor D2 (D2R)-MSNs (D2-MSNs). In addition to control individuals, we also used animals presenting prominent motivational deficits and D2R changes induced by prenatal glucocorticoid (GC) exposure, in order to better understand what is the impact of manipulating these neuronal populations in the context of behavioural dysfunction.

BOX 1 | REWARD

A positive emotional stimulus that promotes increased/repeated behavioural response leading to its acquisition.

BOX 2 | MOTIVATION

A measure of the effort an individual is willing to expend to obtain an object signalling reward delivery or to avoid an object signalling punishment.

In the following sections we will critically discuss the breakthroughs and limitations of this work, and present some ideas on future perspectives that we intend to develop to further advance our knowledge on the role of different striatal populations in reward and motivation.

A new clinically relevant model to study motivation: iuGC animals

Our first study investigated what was the impact on the reward circuit and motivated behaviours in a rat model of *in utero* exposure (iuGC) to the synthetic glucocorticoid dexamethasone (DEX) during gestation days 18 and 19 (iuGC animals).

This model was initially created to mimic the clinical setting of administration of synthetic GCs to pregnant woman in risk of preterm labour[1] to ensure foetal lung maturation. On these circumstances, synthetic GCs (commonly DEX or betamethasone) are administered because they have been shown to reduce the risk for the newborn to develop respiratory distress syndrome, and decrease the prevalence of neonatal death[2]. Yet, despite these obvious beneficial effects in foetal organ maturation, several studies showed a deleterious effect for these synthetic hormones in both animal models and humans. Children exposed to DEX and born at term, have increased

emotionality, unsociability/social withdrawal and general behavioural problems[3], as well as persistent impairments in tests of verbal working memory[4,5]. The offspring of women to whom multiple doses of antenatal GCs were given have reduced head circumferences, and show significantly increased aggressive/violent behaviour and attention deficits[6]. Moreover, *in utero* exposure to GC/stress potentiates the appearance of cognitive, mood, affective and addictive disorders[6–10]. Studies in animal models also showed that synthetic GC exposure induces depressive-like behaviour, anxiety and social deficits[1,11–14]. Nevertheless, it is important to refer that other studies have found no differences in GC-exposed individuals[15,16].

Does this model really mimic the human condition?

The answer to this question is not clear. The current dosage given to pregnant woman consists in 4 doses of DEX 6 mg intramuscularly in a 12-hour period - approximately 0.4 mg kg^{-1} (assuming a 60 kg woman). However, in the past it was common to administer multiple courses of synthetic GCs[2,17–20]. We administer 2 doses of 1 mg kg^{-1} in our rat model, which is higher than the current dosages used in clinics. Yet, this compound has different pharmacokinetic profiles depending on the species to which it is administered. Importantly, it was shown that the half-life of DEX 21-isonicotinate is significantly lower in rats and rabbits in comparison to humans (rat and rabbit: 15-30 minutes, humans: 90-100 minutes)[21–23].

Despite the limitations of a direct translation of this rodent model to humans, some of the anatomical, molecular and behavioural changes observed in iuGC animals are in agreement with human and other primate findings that result from prenatal exposure to DEX (summarized in Table 1)[1,3,5,11–13,24–36].

Table 1. Effect of prenatal exposure to DEX in anatomical, molecular and neurological/behavioural parameters in human (or non-human primates) and rodents.

Prenatal Exposure to Dexamethasone		References
Anatomical		
Rodent	Decreased body and brain weight; hippocampal damage	DeKosky *et al*., 1982
	Decreased body weight	Oliveira *et al*., 2006
	Decreased volume of nucleus accumbens	Leão *et al*., 2007; Rodrigues *et al*., 2012
	Decreased volume of amygdala and increased volume of bed nucleus of stria terminalis	Oliveira *et al*., 2012
	Accumbal neuronal atrophy	Leão *et al*., 2007; Rodrigues *et al*., 2012
Human/non-human primate	Decreased birth weight	Bloom *et al*., 2001
	Decreased hippocampal volume	Uno *et al*., 1990
Molecular		
Rodent	Increased GR expression in the brain	Benesová *et al*., 1989
	Altered HPA axis activity, increased GR expression	Shoener *et al*., 2006
	Decreased corticotropin-releasing factor mRNA in the hypothalamus	Nagano *et al*., 2008
	Impaired HPA axis	Oliveira *et al*., 2006
	Increased expression of D2R in the nucleus accumbens, amygdala and prefrontal cortex	Rodrigues *et al*., 2012; Oliveira *et al*., 2011
	Decrease in TH^+ cells in the ventral tegmental area and decrease in proliferative cells	Leão *et al*., 2007
Human/ non-human primate	Degeneration of hippocampal fibres	Uno *et al*., 1990
	Alterations in the HPA axis response	de Vries *et al*., 2007
	Decrease in neuronal proliferation, calretinin- and calbindin-positive neurons in dentate gyrus	Tauber *et al*., 2006
Neurological/Behavioural		
Rodent	Increased emotional reactivity, hyperactivity	Benesová *et al*., 1989
	Memory deficits and anxiety	DeKosky *et al*., 1982
	Increased anxiety-like behaviour	Nagano *et al*., 2008
	Hyperanxious behaviour	Oliveira *et al*., 2006
	Depressive-like behaviour; anhedonic behaviour; anti-social behaviour	Roque *et al*., 2009; Borges *et al*., 2013
	Drug-seeking behaviour	Rodrigues *et al*., 2012
Human/ non-human primate	Shyness, increased emotionality, less sociability and greater avoidance; no differences in cognitive abilities or behavioural problems	Trautman *et al*., 1995
	Social anxiety and impaired verbal working memory	Hirvikoski *et al*., 2009
	Increased risk of psychomotor impairments	Lee *et al*., 2008
	Neurodevelopmental abnormalities	Spinillo *et al*., 2004
	Altered neurological development	Salokorpi *et al*., 1997
	Decrease in social play; altered motivation for food	Hauser *et al*., 2008

But how relevant and valid is this model for the study of motivation?

To answer this question, it is essential to first describe what is motivation. According to Salamone, motivation is "the set of processes through which organisms regulate the probability, proximity and availability of stimuli"[37]. Motivation is crucial for daily decision-making processes. Everyday we have to take actions based on the outcome; the selection of an appropriate action is dependent on the value attributed to the outcome but it is also dependent on the motivational status of the individual.

A motivated behaviour occurs in phases, in which the individual seeks to approach or avoid the stimulus (commonly designated as appetitive, preparatory, instrumental, approach or seeking phase); and the consummatory phase, in which individual gains access to the goal stimulus (or avoids it). To obtain the goal, individuals often have to work (press a lever, nose poke, run, ...) and the degree of effort reflects their motivational drive.

The most thoroughly studied circuit involved in motivation is the mesolimbic pathway[38–40], comprising dopaminergic projections from the midbrain and in particular the ventral tegmental area (VTA) to the NAc. For long is has been shown that manipulations of the mesolimbic dopaminergic system modifies motivation to take food ("wanting") as well as to take drugs[39,41–43]. Importantly, early life events such as exposure to stressors or increased levels of GCs robustly modify responses towards rewards[13,44–49]. This may arise due to changes in the dynamics of the mesolimbic circuit since it is enriched in GC receptors, and neurons from this circuit are only fully mature 2 weeks post-natal in rats[50] and in adolescence in humans[51]. Indeed, our initial findings showed that iuGC animals present a significant reduction in dopaminergic fibres in the VTA and reduced innervation of the NAc[33]. In this thesis, we further showed that these animals also present significant morphological and dopaminergic abnormalities in the medial prefrontal cortex (mPFC) and orbitofrontal cortex (OFC) (Chapter 2). Importantly, iuGC animals also present reduced NAc volume and altered neuronal morphology[33,49], either this being a direct cause of GC effects or a consequence of altered VTA-NAc projections. In any way, these changes are consistent with anhedonia, depressive- and anxious-like behaviours[1,12,13], social deficits[13], as well as motivational deficits toward natural rewards observed in iuGC animals (Chapter 2).

Motivation levels were evaluated using the Pavlovian-to-instrumental-transfer test (PIT), which relies on the premise that Pavlovian conditioned stimuli (CS) can influence/invigorate instrumental responding to receive a specific reward (reviewed by Cartoni *et al.*[52]). This test measures the

dynamic attribution of incentive salience (BOX 3) to reward-related stimuli, causing them to become motivationally "wanted".

BOX 3 | INCENTIVE SALIENCE

It is a conditioned motivational response usually triggered by and assigned to a reward-related stimulus. Is related, but not reducible, to the stimulus's sensory representation or what has been learned about it. Formally, it is a motivational transformation of a reward-related neural representation, such as a perceived or recalled conditioned stimulus or unconditioned stimulus.
According to Berridge, incentive salience, or 'wanting', is a specific form of Pavlovian-related motivation for rewards, that typically gives a felt 'oomph' to declarative desires, but can also occur unfelt as a relatively unconscious process.

Early studies by Balleine and colleagues identified the NAc as a fundamental substrate underlying PIT since lesions in this area eliminate it[53]. In addition, human studies show that enhanced motivation, measured by increased PIT performance, is positively correlated with increased activity of the NAc[54,55]. iuGC animals presented significant neurochemical and morphological changes in the NAc[33,49] and a decreased in general and selective PIT performance, i.e. present reduced incentive motivation. One paradoxical finding was that, although iuGC animals present decreased motivational drive toward natural rewards (food), they present enhanced conditioning induced by drugs[49]. This may suggest that iuGC exposure causes a bias in incentive/motivational processes that render these animals higher sensitivity to the rewarding effects of drugs of abuse but less so for natural rewards. Interestingly, this behavioural dichotomy is known to be present in cases of drug addiction that highly affect the dopaminergic system, like amphetamines, since addicted individuals prefer them as opposed to natural rewards[39,56–58].

Rescue of motivational deficits of iuGC rats: role of dopamine and D2R

We previously showed that normalization of dopamine levels by systemic levodopa (L-DOPA) administration (dopamine precursor) rescues drug-seeking behaviour[49], and here, we further reported that this treatment is sufficient to normalize motivational drive of iuGC rats for food (Chapter 2). These results are in agreement with previous reports that show that increasing dopamine levels in the NAc is crucial for the regulation of motivational status[59,60].

We further extended these results by showing that D2R, but not D1R, agonist treatment also rescue the motivational deficits of iuGC animals (Chapter 2). These results were interesting because they showed that D2R dysfunction lead to deficits in motivation, contrary to the

predominant view in the literature that highlighted D1R in mediating reward-related responses. Yet, iuGC animals presented D2R rather than D1R changes in several brain regions. Moreover, one study showed that increased expression of D2R in the NAc boosts PIT performance[61]. Interestingly, in humans, approach and avoidance learning toward rewarding or aversive stimuli is dependent on individuals' variability in striatal D2R binding[62], and striatal asymmetries in D2R binding predict reward-dependent learning[63]. In addition, healthy subjects that have higher D2R availability in the striatum show greater incentive motivation[64], further supporting a role for D2R-expressing neurons in reward and motivation processing.

In this view, it is important to refer that exposure to stress (mainly occurring during adolescence) or stress hormones cause severe modifications in D2R binding and/or activity (nicely reviewed by Sinclair *et al.*[65]), which may increase susceptibility to develop motivational deficits. In fact, adolescents reporting low parental care during infancy present lower activity of D2R in the ventral striatum[66]. In schizophrenia, which aetiology has for long been correlated with maternal exposure to stress or stress hormones[67,68], impairment in striatal D2R function or availability is frequently observed in patients[69,70]. Importantly, this striatal D2R dysfunction, together with general dopaminergic improper function, is believed to underlie the patient's attribution of increased incentive salience to otherwise irrelevant stimuli[71]. Additionally, addicted subjects often present alterations in the thresholds required for D2R activation by dopamine[72], which might represent a mechanism through which prenatal stress or prenatal exposure to increased levels of GCs causes drug-seeking and addiction[9,46,73].

It is tempting to speculate that pharmacological manipulation of D2R activity in the context of prenatal GC-triggered deficits in motivation may be a promising approach in the management of some behavioural deficits. Nonetheless, though systemic administration is preferred in a clinical translational perspective, it lacks anatomical specificity. With this limitation in mind, we next decided to use optogenetic tools to manipulate specific neuronal populations of the NAc and evaluate their impact in motivation.

Nevertheless, it is important to state that optogenetic stimulation/inhibition does not necessarily mimic the physiological activation/inhibition of the neuronal population targeted. In fact, in the context of D1- or D2-MSNs, it is important to stress that activation through ligand binding to the receptor is very complex and results in the activation of intracellular signalling cascades (vide general introduction and Fig. 1), whereas optogenetic manipulation of these

neurons relies on the entry of ions that will result in membrane depolarization/hyperpolarization[74]. In this sense, while activation of D2R by dopamine binding for example, may turn the neuron less responsive[75], optogenetic stimulation does the opposite.

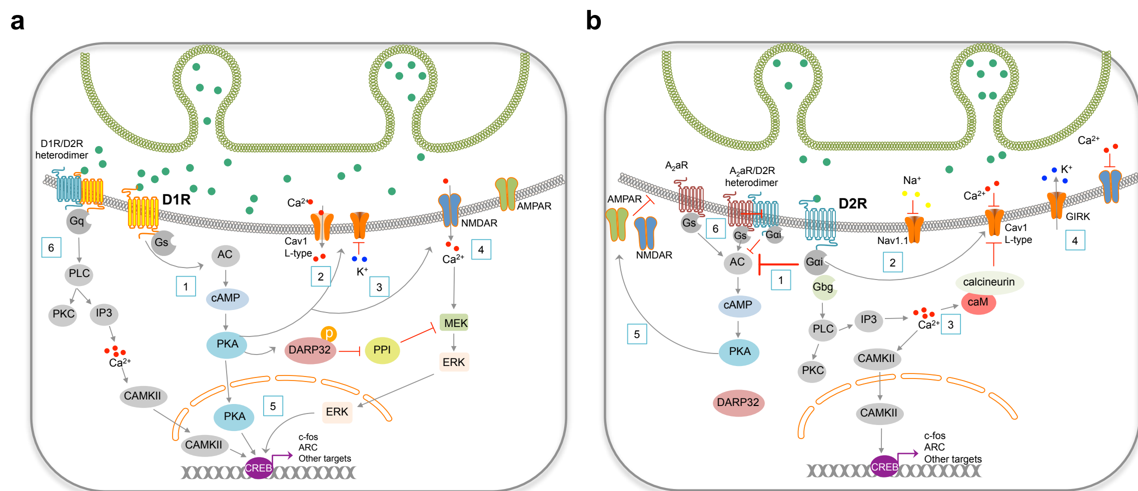

Figure 1. D1R and D2R activation leads to distinct cellular effects. (a) Simplified scheme of D1R signalling. (1) Activation of D1R stimulates G_s proteins that are positively coupled to AC, leading to the production of cAMP and the activation of PKA. PKA induces opening of L-type Ca^{2+} channels and inhibits/closes K^+ channels (2). (3) PKA-dependent phosphorylation of AMPA and NMDA receptors, which enhances membrane expression of these receptors. (4) Stimulation of D1R also increases Ca^{2+} influx through NMDA receptors. This activates a number of signalling pathways, including ERK via the Ras-Raf-MEK cascade. (5) PKA can modulate CREB activity directly or by phosphorylating DARPP-32, inducing the disinhibition of the NMDA/Ras-GRF1/ERK pathway, culminating in the modulation of transcription of target genes. (6) Activation of the G-coupled D1R/D2R heteromer induces a PLC-dependent calcium release, resulting in the activation of CaMKII and its translocation to the nucleus. **(b)** Simplified scheme of D2R signalling. (1) D2R-like receptors activate G_{ai} and G_{ao} proteins, which inhibit AC and limit PKA activation that regulates several voltage-gated K^+, Na^+ and Ca^{2+} channels. (2) Released G_{bg} subunits are capable of reducing Cav1 L-type channel, and activate PLC and PKC, increasing IP3, mobilizing Ca^{2+} stores. (3) Activation of calcineurin can also block Cav1 L-type activity. (4) D2R signalling enhances K^+ channel opening. (5) PKA modulates glutamate receptor incorporation in the membrane. (6) Striatal D2-MSNs co-express A_2aR. A_2aR and D2R are coupled to different G proteins and modulate AC activity in opposing directions. Interestingly, there is also the existence of an antagonistic allosteric A_2aR-D2R interaction.

AC: adenylyl cyclase; cAMP: cyclic adenosine monophosphate; PKA: protein kinase A; AMPAR: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid glutamate receptor; Arc: activity-regulated cytoskeleton-associated protein; CaMKII: calcium/calmodulin-dependent protein kinase type II; CREB: cAMP responsive element binding protein; ERK: extracellular signal-regulated kinases; GIRK: G protein-coupled inwardly-rectifying potassium channels;IP3: inositol 1,4,5-trisphosphate; MEK: mitogen-activated protein kinase; Nav1.1: sodium channel, voltage-gated, type I; Cav1 L-type: Calcium channel, voltage-dependent, L type; PLC: phospholipase C; PKC: protein kinase C; P-DARPP-32: phosphorylated dopamine- and cAMP-regulated phosphoprotein 32 kDa; PP1: protein phosphatase 1; NMDAR: N-methyl-D-aspartate glutamate receptor; PLC: phospholipase C; IP3: inositol (1,4,5)-trisphosphate; D1R: dopamine receptor D1; D2R: dopamine receptor D2; A_2aR: adenosine receptor 2a.

Role of NAc D1R- and D2R-expressing neurons in reward and motivation

Considering the importance of the NAc for reward processing, we were expecting that the rescuing effect observed by the pharmacological manipulation would be due to normalization of dopamine/D2R signalling in this brain region. This NAc centric view, however, lacked confirmation and could be interpreted as too simplistic considering the multitude of brain regions involved in reward/motivated behaviours[76–80]. So, our first approach was to score the activation pattern of different brain regions after two motivation-dependent tasks: PIT and the progressive ratio (PR) schedule of reinforcement test. PIT measures incentive motivation as previously mentioned, and PR test directly measures motivation, by determining the amount of work (breakpoint) that animals are willing to exert to obtain rewards in an operant task[81].

The NAc clearly popped-up has the brain region that mostly contributed for motivational performance (Chapter 3.1), which prompted us to further study this brain region. One caveat however, is that we did not evaluate all the brain regions associated with motivation to date, leaving some brain regions like the lateral habenula (LHb) (reviewed in[82]) out of the analysis.

The NAc is mainly composed by D1- and D2-MSNs, which have been suggested to have opposing functional roles. D1-MSNs are associated with reward and D2-MSNs with aversion[83–85]. However, our behavioural data suggested that D2R impairment is associated with motivational deficits (Chapter 2), which was an enigmatic finding at the time.

Next, we found that both neuronal subtypes were recruited during a motivation-dependent task (PR test) and that their degree of activation was positively correlated with motivational drive of the individual. In agreement, optogenetic stimulation of either D1- or D2-MSNs within the NAc resulted in the same increase in motivation (Chapter 3.1). This was the first report showing the absence of a behavioral dichotomy between the NAc D1- and D2-MSNs in the processing of natural rewards.

Since the stimulation was done during the cue exposure period, we believe that we are enhancing the value attributed to a reward predicting cue, i.e, increasing its incentive salience. Now, it would be interesting to evaluate the effects of stimulating/inhibiting D1- and D2-MSNs in different stages of reward/reinforcement. In fact, these experiments could be complemented with *in vivo* imaging techniques of D1R- and D2R-expressing neuronal populations as previously done for the dorsal striatum by Costa's lab[86].

Taking a step back and reappraising NAc D2-MSN function in reward

To definitely confirm our puzzling findings regarding D2R-expressing neurons in motivation, we used a different strategy in rats that relies on the use of channelrhodopsin (ChR2)/halorhodopsin (eNpHR) under the control of the D2R minimal promoter region (Chapter 3.1). This is particularly relevant because some cre transgenic lines present ectopic expression of the cre protein[87,88]. We replicated the findings observed in mice, i.e., activating NAc D2R-expressing neurons in rats enhances motivation (Fig. 2), and further showed that inhibition of these neurons resulted in the opposite behavioural effect (Chapter 3.1). The specificity of the stimulation was further validated by electrophysiological confirmation in a D2-MSN direct target region, the ventral pallidum (VP) (Fig. 2).

Yet, it is important to refer that the enhancement in motivation may arise from *indirect/downstream* effects since accumbal stimulation of D2R-expressing neurons caused a significant increase in the electrophysiological activity of the VTA (Chapter 3.1) (Fig. 2). It was previously shown that phasic optogenetic activation of dopaminergic VTA neurons induced positive reinforcing actions in a food-seeking operant task, and that its activation is sufficient to reactivate previously extinguished food-seeking behaviour in the absence of external cues[89]. Interestingly, these data suggest that activation of dopaminergic neurons promotes the development of positive reinforcement during reward seeking[89], which is in agreement with what we report here. Interestingly, others showed that phasic activation of VTA dopaminergic neuronal population is sufficient to drive behavioural conditioning and elicit dopamine transients[90]. In fact, one may speculate that the increased motivational status caused by accumbal stimulation of D2R-expressing neurons may prompt an increase in dopamine release in the NAc during the cue exposure period[90,91], through indirect activation of VTA electrophysiological activity, thus increasing cue-induced incentive salience.

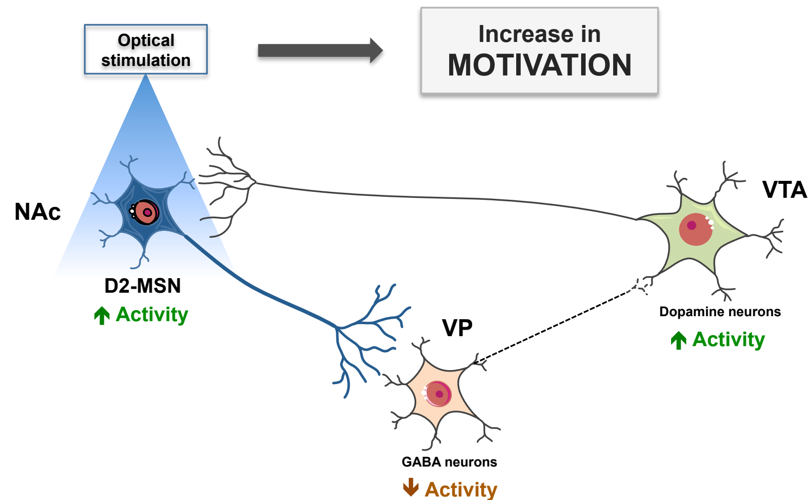

Figure 2. Optical stimulation of D2R-expressing neurons within the nucleus accumbens increases motivation. In normal animals, the NAc acts on the indirect pathway through D2-MSNs (GABAergic) projecting to the VP (a GABAergic population), which in turn will project to the VTA (highly enriched in dopaminergic neurons); optical stimulation of D2-MSNs in the NAc causes a significant increase in the electrophysiological activity of the NAc, a significant decrease in VP and an increase in VTA, which behaviourally results in increase in motivation.

D2-MSN: dopamine receptor D2-expressing medium spiny neuron; NAc: nucleus accumbens; VP: ventral pallidum; VTA: ventral tegmental area; ↑*: increased;* ↓*: decreased.*

In addition, dopaminergic neurons of the VTA are held at a constant hyperpolarized, inactive state, through inhibitory postsynaptic potentials[92] originated by the spontaneously active VP GABAergic neurons[92,93]. Therefore, decreased activity of this brain region (achieved by D2-MSN stimulation) releases VTA dopamine neurons from inhibition and may enable them to fire[92,94] and probably release higher amounts of dopamine to the NAc. This effect may be contributing to the behavioural outcome that we observed. In this perspective, one could use fast-scan cyclic voltammetry (FSCV) together with optogenetic stimulation, in order to assess real-time dopamine release upon optical stimulation of accumbal D2R-expressing neurons in animals performing behaviour[89,90,95].

In further agreement with the hypothesis that activation of D2R-expressing neurons is modulating dopamine release by indirect activation of VTA projections is the fact that blocking dopaminergic action within the NAc by administration of either D1R or D2R antagonists resulted in ablation of the motivational increase caused by accumbal optical stimulation of these neurons (Chapter 3.2). Even without optical stimulation, pharmacological blockage of the action of D1R and D2R in the NAc resulted in decreased breakpoints (Chapter 3.2). Accordingly, previous reports show that either systemic or local administration (in the NAc) of D1R and D2R antagonist greatly

reduces operant responding[96–98], further supporting the relevance of dopaminergic inputs from the VTA to the NAc on modulating the action of MSNs and control reward-related behaviour[99–101].

Further evidence that support the concept that D2R-expressing neurons-dependent increase in motivation is dependent on increased dopamine release by VTA terminals is the fact that inhibition of cholinergic action in dopaminergic terminals (that causes a drop in dopamine release[102–104]) also reduces animal's willingness to work for food (Chapter 3.2). Indeed, the involvement of cholinergic interneurons (CINs) (Fig. 3) in several functions of the brain, such as associative learning, motor control and reward processing has been for long recognized[105–108]. CINs have fast and regulated spontaneous activity and respond to motivationally salient stimuli through a pause and subsequent rebound increase in firing[109–112]. In addition, electrophysiological activity of CINs is closely correlated with phasic activity of the dopaminergic neurons and contributes to the presynaptic regulation of dopamine release in the NAc[103,111,113,114].

NAc cholinergic activity is necessary for the maintenance of motivated behaviours[105–108], and around 80% of CINs express D2R[115], which could be a confounding factor in the interpretation of our results. However, the stimulation parameters we used did not induce a significant activation of CINs (Chapter 3.1). Actually, we observed a similar recruitment of NAc CINs in both stimulated-control and stimulated-ChR2 rats (Chapter 3.1), showing that the behavioural outcome is likely due to D2-MSN activation. One drawback however, is that we only used c-fos as a marker for neuronal activation and the dynamics of gene expression caused by neuronal activation might be far more complex than the one translated by c-fos expression[116,117]. One could use other markers such as egr-1 or Arc to further confirm our results[118]. Patch clamp experiments coupled with optogenetic stimulation of D2R-expressing neurons would also allow determine if non-MSN neurons are being recruited.

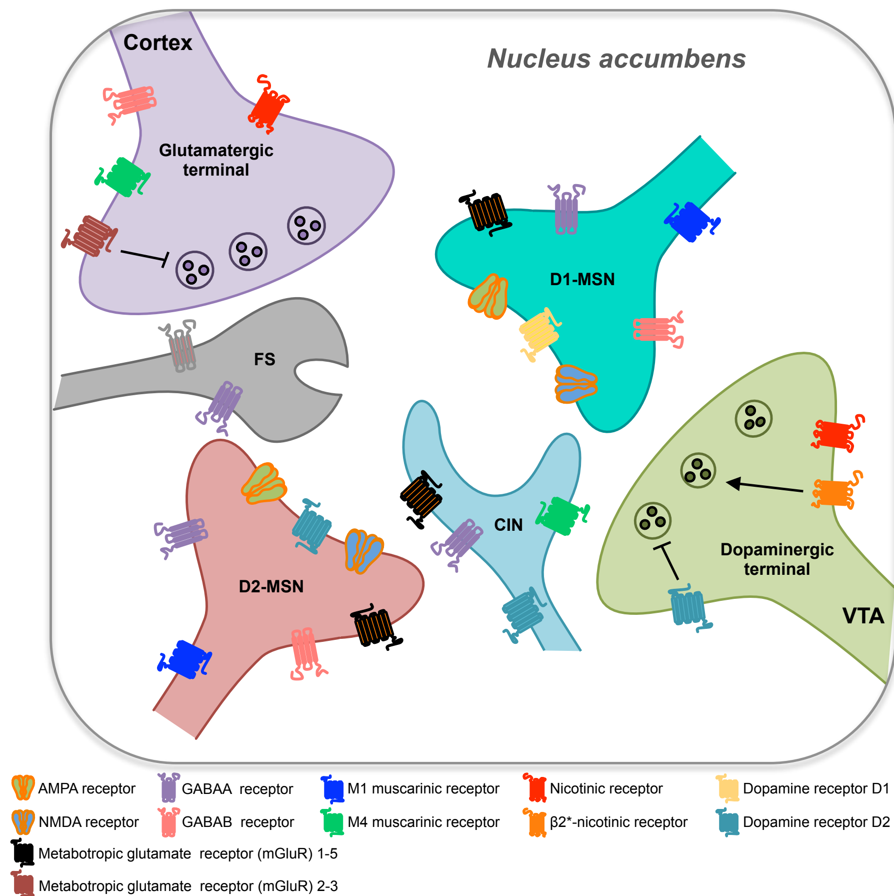

Figure 3. Schematic representation of NAc neuronal populations and expression of specific receptors. The NAc is comprised majorly by D1- and D2-MSNs. These neurons are mainly activated by the release of dopamine from terminals originated in the VTA; however this brain region is also richly innervated by glutamatergic projections arising from the cortex. MSNs express, not only dopamine and glutamate receptors, but also receptors for GABA and ACh. Smaller populations of accumbal neurons are the CINs that also highly express D2R (around 80% of CINs), and the FS GABAergic interneurons. These populations receive GABAergic projections from the MSNs and glutamatergic projections from the cortex. Ach is able to rigorously control the activity of the NAc, since several of accumbal neuronal populations, and glutamatergic and dopaminergic terminals, express receptors for this neurotransmitter. While activation of D2 autoreceptors present in VTA terminals negatively influences the release of dopamine in the NAc, activation of cholinergic β2*-nicotinic receptors increases its release. Activation of mGluR 2-3 receptors expressed in glutamatergic cortical terminals inhibits glutamate release in the NAc.

ACh: acetylcholine; CIN: cholinergic interneuron; D2R: dopamine receptor D2; GABA: gamma-aminobutyric acid; FS: fast spiking interneuron; MSN: medium spiny neuron; NAc: nucleus accumbens; VTA: ventral tegmental area.

Regardless of the previous results obtained in control animals, we were uncertain on the effects of activating D2R-expressing neurons in the context of behavioural dysfunction. iuGC exposure causes significant reward deficits (Fig. 4) in different behavioural paradigms, namely PIT and PR (Chapters 2 and 3.1). Interestingly, previous work form our team showed that these animals present long lasting changes in D2R mRNA and protein expression in the NAc, due to the

altered epigenetic status of the D2R promoter region[49]. Besides affecting its expression, iuGC exposure also causes a decrease in the recruitment of D2R-expressing neurons in the PR test (Chapter 3.1). Specific activation of D2R-expressing neurons in the NAc resulted in the normalization of the motivational levels toward natural rewards (food) in iuGC rats (Fig. 4), a result that was confirmed with two distinct behavioural paradigms (Chapter 3.1). Importantly, our optogenetic manipulation of D2R-expressing neurons resulted in neuronal recruitment to levels comparable with control non-stimulated rats.

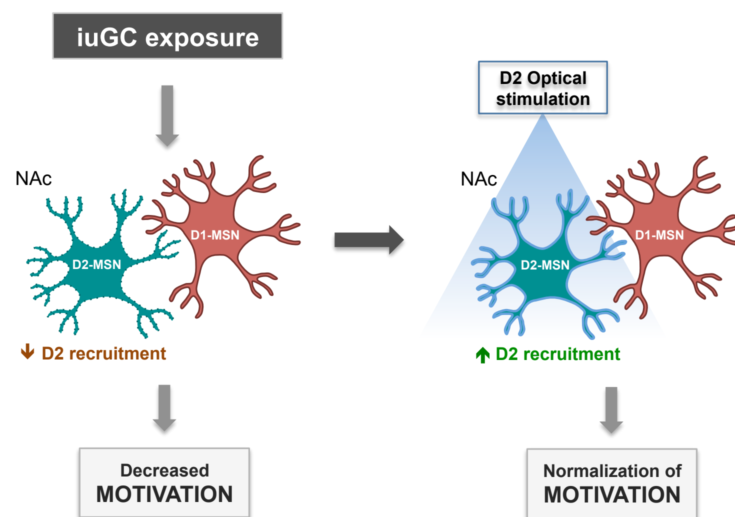

Figure 4. Optical stimulation of D2R-expressing neurons within the nucleus accumbens of iuGC-exposed rats normalizes its motivational deficits. iuGC exposure causes a significant impairment in the recruitment of D2R-expressing neurons, resulting in decreased motivational levels toward natural rewards. Optical stimulation of this specific accumbal neuronal population is sufficient to normalize motivational deficits of iuGC-exposed rats.

D1-MSN: dopamine receptor D1-expressing medium spiny neuron; D2-MSN: dopamine receptor D2-expressing medium spiny neuron; iuGC: in utero glucocorticoid exposed rats; NAc: nucleus accumbens; ↑: increased; ↓: decreased.

In summary, our results suggest that NAc D2-MSNs (and D1-MSNs) play *pro-motivational* actions, at least to what concerns natural rewards.

Same neurons, opposing behavioural effects: the importance of controlling the parameters of optogenetic stimulation

Although we show that that optical stimulation of D1- and D2-MSNs during the exposure of a reward-predicting cue enhances motivation, others have reported somewhat opposing results for these two neuronal populations in both ventral and dorsal striatum (though using different

behavioural paradigms)[84,85]. Apart from anatomical specificities from dorsal and ventral striatum[119,120], one can hypothesize that these neurons can also have distinct roles in different behaviours. Moreover, optical stimulation protocols may greatly influence the behavioural outcomes. Because of this, we tested two additional stimulation protocols of D2R-expressing neurons in the PR test (the same as used by Lobo *et al.*[84] and the same as used by Kravitz *et al.*[85]), which also resulted in motivation enhancement (Chapter 3.1).

However, in the study conducted by Lobo and co-workers[84], activation of NAc D2-MSNs reduced cocaine conditioning in a CPP paradigm (whereas D1-MSN activation enhanced CPP), suggestive of an antagonist role of D2R-expressing neurons in cocaine rewarding effects. Comparing these results with ours requires caution. First, the use of cocaine as reinforcer, which is known to alter neural signalling in the mesolimbic system[121–123], may affect the behavioural outcome. Apart from this, a conditioning paradigm such as the CPP and an instrumental task (PR) evaluate clearly different behavioural dimensions [124–129]. In a conditioning paradigm, the intrinsic reinforcing properties of the optogenetic stimulus are tested (rewarding vs aversive)[84,90,128,129], whereas in the PR, we evaluate willingness to work to obtain a reward. In this last, the intrinsic properties of the stimulus do not necessarily determine the animals' behaviour, but rather serve to influence their response towards a cued action that predicts the delivery of a positive reinforcer[89,125,126].

To further understand the role of D2R-expressing neurons in reward/reinforcement, we tested the animals in an unbiased CPP test, in which one of the chambers was paired with optical stimulation. Brief optical stimulation of NAc D2R-expressing neurons (40 Hz, 40 pulses of 12.5 ms, every minute) was sufficient to induce place preference, i.e., was reinforcing. Electrophysiological measurements showed that this was associated with inhibition of VP neuronal firing and consequent increase in VTA activity (Chapter 3.3). Additionally, inhibition of the same neuronal population caused an aversive effect.

D2-MSNs can regulate basal ganglia activity at least in part through GABAergic monosynaptic connections to the VP (and globus pallidus) [130–134]. This may cause disinhibition of glutamatergic nuclei of the thalamus and cortex[133,135,136], that can result in increased motivational levels. In agreement with this is the fact that pharmacological inhibition of glutamatergic signals to the NAc through specific AMPA and NMDA receptors antagonists normalized the D2R-expressing neuronal-dependent increase in motivational levels (Chapter 3.2).

Interestingly, a more prolonged optical stimulation of D2R-expressing neurons induced the opposite effect, i.e., it caused a significant decrease in preference. This was observed using two different long stimulation protocols (40 Hz, 400 or 2400 light pulses of 12.5 ms duration). Strikingly, we show that the pattern of activation of VP and VTA neurons differs when the stimulation is more prolonged in time: VP activity is reduced in the 1st second of stimulation and increases in the rest of the stimulation period, contrary to the VTA (1st second: increased, rest of period: decreased). Moreover, this aversive effect was further confirmed in a distinct conditioning paradigm, the real-time place preference, in which we showed that rats had reduced time spent in the chamber paired with accumbal optical activation of D2R-expressing neurons.

Interestingly, a previous study showed that activation of D2-MSNs (occurring for 5 minutes, with pulsed light) causes a significant decrease in animals' motivation to work for cocaine in a PR test[130]. However, while the authors associate this behavioural effect with inhibition of VP activity, they only tested the electrophysiological effects of a brief D2-MSN activation (16.6 Hz, 33 pulses of 0.5 ms), and not with the longer stimulation[130].

These results emphasize the importance of cautious interpretation of optogenetic experiments and the need to test different stimulation protocols and to assess the pattern of neuronal activation/inhibition *in loco* and in downstream regions. Different studies showed an aversive role for D2-MSNs in valenced behaviours[84,85], which was paradoxical comparing with some of our data. In this view, part of the differences might be explained by methodological discrepancies. Nevertheless, we do observe a positive effect of stimulation of D2R-expressing neurons in cue-driven enhancement in motivation regardless of the type of stimulation, suggesting that D2-MSNs are more relevant in *pro-motivational* aspects than previously anticipated.

Looking into the future: different questions, models and approaches to improve the study of the reward circuit

At this stage, it is important to present some methodological alternatives to answer the question of this thesis: *What is the role of accumbal D1- and D2-MSNs in motivation? Is there really a dichotomy between accumbal D1R- and D2Rexpressing neurons in rewarded behaviour?*

By using the INTRSECT technique – INTronic Recombinase Sites Enabling Combinatorial Targeting[137] – one could combine different transgenic lines (that express specific reporters, such as cre, flp, dre, rox in specific neurons) to target particular subset of striatal neurons and control

confounding parameters. For example, we could activate D2R-expressing neurons but not those that are $D2R^+ChAT^+$, excluding D2R-expressing CINs neurons from the activation protocol.

Furthermore, although in our work we show that only a very small percentage of D1R-expressing neurons also expressed ChR2 (Chapter 3.1), we could not fully exclude a possible influence of D1R/D2R-coexpressing MSNs from the behavioural outcome observed. In order to exclude the influence of D1R-expressing neurons from the observed behavioural changes it could be interesting to perform stimulation in the D2-MSN terminals in the VP.

In spite of the pivotal role played by pallidal nuclei in reward processing, it is known that NAc projections to other regions of the basal ganglia, such as subthalamic nucleus (STN)[138] and thalamus[135,139] also play a crucial role in reward behaviour. So, it would be interesting to further investigate the effects o NAc D2-MSN optical stimulation in the activity of these brain regions.

In addition to behavioural interpretation of manipulating specifically D1- and D2-MSNs, it would be very interesting to map the activity of these neural circuits. For example, an optogenetic approach together with 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.

In vivo imaging would also be a crucial technique to better dissect the role of different striatal populations in behavioural performance. One example is the use of 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 fibres to monitor neuronal activity along time[86,140–143]. As this technique can be applied in freely moving animals, its application in our context would allow the identification of the recruitment of D1R- and D2R-expressing neurons in precise moments of reward-related behaviours. Interestingly, using this technique, Cui and colleagues showed that both direct and indirect pathways of the dorsal striatum present a transient increase in neural activity when animals initiate actions[86], data that also challenged the classical view of the dichotomous function of dorsal striatum in motor control. More recently, this technique was also used to investigate the role of D2R-expressing neurons in risk-decision making[142]. Interestingly they show that risk-preferring rats convert instantaneously to risk-averse when receiving accumbal D2R-expressing neuronal optical stimulation[142], suggesting that individual differences in risk (unfavourable)-preference can be largely encoded by D2R-expressing neurons in the NAc.

Conclusions

In the present thesis we showed that:

1. Prenatal exposure to high levels of synthetic glucocorticoids (iuGC model) causes a significant impairment in motivation, associated with changes in D2R in the mesocorticolimbic system.
2. Systemic administration of a dopamine precursor (L-DOPA) or D2R agonist (but not D1R agonist) reverts reward-related deficits in iuGC rats.
3. Recruitment of both D1R- and D2R-expressing neurons within the NAc is positively correlated with motivational drive.
4. Optogenetic activation of NAc D1R- or D2R-expressing neurons enhances motivation.
5. The behavioural effect of NAc optical stimulation of D2R-expressing neurons is dependent on cholinergic modulation of VTA dopaminergic terminals.
6. Brief NAc optical stimulation of D2R-expressing neurons is sufficient to induce Pavlovian conditioning, while prolonged stimulation of the same neurons causes aversion. This paradox is probably explained by the observed changes in the electrophysiological activity of target regions caused by different stimulation protocols.

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APPENDICES

APPENDIX 1

Reappraising striatal D1- and D2-neurons in reward and aversion

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Review article

Reappraising striatal D1- and D2-neurons in reward and aversion

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ABSTRACT

The striatum has been involved in complex behaviors such as motor control, learning, decision-making, reward and aversion. The striatum is mainly composed of medium spiny neurons (MSNs), typically divided into those expressing dopamine receptor D1, forming the so-called *direct pathway*, and those expressing D2 receptor (*indirect pathway*). For decades it has been proposed that these two populations exhibit opposing control over motor output, and recently, the same dichotomy has been proposed for valenced behaviors. Whereas D1-MSNs mediate reinforcement and reward, D2-MSNs have been associated with punishment and aversion.

In this review we will discuss pharmacological, genetic and optogenetic studies that indicate that there is still controversy to what concerns the role of striatal D1- and D2-MSNs in this type of behaviors, highlighting the need to reconsider the early view that they mediate *solely* opposing aspects of valenced behaviour.

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1. Introduction

For decades, researchers have been trying to dissect the role of striatal neurons in reward and reinforcement, and more lately in aversion and punishment. The study of the circuits underlying these behavioural dimensions is particularly relevant to better understand the basis of addiction and mood disorders, namely depression. For example, imaging studies showed that discrete circuits that are dependent on key elements such as the ventral tegmental area (VTA) and the ventral striatum suffer deep neuronal and structural changes in addicted individuals (reviewed by Koob and Volkow, 2010). Likewise, patients with mood disorders display reduced nucleus accumbens (NAc) volume and activation (Baumann et al., 1999; Heller et al., 2009). Moreover, animal studies have shown that different striatal populations contribute differently for depressive-like behaviour (Francis et al., 2015).

The development of new transgenic animal models and sophisticated tools such as optogenetics has provided key insights on the role of striatal neurons in behaviour but also raised some questions about the interpretation of earlier pharmacological and lesion studies. For more than 30 years it is hypothesized that medium spiny neurons (MSNs) expressing dopamine receptor 1 (D1R, D1-MSNs) or expressing dopamine receptor 2 (D2R, D2-MSNs) exert opposing control over motor output. Recently, this hypothesis has been extended, proposing that D1-MSNs mediate reinforcement and reward, whilst D2-MSNs mediate punishment and aversion. D1- and D2-MSNs have been classically associated with the basal ganglia direct and indirect pathways, respectively, based on the studies showing segregation of these neuronal populations into distinct circuits. Yet, more recent anatomical and behavioral data argues against this simplistic framework. First, whereas the division of direct and indirect neurons based on the respective expression of D1R and D2R in dorsal striatum is accurate, this does not hold true for the ventral striatum since the indirect pathway contains a mixture of both D1- and D2-MSNs (Kupchik et al., 2015; Lu et al., 1998). In addition, behavioral studies suggest that the same type of neuron but in different striatal locations can selectively drive opposing behaviors, namely reward and aversion (Al-Hasani et al., 2015). Other studies suggest that in fact, both ventral striatal subpopulations can mediate incentive motivation and reinforcement (Soares-Cunha et al., 2016). Similarly, in the dorsal striatum, both subpopulations are also involved in positive reinforcement, but support different action strategies (Vicente et al., 2016).

In this review we will discuss the commonalities and divergences between pharmacological, genetic and optogenetic studies regarding the role of dorsal and ventral striatal neurons in mediating the responses to reward and aversion and provide a critical analysis on the contribution of D1R- or D2R-expressing neurons in this process.

2. Neuroanatomy of the striatum: primate vs. rodent

Despite several bridges that are commonly made between rodent and human anatomy, it is important to highlight the major anatomical sub-regions that constitute the striatum of these two phylogeneticaly distinct species (Fig. 1). The human dorsal striatum is subdivided into caudate nucleus and putamen by the internal capsula (Graybiel and Ragsdale, 1978) in its most dorsal portion. The caudate nucleus receives inputs mostly from cortical regions (prefrontal and orbitofrontal cortices) and putamen receives mainly excitatory inputs from sensorimotor areas (Graybiel and Rauch, 2000; Krack et al., 2010). The human ventral striatum is composed by the NAc (and olfactory tubercle), which receives projections from both prefrontal and limbic structures, and acts as a motor-limbic interface, being involved in motor, emotional and motivational processes (Fig. 1A). Thalamostriatal projections are also different from dorsal and ventral striatum. The central medial nucleus and the dorsolateral parafascicular (PF) of the intralaminar complex innervate the putamen, whereas caudal PF innervates the caudate (McFarland and Haber, 2000; Smith et al., 2009, 2004). Ventral striatum receives projections from rostral PF but also from midline nuclear group nuclei (Giménez-Amaya et al., 1995; Smith et al., 2009, 2004).

Rodent striatum is composed by dorsal striatum and ventral striatum (Voorn et al., 2004). Dorsal striatum is sub-divided into dorsomedial striatum (DMS), which mainly receives inputs from the most ventral region of the prefrontal cortex (infralimbic cortex) and the dorsolateral striatum (homologue to the primate putamen) that mainly receives projections from the motor cortex (Voorn et al., 2004). Similarly as in primates, DLS is mainly innervated by the dorsolateral PF and DMS receives projections from medial PF, whereas the NAc receives projections from midline nuclear group including the parataenial and the intermediodorsal nucleus (Berendse et al., 1992; Haber, 2011; Lanciego et al., 2004).

The rodent ventral striatum is composed by the NAc (Zahm and Brog, 1992; Zahm and Heimer, 1990), subdivided in the core (NAcc) and shell (NAcs) portions, which receives projections from the prelimbic and infralimbic cortex (Lanciego et al., 2004; Russo and Nestler, 2013; Voorn et al., 2004; Yin and Knowlton, 2006) (Fig. 1B). In addition, this striatal region also receives projections from the ventromedial PF (Lanciego et al., 2004; Smith et al., 2004; Voorn et al., 2004) (Fig. 1B).

Importantly, recent studies using transgenic animals, novel viral tools and anterograde/retrograde tracers have provided a comprehensive map of direct inputs to striatal neurons and interneurons, showing a far more complex network than initially anticipated, and which may be determinant for future functional investigation of striatal circuits (Guo et al., 2015; Wall et al., 2013). Moreover, striatal regions strongly project to different regions of the brain, including the basal ganglia and different cortical and thalamic regions (Guo et al., 2015; Haber, 2003; Nauta et al., 1978; Sesack and Grace, 2010; Smith et al., 2004; Watabe-Uchida et al., 2012) (inputs and outputs are summarized in Fig. 1C).

2.1. Striatal compartments

The mammalian striatum is composed of two components, the patches and the matrix, which contain different neurochemical

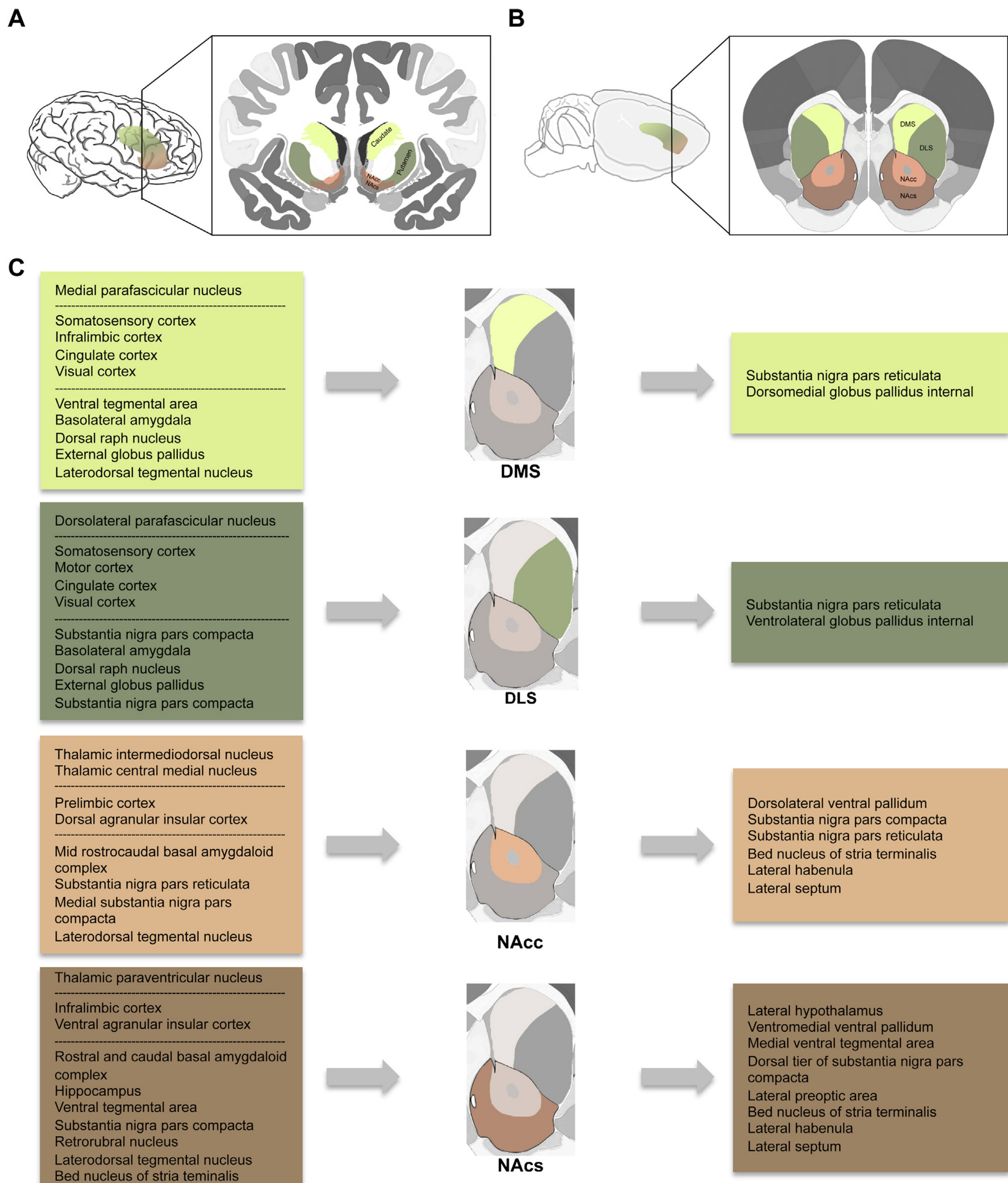

Fig. 1. Simplified neuroanatomical models of human and rodent striatum. (A) Human dorsal striatum includes the caudate and putamen and ventral striatum includes the nucleus accumbens (and olfactory tubercle), which is subdivided in core and shell portions. (B) The rodent striatum is divided in dorsal striatum and ventral striatum – the dorsal striatum is subdivided in dorsomedial and dorsolateral portions, while the ventral striatum is comprised by the nucleus accumbens (core and shell portions). (C) Summary of all brain regions projecting to the striatal sub-divisions, and specific brain targets of these striatal sub-divisions. *NAcc: nucleus accumbens core; NAcs: nucleus accumbens shell; DMS: dorsomedial striatum; DLS: dorsolateral striatum.*

markers (Graybiel and Ragsdale, 1978; Herkenham and Pert, 1981; Olson et al., 1972). Patches (or striosomes) represent around 10% of striatal volume and are densely enriched in μ-opioid receptors, substance P and contain little expression of cholinergic markers (Bolam et al., 1986; Graybiel and Ragsdale, 1978; Herkenham and Pert, 1981). In addition, MSNs contained in the patch compartment receive inputs from limbic and frontal regions (Donoghue and Herkenham, 1986; Gerfen, 1985; Kincaid and Wilson, 1996; Ragsdale and Graybiel, 1988), and from a distinct sub-set of neurons of the substantia nigra pars compacta (SNpc) (Gerfen, 1985;

Gerfen and Young, 1988). The matrix is highly enriched in cholinergic markers, calbindin and somatostatin (Gerfen, 1985, 1984; Graybiel et al., 1986; Graybiel and Ragsdale, 1978), and the MSNs contained in this compartment receive inputs from cortex, thalamus (Fujiyama et al., 2006, 2006; Sadikot et al., 1992) and VTA (Gerfen et al., 1987).

Interestingly, while D1-MSNs (assuming to be those that express dynorphin) projecting to the mesencephalon and D2-MSNs projecting (assuming to be those that express enkephalin) to the substantia nigra pars reticulata (SNr) are contained in the matrix and patches, D2-MSNs projecting to the SNpc are exclusive to patches (Gerfen et al., 1990; Gerfen and Young, 1988).

3. Snapshot of striatal neurons

Striatal neurons represent a homogeneous neuronal population, constituted mainly by GABAergic MSNs, which represent about 95% of all neurons.

Dorsal striatal MSNs are traditionally subdivided into two distinct subtypes based on their axonal targets: striatonigral MSNs which *directly* project to output nuclei of the basal ganglia, namely to the globus pallidus internal (GPi), SNr and VTA (direct pathway; Fig. 2); and the striatopallidal MSNs, which reach output nuclei *indirectly*, by projecting to the GP external (GPe) and subthalamic nuclei (STN) (indirect pathway; Fig. 2B). Activation of the direct pathway briefly supresses pallidum activity allowing thalamocortical activation, facilitating movement, whereas activation of the indirect pathway further inhibits thalamocortical neurons and inhibits movement (Groenewegen, 2003; Parent and Hazrati, 1995; Takakusaki et al., 2004). These two MSN populations express different molecules; striatonigral/direct MSNs express D1R, substance P and dynorphin, and striatopallidal MSNs express D2R, adenosine receptor 2a (A_2aR), and enkephalin (Gerfen, 1992; Heiman et al., 2008; Kawaguchi, 1997; Lobo et al., 2006).

Dorsal striatal MSNs show some degree of topographical segregation. While the most dorsal portion of the dorsal striatum presents a random distribution of D1- and D2-MSNs, the most caudal portion of the dorsal striatum is comprised almost exclusively by D1-MSNs (Gangarossa et al., 2013b).

In the NAc (NAc is assumed as the ventral striatum for the purpose of this review), there is a similar direct/indirect dichotomy, although evidence gathered in the last years shows that the discrete separation of D1-MSNs and D2-MSNs in direct/indirect pathway is not precise (Fig. 2). The direct pathway involves NAc projections to the ventral mesencephalon (SN and VTA) and from there to the mediodorsal thalamus (MDT). The indirect pathway (Fig. 2D) travels through the ventral pallidum (VP) and subthalamic nucleus before reaching the ventral mesencephalon. Whereas NAc core projects to dorsolateral VP (dlVP) and SNr, and NAc shell to ventromedial VP (vmVP) and VTA (Zahm and Heimer, 1990). Akin to dorsal striatum, in the *direct* pathway, NAc-SNr/VTA connections are entirely mediated by D1-MSNs; however, *indirect* pathway NAc-VP projections have major contributions of both types of MSNs (Kupchik et al., 2015; Lu et al., 1998). Because the VP serves as an output nucleus, sending projections outside of the basal ganglia to the MDT, this suggests that both D1- and D2-MSN populations can inhibit/disinhibit thalamic activity, contrary to dorsal striatum (Kupchik et al., 2015).

Similarly to what is observed in the dorsal striatum, accumbal D1-MSNs and D2-MSNs also show differential topographical distribution. D1-MSNs are equally distributed throughout the NAcc and NAcs (Gangarossa et al., 2013a). D2-MSNs are homogeneously distributed in the NAcc, but in the NAcs they are more expressed in the medial and ventral NAcs (Gangarossa et al., 2013a).

Despite the distinctive molecular fingerprinting of the two MSN subpopulations, it has been suggested that 5–15% of dorsal striatum MSNs can express both D1R and D2R (Bertran-Gonzalez et al., 2008; Lester et al., 1993; Perreault et al., 2011). These neurons co-expressing D1R and D2R project to GPe and GPi, as well as SNr and VTA (Deng et al., 2006; Wang et al., 2006, 2007), though their exact role remains undisclosed. Importantly, it has been suggested that >90% the D1R/D2R co-expressing neurons in the NAc present D1R–D2R receptor heterodimers, whereas in dorsal striatum, heterodimers are only detected in ~25% of D1R/D2R co-expressing neurons (Perreault et al., 2010). The function of these heterodimers is still unknown but they have been suggested to exhibit pharmacological and cell-signaling properties distinct from its constituent receptors (Perreault et al., 2014); and these have been associated with depression and addiction (Perreault et al., 2014, 2010). However, the existence of such heterodimers *in vivo* has also been questioned by recent studies (Frederick et al., 2015).

The remaining 5% of striatal neurons are interneurons (Graveland and DiFiglia, 1985) (vide Table 1). Interneurons play a very important role in basal ganglia function, by controlling MSN excitability directly or indirectly, and affecting behaviour (Charara et al., 2003; Gittis and Kreitzer, 2012; Hikida et al., 2001; Kaneko et al., 2000; Tepper et al., 2010; Tritsch and Sabatini, 2012).

Interneurons include large tonically active cholinergic neurons (CIN) (Aosaki et al., 1994; Dautan et al., 2014; Morris et al., 2004) that have been implicated in the control of the activation/modulation of the direct and indirect striatal pathways (Cachope et al., 2012; Centonze et al., 2003; Hikida et al., 2001; Kaneko et al., 2000; Maurice et al., 2004; Pisani et al., 2000; Threlfell et al., 2012; Tozzi et al., 2011; Witten et al., 2010) and constitute the main cholinergic source of this brain region (at least proven to be functional so far) (Bjorklund et al., 1996). Yet, it is important to refer that a neuroanatomical study has shown that the brainstem cholinergic areas pedunculopontine nuclei and the laterodorsal tegmentum also provide a direct innervation of the striatal complex (Dautan et al., 2014). CINs also express dopamine receptors, including D2-R and D5-R reviewed in (Surmeier et al., 2007). Interestingly, specific stimulation of CIN interneurons evokes dopamine release in a β2 nicotinic ACh receptor (nAChR)-dependent manner (Cachope et al., 2012; Maurice et al., 2004; Pisani et al., 2000; Tozzi et al., 2011).

The second class of interneurons are the fast spiking GABAergic (FS) interneurons, which characteristically express parvalbumin and are similar to the FS interneurons present in the cortex and hippocampus (Bevan et al., 1998; Tepper and Bolam, 2004). This particular neuronal population conveys its primary inhibitory synapses to both D1-MSNs and D2-MSNs (Bennett and Bolam, 1994; Gittis et al., 2010; Kreitzer and Malenka, 2008; Lapper et al., 1992; Planert et al., 2010; Sidibé and Smith, 1999; Tepper et al., 2010), providing a strong feed-forward inhibition that shapes the firing patterns of MSNs (Gittis et al., 2010; Planert et al., 2010; Tepper et al., 2008).

Another population of interneurons are the low threshold spiking (LTS) GABAergic interneurons that express somatostatin, neuropeptide Y and nitric oxide synthase, and are involved in long-term plasticity (English et al., 2012; Ibáñez-Sandoval et al., 2015, 2010; Kreitzer, 2009; Sidibé and Smith, 1999; Vuillet et al., 1989). Less studied are the tyrosine hydroxylase and calretinin striatal interneurons, which present sparse connections with MSNs (Table 1).

Despite their low number, interneurons can exert a very significant role in the control of striatal circuit activity and establishment of behavioural conditioning *in vivo*.

It is important to note that there are species differences in the density of these neuronal subtypes within the striatum. For example, primates contain more interneurons than rodents and have

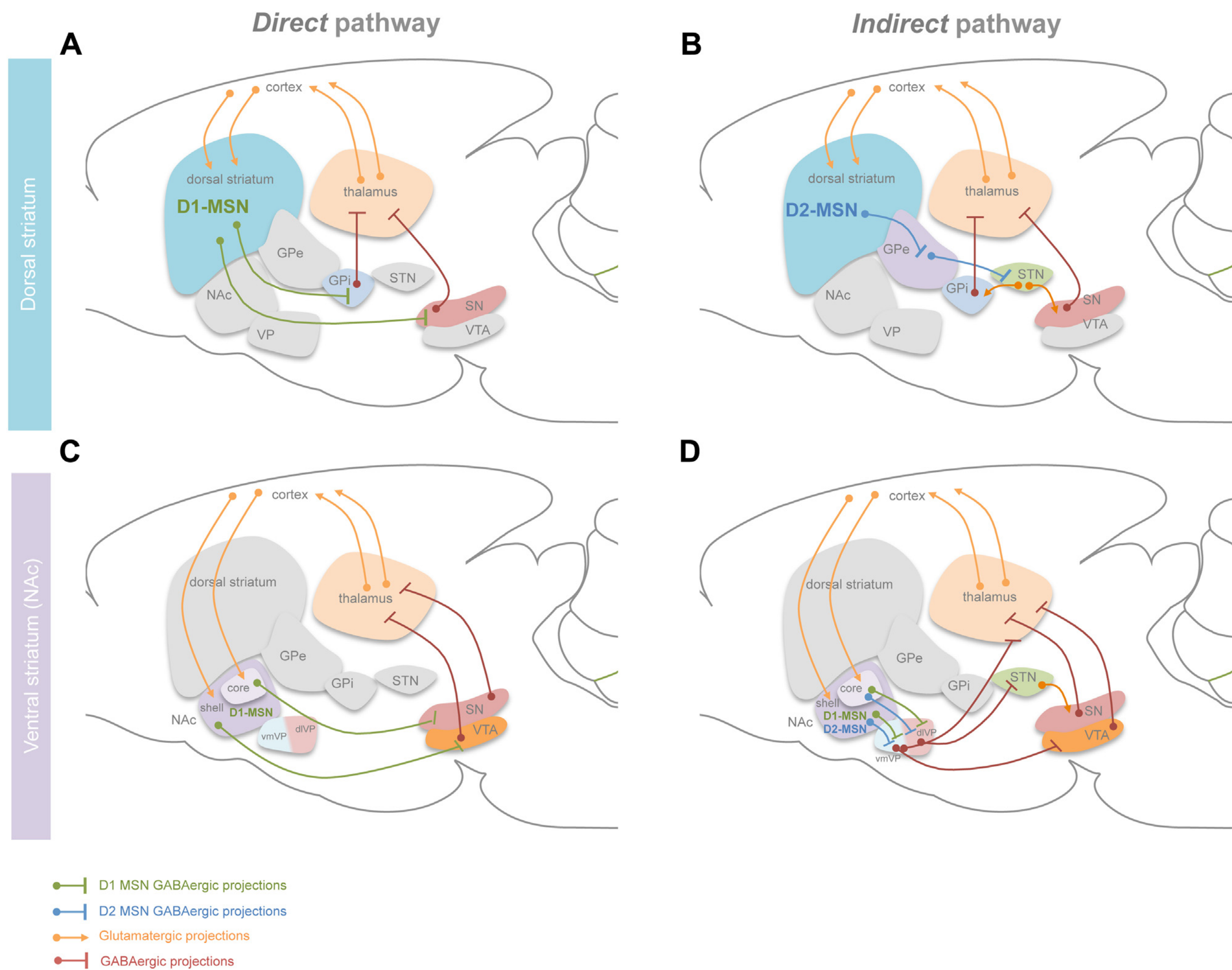

Fig. 2. Direct and indirect striatal pathways. (A) In the dorsal striatum, MSNs from the direct/striatonigral/D1-MSN pathway project *directly* to the basal ganglia output nuclei, the SNr and the GPi. The inhibitory GABAergic effect leads to disinhibition of the thalamus, which in turns projects to the cortex. MSNs from the direct pathway express D1R, but also express M4 cholinergic receptors, dynorphin, and substance P. (B) Activation of indirect/striatopallidal/D2-MSN MSNs, which project *indirectly* to the SNr via the GPe and the STN, inhibits thalamic output to cortex. Dopaminergic input from the SNc modulates corticostriatal transmission by exerting a dual effect on MSNs, depending on their nature (D1R-or D2R-expressing). Indirect pathway MSNs co-express D2R, A_2aR, enkephalin, and neurotensin. There is a part of MSNs that co-express both receptors (<20%), although the functional role of these is still unknown. (C) In the ventral striatum, more specifically within the NAc, there is a similar direct/indirect dichotomy akin the dorsal striatum. The direct pathway involves NAc projections to the SNr/VTA and from there to the mediodorsal thalamus. The direct NAc innervation of the SN/VTA is entirely mediated by D1-MSNs. (D) The indirect circuit projects to the VP and STN before reaching the output nuclei. While the core projects to the dlVP, the shell innervates the vmVP. The dlVP projects to the STN and SN, whereas the vmVP projects to VTA and out of the basal ganglia to the mediodorsal thalamus. Indirect pathway NAc-VP projections have major contributions of both types of MSNs. Moreover, both D1- and D2-MSNs innervate VP output neurons that project to the mediodorsal thalamus, which suggests that both populations can inhibit/disinhibit thalamic activity. *SNr: substantia nigra pars reticulata; GPi: globus pallidus pars interna; GPe: globus pallidus pars externa; STN: subthalamic nucleus; SNc: substantia nigra pars compacta; NAc: nucleus accumbens; VP: ventral pallidum; dlVP: dorsolateral pallidum; vmVP: ventromedial pallidum; D1R: dopamine receptor 1; D2R: dopamine receptor 2; A_2aR: adenosine receptor 2a.*

more calretinin-positive and parvalbumin-positive interneurons in the dorsal striatum in comparison to rodents (Graveland et al., 1985; Wu and Parent, 2000).

Importantly, since interneurons also express dopamine receptors (reviewed in Tritsch and Sabatini, 2012), this complicates the use of specific agonists/antagonists or genetic approaches relying on the expression of these receptors to study the function of D1- and D2-MSNs.

4. Differences and commonalities between D1R and D2R signalling

Dopamine activation of G-protein coupled dopamine receptors (D1R-D5R) excites or inhibits MSNs by modulating the gating and trafficking of voltage-dependent and ligand-gated (ionotropic) ion channels located in the cell membrane (Surmeier et al., 2007). The separation of the two major classes of dopamine receptors is based on intrinsic structural, pharmacological, and signalling properties: D1Rs and D5Rs are grouped in the D1R-like receptor subfamily, while the D2Rs, D3Rs and D4Rs are clustered in the D2R-like receptor subfamily (Beaulieu and Gainetdinov, 2011).

The genes encoding D1R are contained in a single exon; however, the same is not true for D2Rs. These receptors can be alternatively spliced, giving rise to several isoforms with distinct properties and subcellular localization (reviewed in D'souza, 2010). Pharmacological studies show that D2R-like receptors present a higher affinity towards dopamine when compared with the D1R subfamily, which can reach 10- to 100-fold (Beaulieu and Gainetdinov, 2011). D3Rs and D4Rs present the highest affinity to dopamine binding and D1R displays the lowest. Although both dopamine receptor subfamilies are GPCRs, being able to activate heterotrimeric G proteins, the second messengers and effector proteins activated by both receptor classes vary greatly and often mediate opposite effects (Beaulieu and Gainetdinov, 2011).

Table 1
Summary of the different types of striatal cells, their properties and dopamine receptor expression.

Type of striatal cell	Properties	Dopamine receptor expression?	References
Medium spiny neurons (MSNs) (90–95%)			
D1-MSNs	Presence of D1R, dynorphin and substance P. Low input resistance, inward rectification, and a long delay to initial spiking. Synapse with both D1-MSNs and with D2-MSNs.	D1R	Cepeda et al., 2008; Gerfen, 1985; Gertler et al., 2008
D2-MSNs	Presence of D2R, enkephalin and adenosine receptor 2a (A_2a). Low input resistance, inward rectification, and a long delay to initial spiking; increased excitability. Synapse within each other and onto the striatopallidal pathway	D2R	Cepeda et al., 2008; Gerfen, 1985; Gertler et al., 2008
D1/D2-MSN (5–15%)	Co-expression of D1R and D2R, expression of dynorphin and enkephalin. Some neurons present a D1R-D2R heterodimer. Medium-sized spiny projection neurons, with enhanced dendritic branching.	D1R and D2R	Choi et al., 2009; Hasbi et al., 2011; Lee et al., 2004; Perreault et al., 2012
GABAergic Interneurons (3–4%)			
Fast spiking interneurons (FS)	Presence of parvalbumin. Small diameter somata and aspiny dendrites that branch modestly. Short-duration spikes, high frequency firing. Form gap junctions with other FS interneurons; Strong and dense projections to MSNs.	>70% D5R	Bevan et al., 1998; Kawaguchi, 1997;Kreitzer, 2009; Kubota and Kawaguchi, 2000; Tepper et al., 2010; Tepper and Bolam, 2004
NPY neurogliaform neurons	Presence of neuropeptide Y. Compact and highly branched dendritic and local axonal arborizations. Low threshold calcium spikes (LTS) and a prolonged calcium dependent plateau potential. Second major class of projecting interneuron in the striatum.	?	English et al., 2012; Ibáñez-Sandoval et al., 2011; Kawaguchi, 1997; Kreitzer, 2009; Vuillet et al., 1989
Low-Threshold Spiking interneurons (LTS)	Presence of neuropeptide Y, somatostatin and nitric oxide synthase. Contain few dendritic branches and extensive, but low axonal arborisation. High input resistance and a sustained plateau potential that persists after the end of current injection; rebound spiking following hyper polarizations. Synapse with MSNs dendrites.	<10% D1R; >70% D5R	Ibáñez-Sandoval et al., 2011; Kawaguchi, 1997; Kreitzer, 2009; Vuillet et al., 1989
TH interneurons	Presence of tyrosine hydroxylase, but do not seem to produce dopamine. Medium sized interneurons with low branching, varicose dendrites; dense, highly varicose axon collateral fields. Comprise four electrophysiologically distinct neuron types – large variability. Make afferent and efferent GABAergic synaptic connections with MSNs.	?	Ibáñez-Sandoval et al., 2015, 2010
CR interneurons	Presence of calretinin. Medium sized, possess few, aspiny, infrequently branching dendrites. Electrophysiological profile not fully known, but in part similar to LTS interneurons. Sparse connections with MSNs.	50% D5R	English et al., 2012; Kreitzer, 2009; Tepper and Bolam, 2004
Cholinergic interneurons (0.5–1%)			
Cholinergic interneurons (CIN)	Presence of acetylcholine markers. Aspiny neurons with large cell bodies and widespread axonal fields. Hyperpolarization-activated current and broad spikes with long spike after hyper polarizations; tonic low-frequency activity. Synapse onto MSNs.	<20% D1R; >80% D2R; >80% D5R	Aosaki et al., 1994; Kreitzer, 2009; Morris et al., 2004; Tepper and Bolam, 2004; Witten et al., 2010; Kaneko et al., 2000; Hikida et al., 2001

D1Rs stimulate the heteromeric GPCRs Gs/olf, which are coupled to adenylyl cyclase (AC) (Hervé et al., 1995) (Fig. 3A). The increase in cytosolic cyclic adenosine monophosphate (cAMP) levels causes activation of protein kinase A (PKA) and phosphorylation of its intracellular targets, such as voltage gated K^+, Na^+ and Ca^{2+} channels, ionotropic glutamate, GABA receptors and various transcription factors. One of the major targets of PKA is the dopamine and cAMP-regulated phosphoprotein DARPP-32, which is highly expressed in striatal neurons and plays a critical role in the modulation of downstream signals. DARPP-32 can integrate signals from different neurotransmitters and bidirectionally modulate PKA. If PKA phosphorylates DARPP-32 this will amplify PKA signalling by inhibiting protein phosphatase 1 (PP1), which counteracts PKA's activity (Svenningsson et al., 2004). On the other hand, dephosphorylating of DARPP-32 by the calmodulin-dependent protein phosphatase 2B (PP2B), upon D2R-like receptor activation, will convert DARPP-32 into a potent PKA signalling inhibitor and so, completely change neuronal function (Svenningsson et al., 2004). PKA activation, through D1Rs, enhances surface expression of both AMPA and NMDA receptors (Hallett et al., 2006; Snyder et al., 2000). Furthermore, D1R signalled PKA phosphorylation of the somatic Nav1.1 Na^+ channels promotes activity-dependent entry into an inactivated state (Carr et al., 2003; Scheuer and Catterall, 2006). D1R stimulation is also responsible for inactivation of several K^+ channels (e.g. Kirk2, Kv1 and Kv4) (Hernandez-Lopez et al., 2000). PKA phosphorylation followed by D1R activation enhances the opening of some Ca^{2+} channels (e.g. $Ca_v1.3$), but it reduces the opening of others (e.g. Ca_v2 Ca^{2+}) (Surmeier et al., 1995; Vilchis et al., 2000). In summary, D1R signalling increases the responsiveness of striatonigral neurons to sustained release of glutamate, by enhancing surface expression of its receptors and by modulating ion channel function to promote MSN *up state* reviewed in (Surmeier et al., 2007).

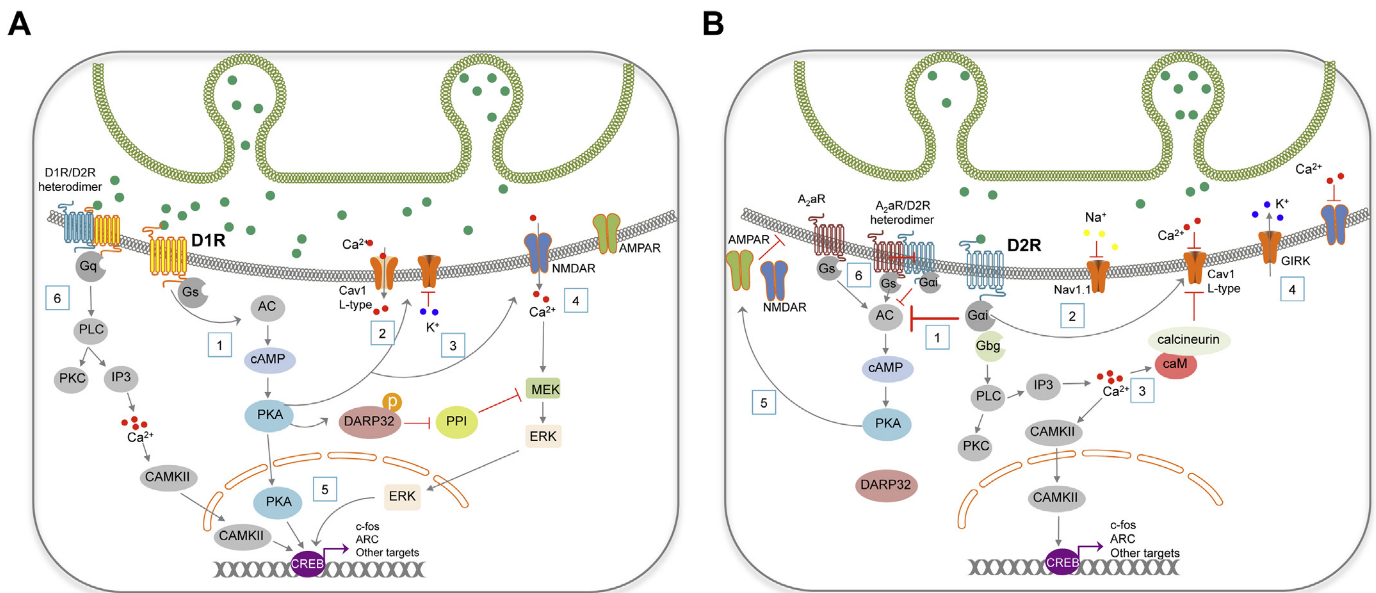

Fig. 3. D1R and D2R activation leads to distinct cellular effects. (A) Simplified scheme of D1R signalling. (1) Activation of D1R stimulates G_s proteins that are positively coupled to AC, leading to the production of cAMP and the activation of PKA. PKA induces opening of L-type Ca^{2+} channels and inhibits/closes K^+ channels (2). (3) PKA-dependent phoshorylation of AMPA and NMDA receptors, which enhances membrane expression of these receptors. (4) Stimulation of D1R also increases Ca^{2+} influx through NMDA receptors. This activates a number of signalling pathways, including ERK via the Ras-Raf-MEK cascade. (5) PKA can modulate CREB activity directly or by phosphorylating DARPP-32, inducing the disinhibition of the NMDA/Ras-GRF1/ERK pathway, culminating in the modulation of transcription of target genes. (6) Activation of the G-coupled D1R/D2R heteromer induces a PLC-dependent calcium release, resulting in the activation of CaMKII and its translocation to the nucleus. (B) Simplified scheme of D2R signalling. (1) D2R-like receptors activate Gαi and Gαo proteins, which inhibit AC and limit PKA activation that regulates several voltage-gated K^+, Na^+ and Ca^{2+} channels. (2) Released Gβγ subunits are capable of reducing Cav1 L-type channel, and activate PLC and PKC, increasing IP3, mobilizing Ca^{2+} stores. (3) Activation of calcineurin can also block Cav1 L-type activity. (4) D2R signalling enhances K^+ channel opening. (5) PKA modulates glutamate receptor incorporation in the membrane. (6) Striatal D2-MSNs co-express A_2aR. A_2aR and D2R are coupled to different G proteins and modulate AC activity in opposing directions. Interestingly, there is also the existence of an antagonistic allosteric A_2aR-D2R interaction. *AC: adenylyl cyclase; cAMP: cyclic adenosine monophosphate; PKA: protein kinase A; AMPAR: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid glutamate receptor; Arc: activity-regulated cytoskeleton-associated protein; CaMKII: calcium/calmodulin-dependent protein kinase type II; CREB: cAMP responsive element binding protein; ERK: extracellular signal-regulated kinases; GIRK: G protein-coupled inwardly-rectifying potassium channels;IP3: inositol 1,4,5-trisphosphate; MEK: mitogen-activated protein kinase; Nav1.1: sodium channel, voltage-gated, type I; Cav1 L-type: Calcium channel, voltage-dependent, L type; PLC: phospholipase C; PKC: protein kinase C; P-DARPP-32: phosphorylated dopamine- and cAMP-regulated phosphoprotein 32 kDa; PP1: protein phosphatase 1; NMDAR: N- methyl-D-aspartate glutamate receptor; PLC: phospholipase C; IP3: inositol (1,4,5)-trisphosphate; D1R: dopamine receptor 1; D2R: dopamine receptor 2; A_2aR: adenosine receptor 2a.*

D2Rs are coupled to Gi/o proteins, inhibiting AC and limiting PKA activity (Stoof and Kebabian, 1984) (Fig. 3B). Additionally, D2R activation is also responsible for the transactivation of tyrosine kinases (TK) (Nair and Sealfon, 2003). Similarly to what occurs with D1R, D2R signalling alters glutamate receptor function in dorsal striatum MSNs, through dephosphorylation of GluR1 subunit that will promote the removal of AMPA receptors from the plasma membrane (Håkansson et al., 2006). D2R-mediated recruitment of intracellular Ca^{2+} leads to negative modulation of $Ca_V1.3$ Ca^{2+} channels through calcineurin-dependent mechanism (Hernandez-Lopez et al., 2000; Olson et al., 2005). Additionally, activation of D2R receptors reduces the opening of Na^+ channels, dependently of PKC-mediated enhancement of slow inactivation (Surmeier et al., 1992). Moreover, D2R receptor signalling acts by promoting the opening of K^+ ($K_{ir}3$) channels (Greif et al., 1995), which will change the electrophysiological properties of neurons and generally cause a transient decrease in neuronal excitability (Hernandez-Lopez et al., 2000). In brief, studies are consistent with a role for D2R to reduce the responsiveness of striatopallidal neurons, through coordinated modulation of ion channels function and glutamate receptors expression at the surface.

Importantly, D2Rs are also found presynaptically, where they can modulate neurotransmitter release (Benoit-Marand et al., 2001). In the striatum, D2Rs are present on corticostriatal inputs and function to decrease glutamate release by presynaptic mechanisms (Kornhuber and Kornhuber, 1986; Maura et al., 1988; Yamamoto and Davy, 1992). Additionally, these Gi/o –coupled inhibitory receptors also play a major part in shaping dopamine transmission (Benoit-Marand et al., 2001). Found at both somato-dendritic and axonal sites of the VTA, D2R autoreceptors regulate the firing patterns of dopamine neurons and control the timing and amount of dopamine released within the striatum (Ford, 2014). In addition, D2Rs are also expressed in up to 80% of CINs (Maurice et al., 2004).

It is important to keep in mind that D2Rs present a mechanism of alternative splicing, and thus, the same gene encodes two distinct isoforms of the same receptor, D2S and D2L (Picetti et al., 1997). Interestingly, these two alternatively spliced receptors have distinct functions *in vivo*; while D2L acts mainly at postsynaptic sites, D2S serves presynaptic autoreceptor functions (Usiello et al., 2000).

Despite the simplistic vision given above on the mechanism of action of the distinct dopamine receptors within the striatum, it is important also to note that the activity of these receptors goes much beyond the specific activation of its signalling cascades. Although it falls outside the scope of the present review, it is important to keep in mind the existence of interactions between dopamine receptors and other neurotransmitter receptors, known to be relevant for motor control, cognition and memory, neurodegenerative disorders, schizophrenia or even addiction (Calabresi et al., 2000; Chesselet and Delfs, 1996; Fuxe et al., 2010; Graybiel, 1995; Rolls, 1994; Schultz, 1997). Interestingly, these interactions do not occur only at the intracellular level via second messenger pathways, but it also can occur through physical contact. For example, physical contact exists between dopamine receptors and NMDA at the membrane and cytoplasm levels (Freund et al., 1984; Smith and Bolam, 1990). In addition, a direct interaction between the D5R and $GABA_A$ receptors has also been reported, which may represent a mechanism controlling synaptic efficacy (Liu et al., 2000). Also, D2R is

also known to directly interact with A_2aR, converging in the control of AC function (reviewed in Fuxe et al., 2010). In addition to a negative and positive coupling of D2R and A_2aR to the adenylate cyclase activity, respectively, there is also an antagonistic allosteric A_2aR–D2R interaction (Fig. 3B).

5. The conundrum: one ligand, different neuronal responses

In addition to different dopamine receptors that can activate distinctive signalling cascades, in the past years a great deal of attention has been given to the dynamics of striatal dopamine release. Dopaminergic neurons fire in an asynchronous tonic pacemaker manner that can switch to transient synchronous bursts (consisting of a series of consecutive spikes) upon exposure to unexpected or anticipated rewards (Redgrave and Gurney, 2006; Schultz, 2002). Accumulating evidence shows that striatal dopamine neurotransmission occurs in these two dissociable temporal modes, "tonic" and "phasic", both of which are highly regulated by glutamatergic forebrain inputs and by cholinergic and GABAergic inputs within the striatum (Grace, 2000, 1991; Moore et al., 1999).

Tonic dopamine transmission constitutes the extra-synaptic pool of steady-state dopamine levels. These levels are strongly regulated and maintained within a narrow concentration in the striatum (Parsons and Justice, 1992). Phasic dopamine, which is proposed to be the signal that mediates rapid behaviourally relevant activation of the dopamine system by relevant stimuli, is a high-amplitude transient signal that results from a cell-burst firing and consequent uptake of high levels of dopamine by the synapse (Garris et al., 1994).

Phasic dopamine transients that result from synchronous burst firing are mainly thought to activate the low-affinity striatal D1Rs, while tonic dopamine levels arising from pacemaker firing maintain steady-state activation of high-affinity D2Rs (Grace et al., 2007; Grace and Bunney, 1984; Keefe et al., 1993; Richfield et al., 1989; Surmeier et al., 2011). However, recent studies show that high-affinity D2Rs are also activated by phasic dopamine transients (Marcott et al., 2014), which raises new possibilities to the actual contribution of the D2-MSNs pathway in the control of behaviour.

6. Striatal activity during reward and aversion

6.1. Ventral striatum

Pioneering work by Schultz and colleagues using electrophysiological recordings from the primate striatum revealed excitations in response to cues that predict reward (Bowman et al., 1996; Cromwell and Schultz, 2003; Hassani et al., 2001; Hollerman et al., 1998; Schultz et al., 1997), that depended strongly on the predictive value of the cue (Hassani et al., 2001), the magnitude of the predicted reward (Cromwell and Schultz, 2003), and the temporal proximity to the reward (Shidara et al., 1998). Additionally, it was also shown that the magnitude of the excitation evoked by predictive cues is positively correlated with the motor activity needed to obtain the reward (Cromwell and Schultz, 2003; Hassani et al., 2001; Hollerman et al., 1998). Interestingly, cue-evoked excitation or inhibition of NAc neurons is larger for cues that predict rewards than for cues that do not predict a reward (Ghitza et al., 2003; Nicola, 2007) and larger when the cue elicits a behavioural response (Nicola, 2007).

Intraoral delivery of a sucrose solution increases dopamine release within the NAc, that is time-matched with the infusion onset (Roitman et al., 2005), and NAc neurons respond by decreasing their activity (Roitman et al., 2005). This transient inhibition has also been observed in animals performing behavioral tasks that require operant response like self-administration of many different types of rewarding stimuli such as cocaine (Peoples and West, 1996), heroin (Chang et al., 1998), ethanol (Janak et al., 1999), sucrose (Nicola et al., 2004) and food (Carelli, 2002). On the other hand, oral administration of quinone (an aversive flavor) evokes the opposite response (i.e. suppression of dopamine transients). In addition, NAc neurons are highly responsive to aversive stimuli and respond by increasing their activity (Roitman et al., 2005).

So, in a simplistic perspective, one can assume that rewarding stimuli evoke an instantaneous increase in dopamine release in the NAc and a decrease in the firing rate of NAc neurons. Aversive stimuli evoke the opposite response by suppressing dopamine transients and increasing the firing rate of NAc neurons. Less explored is the role of D1-MSNs and D2-MSNs in this process due to the inherent technical difficulties of separating these populations using *in vivo* electrophysiological recordings.

6.2. Dorsal striatum

Single cell recordings performed in behaving primates showed that dorsal striatal neurons increase activity selectively during the expectation of reward (Apicella et al., 1992; Hikosaka et al., 1989; Schultz et al., 1992; Schultz and Romo, 1988), akin to NAc. Dorsal striatum neurons respond to task-related sensory events by becoming active before task-dependent motor behaviours and maintain a tonic activity until the expected reward is delivered (Hikosaka et al., 1989; Hollerman et al., 1998). Interestingly, the activity of these neurons is modulated by the expected presence, amount or probability of reward or by the amount of attention or memory required to execute the task (Cromwell and Schultz, 2003; Kawagoe et al., 1998). Thus, the presence of sensorimotor, cognitive and motivational signals in single striatal neurons further supports a prominent role of dorsal striatum in learning (Balleine et al., 2007).

Other studies showed that dorsal caudate neurons are activated when different food rewards are shown to the animal (Nishino et al., 1984). Interestingly, this study showed that different neuronal population of the caudate responded to visual presentation of food- and non-food-associated cues and the magnitude of electrophysiological responses was dependent on the nature of the food and was inversely related to the latency of the onset of lever pressing (Nishino et al., 1984). In addition, other neuronal populations also responded during lever pressing and during food retrieval and consumption, thus suggesting that the primate dorsal striatum is constituted by a complex neuronal network that controls sensory, non-motor and motor responses in operant-dependent rewarding events (Nishino et al., 1984). In addition, in a task where a drop of liquid was given each time the animal correctly executed or withheld an arm movement in reaction to a visual stimulus (Go/No-Go task) it was shown that neurons of the dorsal striatum increased activity upon liquid delivery (Apicella et al., 1991), supporting a role in the integration of primary reward information (Apicella et al., 1992, 1991). Additionally, rodents performing a multiple T-maze task with the goal of finding the correct locations to obtain a food reward increase striatal electrical responses not only during motor behaviour, but also during the delivery of food (Schmitzer-Torbert and Redish, 2004).

Apart from reward expectation and consumption, dorsal striatal neurons play a crucial role in decision-making processes, particularly those encoding specific action–outcome associations and the selection of actions on the basis of their currently expected reward value (Gremel and Costa, 2013; Kimchi et al., 2009; Miyachi et al., 2002; Tang et al., 2007). Whereas DMS neurons seem to be crucial in the early acquisition and performance of an instrumental task to receive a reward, i.e., goal-directed behaviour, DLS neurons appear to be preferentially recruited in habitual behaviours that appear

after overtraining (Gremel and Costa, 2013; Kimchi et al., 2009; Miyachi et al., 2002; Tang et al., 2007).

To what concerns neuronal activity of the dorsal striatum in aversive behaviour in freely moving animals, we did not find any study. Nevertheless, it is important to refer that the SN, which densely innervates dorsal striatum, is not commonly recruited or inhibited by aversive stimuli (Brown et al., 2009), though lesions of dorsal striatum have been shown to produce deficits in conditioned freezing and passive and active avoidance (White and Salinas, 2003; Winocur and Mills, 1969).

Promising tools such as *in vivo* calcium imaging may shed some light on the activity of different striatal populations in reward and aversion. For example, by using a Cre-dependent viral expression of the calcium indicator GCaMP3 in the dorsal striatum of D1-Cre and A_2a-Cre (A_2aR is co-expressed with D2R) mice, researchers showed that both neuronal populations were activated when animals initiated actions but not in inactive periods (Cui et al., 2013). By using microprobe optical imaging to assess the dynamic changes in intracellular calcium, researchers have shown that cocaine-rewarding effects induce activation of direct-pathway neurons and a slower deactivation of indirect-pathway neurons (Luo et al., 2011).

7. Role of D1- and D2-MSNs in reward and aversion

Despite the known cytoarchitecture of the striatum, its heterogeneity poses a major challenge for the attribution of specific biological roles to each type of MSN in behaviour. Traditionally, D1-MSNs are thought to mediate reward and positive reinforcement whereas D2-MSNs encode aversion and negative reinforcement (Kravitz et al., 2012; Lobo et al., 2010; Volman et al., 2013); however, some recent data challenges this simple dichotomy (Cazorla et al., 2014; Kupchik et al., 2015; Saunders et al., 2015; Smith et al., 2013; Soares-Cunha et al., 2016).

Having this in mind, in the following sections, we will discuss pharmacological, genetic and optogenetic studies that have been applied to try to clarify the biological role of each type of MSN in reward and in aversion, and highlight the differences between dorsal striatum and ventral striatum neuronal populations in the modulation of these behaviours.

7.1. Pharmacological approaches

Initial pharmacological manipulations using selective agonists and antagonists allowed a breakthrough in the understanding of the role of D1R and D2R in reward and, less so, in aversive behaviors. Because systemic administration of these compounds challenges the interpretation of the data, in this review we will only focus on intra-striatal administration of D1R and D2R agonists/antagonists.

7.1.1. NAc D1R and D2R in reward/reinforcement

Different groups showed that NAc local application of both D1R and D2R agonists stimulated locomotor activity (Breese et al., 1987; Dreher and Jackson, 1989; Fink et al., 1991). Regarding reward/reinforcement, the literature is inconsistent. Initial studies have shown that NAc administration of D1R and D2R agonists is reinforcing (White et al., 1991). Conversely, others have shown that intra-accumbal administration of D1R or D2R agonists is not reinforcing *per se*, because animals do not readily self-administer these compounds. However, concurrent activation of both D1R and D2R in the NAc shell had a cooperative effect, inducing self-administration (Ikemoto et al., 1997). In accordance, recent studies have shown that both D1R and D2R in the NAc are required for mediating the reinforcing properties of optogenetic self-stimulation of VTA-NAc projections (Steinberg et al., 2014).

Regarding the effects of these receptors in mediating natural reward-related responses, it was shown that in hungry rats, pharmacological blockade of D1Rs or D2Rs in the NAc affects the amount and duration of feeding, but it does not reduce the amount of food consumed (Baldo et al., 2002). In agreement, others have shown that blockade of D1Rs or D2Rs within the NAc core or shell decreased lever pressing for food reinforcers, but rats remained directed towards the acquisition and consumption of food (Nowend et al., 2001). This data indicates that both D1R and D2R signalling are relevant for the motivational drive to work to get a food pellet. In fact, using a Pavlovian-to-Instrumental-transfer test (PIT), which indirectly measures “wanting”/incentive salience, it was shown that selective D1R and D2R blockade in the NAc reduced performance (Dickinson et al., 2000; Lex and Hauber, 2008). In accordance, we have shown that D2R dysfunction within the NAc impairs PIT performance, and that systemic administration of a D2R agonists improved behaviour (Soares-Cunha et al., 2014). In agreement, others have shown that microinjection of D1R or D2R antagonists into the NAc reduce the proportion of cues to which the animal responded to get a food pellet (Wakabayashi et al., 2004). Effort-related choice tests also revealed that effort discounting (an indirect measure of motivation) was altered by administration of D2R antagonists, shifting the choice towards the low reinforcement/low cost arm and this was reverted by increased dopamine transmission (Nunes et al., 2010).

Pharmacological targeting of NAc D1R and D2R signaling has also been used to tackle the role of these neurons in the response to drugs of abuse. Most of the studies point for a *pro-rewarding/reinforcing* role of D1R and a null or attenuating effect of D2Rs. For example, rodents pre-treated with a D1R (but not D2R) antagonist showed an attenuated locomotor response to acute cocaine challenge (Anderson et al., 2003), and intra-accumbal administration of D1R antagonist prevents cocaine (Baker et al., 1998) and ethanol place preference (Pina and Cunningham, 2014; Young et al., 2014). On the contrary, others have shown that both D1R and D2R antagonists significantly block the development of amphetamine place preference (Liao, 2008). Later studies have suggested that activation of accumbal D2Rs does not cause primary rewarding effects, but it can facilitate the previous associated rewarding effects of environmental stimuli (Nunes et al., 2010; Self, 2010). In fact, administration of D2R agonists has prominent reinforcing and enhancing effects in cocaine self-administration in animals with extensive experience in this behaviour (Caine et al., 2002). Interestingly, the observed discrepancies in the studies may be related to drug-specific effects, the use of different dosages of antagonists, subtle differences in the behavioural paradigms or a result of the complex distribution of D2R.

7.1.2. NAc D1R and D2R in aversion

The literature is sparser regarding the role of accumbal D1R and D2R antagonists in aversion. An aversive event induces a negative hedonic state; however, due to technical limitations in evaluating the hedonic state of animals, researchers frequently use avoidance behaviour as a representation of aversion (Kravitz and Kreitzer, 2012). Using the two-way active avoidance task, researchers showed that D2R antagonist infusion into the NAc (but not in dorsal striatum) slightly decreased the number of conditioned avoidance responses (Boschen et al., 2011); a similar effect was observed with D1R antagonists (Wietzikoski et al., 2012). Such effects may be partially due to the D1R and D2R contribution for early consolidation of aversive memory (Managò et al., 2009).

7.1.3. Dorsal striatum D1R and D2R in reward and aversion

Studies evaluating the impact of local dorsal striatum pharmacological inhibition/activation of D1R/D2R in reward and aversion are limited. In primates, D1R antagonism in the caudate nucleus (dorsal striatum part in primates) attenuated the reward-dependent saccadic reaction time changes, in contrast to what was

found with D2R antagonism (Nakamura and Hikosaka, 2006). On the contrary, expression of amphetamine place preference is not blocked by infusion in the dorsal striatum of D1R and D2R antagonists (Hiroi and White, 1991). Regarding aversion, as stated before, dorsal striatum D2R antagonism did not impact the number of conditioned avoidance responses (Boschen et al., 2011).

Altogether, these studies seem to indicate that NAc D1R pathway promotes reward/reinforcement whereas D2R signalling has an opposing effect, although disparate results have also been found. Nevertheless it is important to refer that pharmacological activation/inactivation of D1R or D2R is not a direct proxy of D1-MSN or D2-MSN activation/inactivation. In addition, the use of agonists/antagonists raises different questions: (i) the existence of $D1^+/D2^+$-MSNs is not taken into consideration, (ii) other types of striatal cells namely interneurons also possess D1R and D2R, (iii) the existence of pre-synaptic D2Rs in dopaminergic terminals that strongly modulate striatal neuronal activity, (iv) drugs are rarely specific to one type of receptor, (v) pharmacological approaches have poor temporal resolution and diffusion to other brain regions can occur, challenging the interpretation of the data. Such limitations triggered other approaches to elucidate these questions that we will discuss in the next sections.

7.2. Genetic models

The use of genetic methods can provide an alternative for understanding the importance of a specific receptor/pathway. However, it also poses some problems: conventional knockouts often present compensatory mechanisms, and the deletion of D1R or D2R throughout the brain defies the identification of the biological role of striatal neurons *per se* in behavior. Later studies include specific striatal ablation of these receptors, providing a more elegant approach.

7.2.1. D1R knockout models and reward/reinforcement

The first constitutive D1R knockout (KO) transgenic mice lines were obtained by deletion of either part (Drago et al., 1996) or most of the D1R coding region (Xu et al., 1994) of the genome. D1R KO mice have abnormal locomotor behaviour that ranges from hypoactive to hyperactive depending on the experimental conditions. Although in several studies it was reported that these mice were hyperactive in a novel environment and during the dark phase of the light-dark cycle (Granado et al., 2008; Xu et al., 1994), others reported an increased latency to move in an open field consistent with a hypoactive phenotype (Smith et al., 1998).

Although these animals failed to feed normally after weaning (Wall et al., 2011), this phenotype could be rescued by providing KO mice free access to a palatable food, suggesting that the absence of D1R is more related to a motor deficit than to a reward deficit (Drago et al., 1994; Xu et al., 1994). However, more recent studies showed that D1R KO animals present greatly reduced motivation to work for a food reward in a sucrose preference test, which demonstrates that dopamine signalling through D1R-expressing neurons possibly plays an important role in the modulation of motivational processing (El-Ghundi et al., 2003; Gallardo et al., 2014). D1R KO mice also failed to perform a Pavlovian conditioning task (Parker et al., 2010).

The response to drugs of abuse is also compromised in D1R KO animals (nicely reviewed in Lobo and Nestler, 2011). D1R KO mice performing a cocaine self-administration task presented decreased preference for the active lever and reduced overall responses (Caine et al., 2007). These animals also present diminished ethanol consumption (El-Ghundi et al., 1998).

7.2.2. D2R knockout models and reward/reinforcement

Constitutive D2R KO mice display decreased body weight and reduced spontaneous locomotion (Baik et al., 1995; Kobayashi et al., 2004; Nakamura et al., 2014). These animals also respond less for food rewards in instrumental tasks, which could either be attributed to their hypolocomotion or to impairment in motivated responses (Risinger et al., 2000).

The effects of D2R deletion in the response to drugs of abuse resemble those for natural rewards. D2R KO have blunted response to the rewarding (Maldonado et al., 1997) and reinforcing properties of morphine (Elmer et al., 2002), ethanol (Cunningham et al., 2000) and cocaine (Welter et al., 2007).

Other genetic studies have targeted different aspects of D2-MSNs intracellular signaling. Lobo and co-workers knocked down expression of the sphingosine-1-phosphate receptor Gpr6 (which can stimulate cAMP production—in opposition to D2R activation) in striatopallidal cells (indirect pathway) (Lobo et al., 2007). These animals have a faster acquisition of operant lever pressing for sugar pellets, suggesting enhanced motivation in a situation that partially mimics increased D2R activity (Lobo et al., 2007). Also, overexpression of D2R in the NAc using viral gene transfer, selectively increased motivation for food (in a progressive ratio schedule) without altering consummatory behaviour, the representation of the value of the reinforcer, or the capacity to use reward-associated cues in flexible ways, contrary to dorsal striatum D2R overexpression which did not alter performance in any of the tasks (Trifilieff et al., 2013).

7.2.3. D1R and D2R knockout models and aversion

A few studies have focused on the role of D1R and D2R neurons in aversion. It has been reported that D1R ablation (using constitutive D1R KO mice) prevented the expression of conditioned aversive stimuli, which may indicate that D1R activation is also required for the acquisition and expression of taste aversion learning (Cannon et al., 2005). A mice model lacking D2L (post-synaptic) but expressing functional D2S, developed place preference to cocaine (but not morphine), and did not acquire place aversion to morphine withdrawal. These animals also failed to acquire avoidance behavior in response to electrical stimuli (Smith et al., 2002).

In summary, data from genetic models suggest that whereas the response to natural rewards appears to be modulated by both types of receptors in a similar/synergistic manner, the same does not seem to be true regarding drugs of abuse, with D1R promoting reward and sensitizing responses to psychostimulants and D2R dampening these behaviors. This paradoxical finding is far from being understood and highlights the importance of distinguish natural vs non-natural rewards and associated responses. Regarding aversion, the few studies seem to pinpoint a predominant role for D2Rs in aversive learning.

7.3. Specific striatal manipulations

An elegant approach using a reversible neurotransmission blocking technique has shown that the striatonigral pathway is critical for reward learning whereas the striatopallidal pathway was important aversive learning (Hikida et al., 2010). Later, these researchers have combined this methodology with local infusion of a receptor-specific agonist or antagonist in the NAc. They showed that bilateral blockade of the direct pathway in the NAc, but not that of the indirect pathway, significantly reduced cocaine-induced place preference and decreased expression of appetitive reward learning. In addition, mice lacking the D2L receptors (postsynaptic) present a significant impairment in aversive learning (Hikida et al., 2013). Interestingly, this data was supported by the fact that specific inhibition of D2R-dependent neurotransmission within the

NAc also causes impairment in aversive learning in a one-trial inhibitory avoidance task (Hikida et al., 2013).

Others have shown that ablation of NAc A_2aR-expressing neurons (A_2aR is co-expressed with D2R receptor in striatum) induced an increase in amphetamine conditioned place preference, suggesting that these neurons inhibit drug reinforcement (Durieux et al., 2009).

7.4. Pharmacogenetic studies

Recent studies used viral-mediated expression of a designer receptor exclusively activated by a synthetic drug (DREADDs); activation the DREADD hM4D receptor potently reduces striatal excitability. With this technique, researchers found that transient disruption of striatopallidal (D2R) neuronal activity facilitated behavioural sensitization to amphetamine, whereas decreasing excitability of striatonigral (D1R) neurons impaired its persistence (Ferguson et al., 2011).

7.5. Optogenetic studies

Optogenetic studies have provided an elegant tool to manipulate different populations of striatal neurons in a fast, specific and reversible manner in order to overcome some caveats of earlier studies. However, the use of these tools also present clear limitations: (i) viral expression of opsins relies on the use of *specific* promoters that sometimes are not fully characterized, (ii) different stimulation protocols may activate/inhibit distinctive neuronal populations, leading to idiosyncratic behavioral outcomes (this is particularly important considering the unique roles of phasic vs tonic dopamine signaling), (iii) the use of transgenic cre strains to drive opsin expression may present ectopic expression of cre protein (Gong et al., 2007; Stuber et al., 2015) and last, but not least, (iv) optical neuronal activation does not necessarily mimic p*hysiological* activity.

7.5.1. Optogenetic activation of D1-MSNs: NAc versus dorsal striatum

Most of the studies use cre-dependent opsins packed in viral particles in combination with cre transgenic models. Optogenetic stimulation of D1-MSNs within the NAc does not have a major effect on locomotor activity (Francis et al., 2015; Soares-Cunha et al., 2016), and is sufficient to increase the willingness to work for food (Soares-Cunha et al., 2016), suggesting a *pro-reward* role. This is in line with studies indicating that activation of NAc D1-MSNs is sufficient to increase cocaine sensitization and cocaine place preference (Lobo et al., 2010). In agreement, optogenetic inactivation of D1-MSNs supresses cocaine sensitization (Chandra et al., 2013).

Contrary to NAc, activation of D1-MSNs in the dorsal striatum increased spontaneous locomotion as expected. Dorsal striatum D1-MSNs activation was sufficient to induce an operant behaviour in order to receive optic stimulation of these neurons, which confirms its reinforcing properties (Kravitz et al., 2010). Others have shown that unilateral stimulation of D1-MSNs shifts responses for reward toward the contralateral side of the stimulation (Tai et al., 2012). The authors attributed this bias to the positive effects of activation of the direct pathways within the dorsal striatum on motivated responses (Tai et al., 2012). Interestingly, stimulation of D1-MSNs of the dorsal striatum also results in an increase in cocaine place conditioning (Kravitz et al., 2012) emphasizing the role of D1-MSNs in reinforcement. Recently it was shown that although both activation of D1- and D2-MSNs within the DMS result in increased self-stimulation, optical stimulation of D1-MSNs was shown to be involved with actions that support goal-directed behaviours, while activation of D2-MSNs resulted in actions that support the formation of stimulus-response habits (Vicente et al., 2016).

7.5.2. Optogenetic activation of D2-MSNs: NAc versus dorsal striatum

Less concordant is the contribution of NAc D2-MSNs in locomotion, reward and aversion. Activating cre-dependent ChR2 in a D2-cre transgenic mouse line did not trigger a major impact in locomotion (Francis et al., 2015; Lobo et al., 2010), although others reported a decrease in basal locomotor activity (Song et al., 2014).

Stimulation of NAc D2-MSNs attenuated cocaine conditioned place preference (Lobo et al., 2010), and stimulation of A_2aR-containing neurons (supposedly D2-MSNs) and supressed cocaine self-administration (Bock et al., 2013). This *anti-reward* role was challenged by evidence showing that stimulation of NAc D2-MSNs neither affected the acquisition or expression of cocaine-induced behavioral sensitization (Song et al., 2014). In addition, we have shown that accumbal D2-MSNs stimulation strongly enhances motivation/willingness to work for food and is able to induce place conditioning *per se* (Soares-Cunha et al., 2016; Soares-Cunha et al., unpublished observations).

Moreover, it was shown that the reinforcing properties of optogenetic activation of VTA-NAc dopaminergic terminals requires both D1-MSNs or D2-MSNs signaling (Steinberg et al., 2014). The discrepancy in the results might be attributed to technical differences in the experimental protocols. However, one cannot exclude that D2-MSNs may contribute differentially to responses to natural rewards and to drugs of abuse, an aspect that needs to be explored in future works.

Less controversy exists regarding the role of dorsal striatum D2-MSNs on motor activity and reward/aversion. As expected, stimulation of dorsal striatum D2-MSNs (using A_2a-cre line) decreases locomotor activity (Kravitz et al., 2012). Importantly, in this same study, it was shown that D2-MSNs activation caused *punishment*, by inducing a transient period of avoidance towards the trigger that originated D2-MSNs stimulation (Kravitz et al., 2012). Using a D2-cre line, optogenetic stimulation of dorsal striatum D2-MSNs neurons shifted operant responses for food rewards toward the ispilateral side of the stimulation, resembling a decrease in the value for the reward (Tai et al., 2012).

In summary, optogenetic studies seem to support the established view of pro-reward role of D1-MSNs both in the ventral striatum and dorsal striatum. Less explicit is the role of D2-MSNs in reward and aversion, since conflicting findings have been described, which suggests that further studies are needed to understand the contribution of these MSNs for these behavioural dimensions.

8. Striatal D1R and D2R in human behavior

Human imaging studies have also provided some insights on the role of striatal dopaminergic signaling in reward behaviors, namely in the control of motivation, in incentive salience and reinforcement, and to a lesser extent in aversion/punishment.

Similar to findings in animal models, earlier human studies have shown that dopamine is released in the ventral striatum in anticipation to monetary or taste rewards (Knutson et al., 2001; O'Doherty et al., 2002). Later studies have refined the paradigms and were able to show that the striatum responds (albeit differently) to monetary outcomes of different valence (reward and punishment) (Delgado, 2007; Delgado et al., 2000). Interestingly, an anteroposterior valence-specific gradient seems to exist at least in the NAc, with more anterior regions showing relative selectivity for rewards and posterior regions for losses (Seymour et al., 2007). In addition to anatomical specificities, in non-human primates,

separate neural striatal populations coding for actions that are probabilistically associated with positive and negative outcomes exist (Samejima et al., 2005). Several other works have supported the co-existence of appetitive and aversive prediction-error signals within the dopaminergic mesolimbic system, which shifts the view of the canonical "reward pathway" to a value-based decision making system (reviewed in Brooks and Berns, 2013; Liu et al., 2011).

But how can a single molecule mediate two opposing behaviors? The simplest response points for the selective activation of different dopamine receptors. A current popular model argues that burst (phasic) dopamine signals that encode unexpected rewards are associated with behavioral activation (Go) through D1R (low affinity), whereas aversion/avoidance (No Go) learning is mediated through D2R (high affinity) in a U-shaped manner (Frank et al., 2004). In this perspective, it is interesting that individual differences in human approach and avoidance learning are predicted by variability in striatal D1R and D2R binding, respectively (Cox et al., 2015). Adding further complexity to the interpretation of the findings, it has been shown that striatal (and frontal) asymmetries in D2R binding (rather than absolute binding levels) predict individual differences in learning from reward versus punishment (Tomer et al., 2013). Importantly, in drug abusers, low striatal D2R availability is predictive of a pleasurable response to intravenous stimulant administration, while high binding was associated with an aversive experience (Volkow et al., 2002, 1999). Paradoxical findings have also been found, showing that D2R is decisive for measures of choice performance rather than for aversive instrumental learning (Eisenegger et al., 2014).

Natural genetic variants resulting in either increased (C957T polymorphism) or reduced (A1 alelle) striatal D2R also modulate valenced responses. For example, C957T polymorphism is associated with enhanced learning from negative outcomes (Frank et al., 2007). A1 carriers, (lower striatal D2R expression but increased striatal dopamine), are less successful in predicting negative outcomes and showing diminished recruitment of ventral striatum during negative feedback processing and reversal learning (Jocham et al., 2009; Klein et al., 2007). All of these findings support a bidirectional modulatory role for striatal dopamine in reward – via D1R – and aversion learning – via D2R.

In addition to the prevalent view that (phasic) dopamine encodes prediction error signals that mediate reinforcement learning (Bayer and Glimcher, 2005; Schultz et al., 1997), there is also evidence that (tonic) dopamine may play a role in response vigor and motivational aspects of reinforcement, and this may rely on the activation of different dopamine receptors at the striatum level (Bromberg-Martin et al., 2010; Niv et al., 2006). For example, higher D2R availability in the left striatum is associated with greater positive incentive motivation in healthy controls (Tomer et al., 2008). Both healthy volunteers and schizophrenics treated with atypical neuroleptics showed ventral striatum activation in response to reward-predicting cues; however, in patients treated with typical neuroleptics, which block D2R, this response is blunted and is correlated with motivational deficits and the severity of negative symptoms (Juckel et al., 2006).

9. Conclusion

Although different approaches have provided insights about the role of striatal D1-MSNs and D2-MSNs in reward/aversion, the complexity of these behaviors challenges the interpretation of the data. It is remarkably difficult to separate and evaluate distinct aspects of reward/aversion without interference. Moreover, it is important to highlight that clear differences exist between dorsal striatum and NAc MSNs, and the lack of segregation of the direct/indirect pathways based on D1R and D2R expression in the NAc.

Evidence strongly suggests that the canonical view of striatal D1R signalling as pro-reward/reinforcing and D2R signalling as pro-aversive is too simplistic. It is naïve to assume that D1R- and D2R-expressing neurons play completely independent (and contrasting) roles. We believe that there is a coordinated (and not necessarily antagonic) action between the two neuronal subpopulations in the control of these behaviours. Interestingly, some recent studies have also questioned the pro- or anti-kinetic dichotomy of D1-MSNs/direct or D2-MSNs/indirect pathways in motor control (Cui et al., 2013), emphasizing the need to re-evaluate the early view that striatal D1- and D2-MSNs mediate exclusively opposing aspects of behaviour.

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