THE ROLE OF D1R/D2R CO-EXPRESSING STRIATAL MEDIUM SPINY NEURONS: IMPLICATIONS FOR PSYCHOSTIMULANT RESPONSE

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TABLE OF CONTENTS

ABSTRACT

Dopamine (DA) is one of the principal catecholaminergic neurotransmitters in the brain. Initially thought as a mere precursor to norepinephrine synthesis in the 1950s, it has been demonstrated to be an essential part of the mammalian central nervous system. DA is synthesized in the substantia nigra (SN) and the ventral tegmental area (VTA) by mesencephalic neurons, projecting to the striatum and the limbic system, among other targets (Tritsch & Sabatini, 2012). The striatum is largely influenced by DA activity. The dorsal striatum, also known as the caudate putamen (CPu), is the target of the nigrastriatal DA pathway originating from the SN, often implicated in motor function; the ventral striatum, which is primarily composed of the nucleus accumbens (NAc), is the target of the mesolimbic DA projection from the VTA and is linked to reward-related behaviours (Haber, 2014).

The largest represented type of striatal neurons, the medium spiny neurons (MSNs), express DA receptors and are generally thought to be divided into two classes based on the family of DA receptors they express: Dopamine receptor 1 (D1R) and dopamine receptor 2 (D2R; Gerfen & Surmeier, 2011). Although the segregation of D1R MSNs and D2R MSNs is a well-established model to study striatal functions, an increasing number of studies indicate that some MSNs could co-express both D1R and D2R, suggesting an alternative mechanism in the striatum. The present study aims to characterize the nature of D1R/D2R co-expressing striatal MSNs. By using a $D1R^{lox}A2a^{cre}$ mouse knockout (KO) model intended to remove D1R from this subpopulation of neurons, this work intends to elucidate the cellular and behavioural functions of these D1R/D2R co-expressing MSNs and clarify their role in psychostimulant drug response.

RÉSUMÉ

La dopamine (DA) est l'un des principaux neurotransmetteurs catécholaminergiques. Initialement considérée dans les années 50 comme un simple précurseur de la synthèse de noradrénaline, il fut depuis démontré qu'elle est essentielle au fonctionnement du système nerveux central chez les mammifères. La DA est synthétisée dans la substance noire (SN) et dans l'aire tegmentale ventrale (VTA) par les neurones mésencéphaliques projetant vers le striatum et le système lymbique (Tritsch & Sabatini, 2012). Le striatum est grandement influencé par l'activité de la DA. Le striatum dorsal, aussi connu comme le putamen caudé (CPu), est la cible de la voie de la DA nigrostriatale provenant de la SN : une voie qui s'avère être souvent impliquée dans la fonction motrice. Le striatum ventral, composé principalement du noyau accumbens (NAc), est quant à lui cible des projections mésolimbiques de DA provenant de la VTA et est lié aux comportements de récompense.

Le type de neurones les plus retrouvés dans le striatum, les neurones épineux moyens (MSNs), expriment les récepteurs à la DA et sont généralement considérés comme étant divisés en deux classes selon la famille de récepteurs qu'elles expriment : les récepteurs à dopamine de type 1 (D1R) et les récepteurs à la dopamine de type 2 (D2R ; Gerfen & Surmeier, 2011). Même si cette ségrégation en MSNs D1R et D2R a permis d'établir un premier modèle d'étude des fonctions striatales, un nombre croissant d'études proposent que certains de ces MSNs pourraient co-exprimer D1R et D2R, suggérant un niveau de fonctionnement plus complexe au sein du striatum. Cette étude a pour but de caractériser les MSNs co-exprimant les D1R/D2R dans le striatum. A l'aide d'un modèle de souris knock-out (KO) permettant de supprimer l'expression du D1R de dans cette sous-population de neurones, cette étude tente d'élucider les fonctions

cellulaires et comportementales des MSNs co-exprimant D1R/D2R et de clarifier leur rôle dans la réponse aux psychostimulants.

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INTRODUCTION AND LITERATURE REVIEW

1. Overview of Dopamine and Striatal MSNs

Dopamine (DA, also known as 3-hydroxytyramine), a metabolite of the amino acid tyrosine, is a catecholaminergic neurotransmitter implicated in a variety of physiological and psychological functions, including attention, memory, learning, voluntary movement, and responses to reward (Beaulieu & Gainetdinov, 2011). There are five types of G-protein coupled DA receptors, divided into two classes: D1-class receptors (D1R and D5R subtypes) activate $G_{s/olf}$ and stimulate cAMP production via activating adenylyl cyclase (AC) activity, while D2-class receptors (D2R, D3R, and D4R subtypes) activate $G_{i/o}$ and reduce cAMP production via inhibiting AC activity (Beaulieu & Gainetdinov, 2011). The D1R is expressed mostly postsynaptically and is localized in nigrostriatal, mesolimbic and mesocortical pathway structures, including the striatum (CPu and NAc), SN, olfactory bulb, amygdala, hippocampus and the frontal cortex. In contrast, the D2R subtype is expressed either presynaptically on DA neurons or postsynaptically on DA target cells. Many D2R are concentrated in the striatum and the olfactory tubercle, though they can also be found in the SN, VTA, hypothalamus, cortex and hippocampus, amongst other areas (Beaulieu & Gainetdinov, 2011)

Targeted by the DA neurons projecting from the VTA and the SN, the striatum is one of the main brain structures influenced by dopamine activity. It is generally divided into two parts; the dorsal area is referred to as the caudate-putamen (CPu) and the ventral area forms the nucleus accumbens (NAc). The CPu is the target of the nigrostriatal DA pathway originating from the SN; this pathway has been frequently linked to motor function. The NAc is the target of the mesolimbic DA pathway from the VTA; this pathway is primary implicated in reward-related behaviours (Haber, 2014). In the striatum, the largest neuronal population is composed by

GABAergic medium spiny neurons (MSNs), representing about 90% of the total population characterized by their high spine density, negative resting potential, and low firing rates *in vivo*. The remaining neurons are interneurons, which can be divided into three main categories: GABAergic fast spiking cells (positive for parvalbumin), GABAergic low-threshold spiking cells (positive for somatostatin, nitric oxide synthase, or neuropeptide Y), and cholinergic tonically active cells with large cell bodies and dense axonal arborizations (Kreitzer & Malenka, 2008). This large population of striatal MSNs is classified into two distinct populations: D1R-expressing striatonigral MSNs leading to the direct pathway, and D2R-expressing striatopallidal MSNs leading to the indirect pathway (Gerfen & Surmeier, 2011). The direct pathway D1R MSNs receive excitatory glutamatergic inputs from the thalamus and cortex, and these MSNs send inhibitory GABAergic input to the globus pallidus internus (GPi) and the substantia nigra pars reticulata (SNr), structures that inhibit the motor nuclei of the thalamus. Thus, activating the direct pathway D1R MSNs disinhibits the thalamus and allows it to facilitate movement via its cortical connections. Conversely, the indirect pathway D2R MSNs projects inhibitory GABAergic connections to pallidal neurons, which are in turn responsible for inhibiting the subthalamic nucleus (STN). The STN has excitatory glutamatergic input to the GPi and SNr structures, which inhibits the thalamic motor nucleus as previously mentioned. Therefore, activating the indirect pathway D2R MSNs has a net effect of disinhibiting the STN and allowing it to excite GPi and SNr, which ultimately inhibits the thalamus and reduce motor output through its cortical connections (Kreitzer & Malenka, 2008). The D1R MSNs can be identified by their co-expression of substance P, the peptide dynorphin, and M4 muscarinic acetylcholine receptors, while the D2R MSNs are marked by their co-expression of enkephalin as well as adenosine 2a (A2a) receptors (Valjent et al., 2009; Ena et al., 2011).

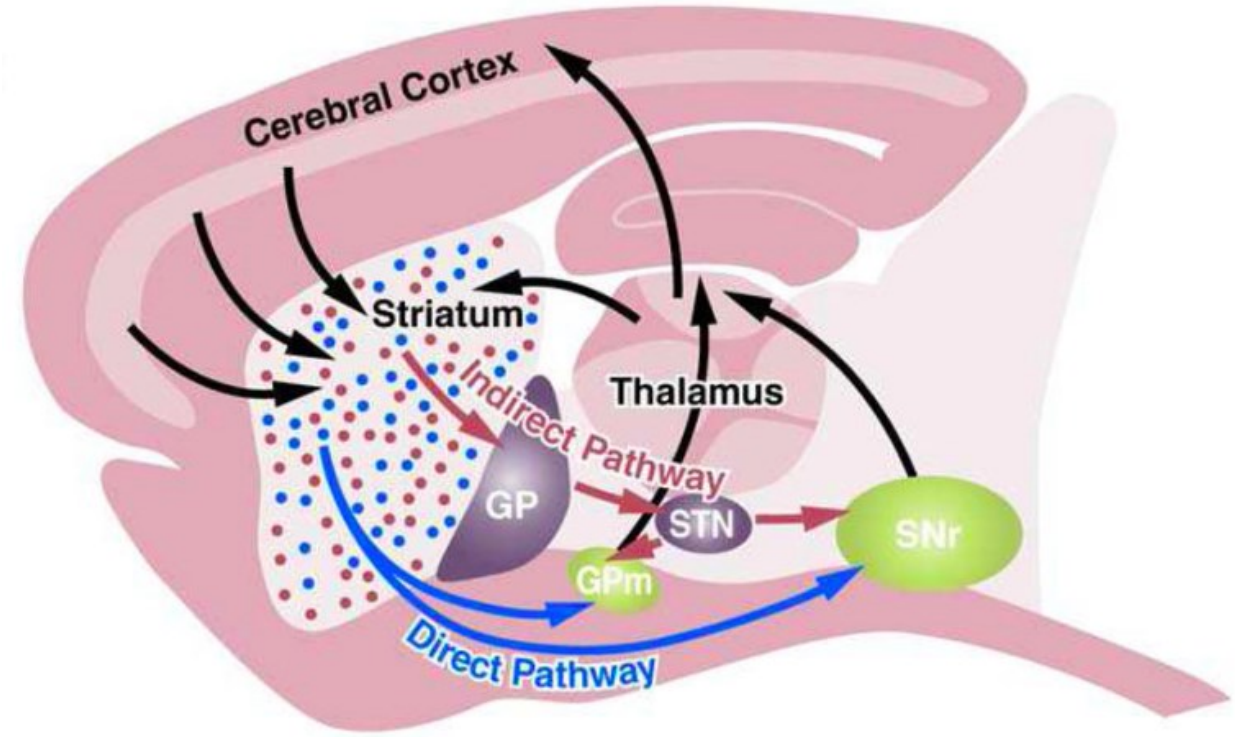

Figure 1. Schematic representation of the direct and indirect pathways and related brain structures on a sagittal view of the mouse brain. Blue arrows represent the striatonigral projections of the direct pathway MSNs, and the red arrows represent the striatopallidal projections of the indirect pathway MSNs (reprinted from Kreitzer & Malenka, 2008).

2. D1R and D2R co-expression on striatal MSNs

Adding more complexity, emerging evidence are challenging the traditional view binary view that the D1R MSNs are specific to the direct pathway and D2R MSNs are specific to the indirect pathway. Some D1R MSNs project into the indirect pathway as well, while there can also be convergence of the direct and indirect pathways on the same neuron (Kupchik & Kalivas, 2017). Studies in the past two decades have suggested that D1R and D2R receptors may be co-expressed in some of the MSNs. However, the certainty and the extent of this co-expression remains unclear.

Early work using single-neuron RT-PCR analysis suggested that almost half of the cells in the dorsal striatum express both D1-like and D2-like receptor mRNA, indicating that co-expression of D1R and D2R on the same MSN is possible (Surmeier et al., 1996). Immunostaining with D1R and D2R fluorescent antibodies generated evidence of near complete D1R/D2R co-localization in cultured striatal MSNs as well as in the MSNs on CPu and NAc brain sections (Aizman et al., 2000). Some presynaptic varicosities of NAc MSNs respond to both D1R and D2R agonism, suggesting that the MSNs that synapse with these varicosities may be able to receive both D1R and D2R signals, implicating D1R/D2R co-localization (Mizuno et al., 2007).

2.1 Transgenic mice models

To further study this cell population, bacterial artificial chromosome (BAC) transgenic mice models were developed. Mice engineered with the td-Tomato fluorescent reporter protein linked to the D1R gene promoter (Drd1a-tdTomato mice) was crossed with other mice expressing green fluorescent protein (GFP) linked to the D2R gene promoter (Drd2-eGFP), such that MSNs co-expressing D1R and D2R receptors expressed both fluorescent proteins. Initial findings with this model showed < 1% co-localization of D1R and D2R reporter proteins in the same cells (Shuen et al., 2008). Another study using the same model found a small degree of reporter protein co-localization (5-7% NAc shell, 2-3% NAc core, 2-7% CPu), though direct analysis of D1R and D2R in the same study did not bring clarity as to whether D1R/D2R co-localization exists in these cells (Frederick et al., 2015). Notably, Thibault et al. (2013) used this reporter co-localization model to examine D1R/D2R distribution throughout development, demonstrating that striatal D1R/D2R co-localization (based on the reporters) was around 9% prenatally and decreases over time of development to reach about 3% by post-natal day 14. This

suggested that a higher D1R/D2R co-localization is developmentally significant and marks a transitional stage that become refined over the life of an organism.

An alternative BAC transgenic model aimed to address D1R/D2R co-localization involved engineered mice with the GFP linked to the D1R gene promoter (Drd1a-eGFP), GFP linked to the D2R gene promoter (Drd2-eGFP), and the offspring of these two lines. The proportion sum of the marked MSNs in D1R GFP reporter line and those in the D2R GFP reporter line exceeds 100%, which led to estimates of co-localization (17% NAc shell, 6% NAc core, 5% CPu) based on the observation that all MSNs express at least one of the D1R and D2R (Bertran-Gonzalez et al., 2008; Matamales et al., 2009). Particularly, the caudomedial aspect of the NAc showed 38% of estimated D1R/D2R reporter co-localization (Gangarossa et al., 2013a).

The abovementioned *in vivo* transgenic mice models improve upon the initial in vitro studies of D1R/D2R co-localization, though their use of genetically linked reporters was not a direct assessment of D1R and D2R levels. As such, the exact percentage of D1R and D2R co-expressing MSN in the striatum remains a point of contention in the field, due to the mixed results of co-localization percentage estimates in existing studies.

2.2 Co-localization as D1R/D2R heteromers?

Taking one step further than co-expression, there have been evidence mostly from Susan George's research group suggesting that D1R and D2R form a heteromer in certain striatal MSNs (Rashid et al., 2007; Hasbi et al., 2009; Perreault et al., 2011; Shen et al., 2015). In particular, the formation of this D1R-D2R oligomer has been implicated to follow a G-protein pathway differentiated from the direct and indirect pathways; this pathway involves the increase of calcium/calmodulin-dependent protein kinase II (CaMKII) activity in NAc and can be activated by the proposed heteromer agonist SKF 83959 (Rashid et al., 2007). There has been

some other evidence in support of the heteromer theory, demonstrating that D1R and D2R heteromer coupling occurred at higher levels in depressed human post-mortem brain samples, and that using an interfering peptide to disrupt D1R/D2R coupling led to antidepressant-like effects in rodents (Pei et al., 2010; Shen et al., 2015).

However, the presence of this heteromer remains controversial. Recent evidence indicates that the mechanism of the proposed D1R-D2R heteromer agonist SKF 83959 was only dependent on D1R and not on D2R, suggesting that the interaction between the two receptors may not be necessarily present (Frederick et al., 2015). The same study also found that little to no heteromer interaction existed between the D1R and D2R in the adult rodent striatum; this was found by using proximity ligation assay, a technique that produces a signal only when two proteins are sufficiently close to form a heteromer (Frederick et al., 2015).

Whether the D1R-D2R heteromer exists remains a source of scientific interest. If D1R-D2R heteromers are a naturalistic phenomenon, then D1R and D2R are necessarily co-expressed on a subpopulation of striatal MSNs. Even in the case that they do not form heteromers, the possibility that D1R and D2R are co-expressed on the same MSN is not eliminated. As such, debate over the D1R-D2R heteromer indicates that significant scientific interest exists over the extent of interaction between the D1R system and the D2R system in the striatum.

3. DA receptors in psychostimulant response

Drugs of abuse (such as psychostimulants, opiates, ethanol, and nicotine) increased extracellular concentrations of DA in the striatum, especially in the NAc (Di Chiara & Imperato, 1988). The striatum, particularly the mesolimbic VTA-NAc pathway, has been identified as the main pathway for reward response and addiction (Nestler, 2005) and is an important site that

mediates the effects of psychostimulants like cocaine and amphetamine. Both drugs act by increasing extracelluar DA levels: cocaine blocks the reuptake of DA from the synaptic cleft by binding to the dopamine transporter (DAT), while amphetamine interferes with DAT reuptake while also causing more DA to exit the cell through DAT-mediated reverse transport (Dela Peña et al., 2015). Psychotimulants such as cocaine and amphetamine, in addition to eliciting reward, led to hypermotility; it also led to locomotor sensitization, defined as the escalating effect of a drug (e.g., psychostimulants) resulting from repeated administration at a given dose (Di Chiara, 1995). Upon the administration of psychostimulants, D1R and D2R exerted specific influences over locomotor- and reward-based behaviours, and these influences have been investigated through a wide range of methodologies (Baik, 2013).

3.1 D1R in psychostimulant response

The full constitutive D1R knockout (KO) transgenic mice model has clarified some aspects of D1R involvement in psychostimulant response. D1R KO mice displayed basal hyperlocomotor activity compared to WT but failed to show the psychostimulant-induced hypermotility exhibited by WT (Xu et al., 1994; Crawford et al., 1997; Karlsson et al., 2008). As such, this phenomenon was D1R-dependent, reinforcing similar findings from previous studies that analyzed psychostimulant response in the presence of D1R antagonist SCH 23390 (Cabib et al., 1991; Hummel & Unterwald, 2002). D1R KO showed reduced to no locomotor sensitization to psychostimulants (Karlsson et al., 2008). Moreover, when D1R-MSNs were specifically disrupted via toxins or optogenetics, cocaine sensitization was also diminished (Hikida et al., 2010; Chandra et al., 2013). Cocaine conditioned place preference (CPP) was not affected in the D1R KO (Miner et al., 1995), though cocaine self-administration and intracranial self-

stimulation behaviours were D1R-dependent (Tran et al., 2005; Caine et al., 2007), suggesting that D1R may be involved in specific aspects of reward processing.

Transcription factors including c-Fos and CREB appears 1-2 hours after drug administration (Ruffle, 2014), which were often used as markers to detect drug-activated cells. Cocaine and amphetamine has been shown to induce expression of c-Fos in the striatum (Graybiel et al., 1990). This c-Fos increase was preferentially occurring in D1R-expressing MSNs, indicating that the D1R pathway specifically play a role in psychostimulant-induced physiological changes (Bertran-Gonzalez et al., 2008). Another transcription factor ΔFosB peaks in the striatum approximately 6 hours after acute psychostimulant administration, though many of its isoforms remain stable and will persist for a longer period, thereby accumulating in the case of chronic psychostimulant administration (Nestler et al., 2001). As such, ΔFosB is a potential molecular mechanism through which gene expression changes following psychostimulant abuse are maintained long-term. Given the D1R-dependent nature of c-Fos expression, this ΔFosB accumulation following chronic psychostimulant exposure may also be linked to a D1R mechanism.

3.2 D2R in psychostimulant response

D2R antagonists do not affect the cocaine-induced hypermotility (Cabib et al., 1991), though D2R KO transgenic mice demonstrated a hypomobility at baseline and showed a decreased motor response to cocaine compared to WT (Chausmer et al., 2002; Welter et al., 2007). Locomotor sensitization to stimulants was not significantly changed in D2R KO; similarly, NAc-specific alterations of D2R activity did not alter cocaine-based locomotor sensitization (Lobo et al., 2010, Sim et al., 2013) suggesting that D2R was not critical for this phenomenon. However, there were some evidence that D2R inhibition (via toxins on D2R-

MSNs) can inhibit cocaine sensitization (Hikida et al., 2010). Cocaine-induced CPP was not D2R-dependent based on D2R antagonist and D2R KO studies, while similar results were also seen in studies involving NAc-specific D2R inhibition (Cervo & Samanin, 1995; Welter et al., 2007; Hikida et al., 2010; Sim et al., 2013). Interestingly, optogenetic activation of D2R-expressing MSNs specifically in the NAc reduced cocaine CPP, while the conditioned KO of D2 autoreceptors demonstrated an enhancement of cocaine CPP (Lobo et al., 2010; Bello et al., 2011), suggesting that certain D2R subpopulations may serve as an inhibitory control mechanism over reward-seeking. Cocaine self-administration was D2R-dependent, such that D2R KOs showed an increase in self-administration while activation of D2R-MSNs in the NAc induced a decrease in self-administration (Caine et al., 2002; Bock et al., 2013); similar to the CPP findings, D2R transmission may serve as an inhibitor of drug-seeking behaviours.

3.3 D1R/D2R integration?

Based on the previous findings, it appears that in the case of psychostimulant response, striatal D1R MSNs play a stronger role in modulated locomotor-based effects, while the D2R MSNs are more involved in reward response. However, these effects were not completely mutually exclusive, suggesting that there is some significant degree of interaction between the D1R and the D2R systems.

Interestingly, the primarily D1R-MSN-based c-Fos activation after psychostimulant administration (as discussed above in Bertran-Gonzalez et al., 2008) was true in familiar environments such as the home cage or a habituated context, but both D1R and D2R MSNs (as labelled by D1R and D2R mRNA) showed c-Fos activation if the animals received the psychostimulant in a novel environment (Badiani et al, 1999). Therefore it is possible that co-activation of D1R and D2R systems is a critical process when integrating sensorimotor and

reward-based information at the onset of psychostimulant administration. There was some evidence that D1R/D2R co-activation was required for the expression of the locomotor sensitization effect in rodents previously conditioned with cocaine; either D1R or D2R agonism alone was not sufficient to manifest the sensitized effect (Capper-Loup et al., 2002). Similarly, as discussed previously, the psychostimulant-induced locomotor effect was predominantly D1R-dependent, though changes in D2R can also reduce this effect. This reinforces the possibility that the cooperation between D1R and D2R systems is necessary to produce the psychostimulant-induced changes *in vivo*.

If D1R/D2R co-activation is a foundation of psychostimulant effects, the subset of D1R/D2R co-expressing MSNs could be an important site for this signal integration. This integration is supported by the D1R and D2R studies summarized previously, since a significant overlap of function is present in the two systems. As such, the relative expression of DA receptors located on striatal D1R/D2R MSNs may be particularly relevant for this signal integration. The goal of the present study is to examine the D1R on this D1R/D2R co-expressing MSN subpopulation and to investigate its role in the regulation of psychostimulant response.

HYPOTHESIS

Do the D1R/D2R co-expressing MSNs plays a role in regulating the response to addictive psychostimulants?

OBJECTIVES

1. Characterize the D1R/D2R co-expression in the striatal MSNs.
2. Evaluate the role of D1R/D2R co-expressing MSNs in regulating behavioural response to psychostimulants.
3. Evaluate the role of D1R/D2R co-expressing MSNs in generating psychostimulant-induced neuronal changes.

METHODS

1. Animals

All work related to animal care and breeding were conducted in accordance to the Canadian Council on Animal Care guidelines and the Douglas Institute Research Center Animal Care Committee. The mice were lived under standard conditions of 22±1°C and 60% relative humidity under a 12-h light-dark cycle. Animals were housed in groups of 4-5 in cages where food and water were available *ad libitum*.

Genetically modified mice were created to remove D1R in the cells that co-express D1R and D2R receptors using the cre-lox system. To create such mice, heterozygous D1R floxed mice ($D1R^{lox+/-}$) were crossed with heterozygous A2a cre mice ($A2a^{cre/+}$) to obtain heterozygous (HET) offspring for both genotypes ($D1R^{lox+/-}/A2a^{cre/+}$). Double HET animals were then crossed to obtain KO animals ($D1R^{lox+/+}/A2a^{cre/+}$) and littermate wildtype (WT) animals. In the KO animals, the floxed sequence of the D1R gene is removed only in cells where cre is expressed, which is any cell that expresses A2a. As aforementioned, A2a marks D2R-expressing MSNs, as the A2a receptor exhibits reliable co-expression with D2R (Gangarossa et al., 2013b). D2R itself is more widely expressed early in development, such that using D2R as the cre site may cause more removal of D1R than intended; A2a was chosen as the cre site due to its later expression in development (Durieux et al., 2009). For all experiments, $D1R^{lox}A2a^{cre}$ KO and littermate WT ($D1R^{lox-/-}/A2a^{cre/+}$) mice were used at 2-5 months of age.

2. D1R/D2R fluorescent *in-situ* hybridization

2.1 Slide preparation

Fresh brains were harvested from $D1R^{lox}A2a^{cre}$ KO and WT animals after deep anesthesia with isoflurane, frozen in isopentane at -40°C, and stored at -80°C. Striatum slices were collected using a cryostat (10 μm thickness) and immediately mounted on slides. Additional fresh tissue is collected using the same protocol from C57bl/6 pups (0, 7, 14, and 21 days old). Digoxigenin-labelled D1R mRNA probe and fluorescein-labelled D2R mRNA probe were made prior to the experiment. The complementary DNAs for both receptor mRNAs were amplified with PCRs, and the resulting product was run on a 1% agarose gel and extracted. The PCR product from the D1R mRNA was incubated with 10X digoxigenin-RNA labelling mix, along with RNA polymerase, 5X transcription buffer, dithiothreitol (DTT), RNAsin, and water (the D2R probes were made using the same protocol, substituting for the D2R mRNA PCR product and the 10X fluorescein-RNA labelling mix). This mixture was purified using ProbeQuant G-50 micro columns. The concentration of probes was measured, and the quality of the probes was assessed using 1% agarose gel.

Striatal sections were washed in RNAse-free PBS and immerged in 4% formaldehyde for 10 minutes. They were then washed in PBS and exposed to a fresh acetylation solution (18.5 mg/ml triethanolamine, 2.24 μl/ml NaOH 10N, and 2.5 μl/ml acetic acid anhydride dissolved in RNAse-free water) for 10 minutes. The slices underwent another wash in PBS and were incubated in a 5X SSC humid chamber for 30 minutes with hybridization buffer (0.25 mg/ml E.coli tRNA diluted in the following solution by volume: 50% formamide, 25% 20X SSC, 10% RNAse-free water, 10% Denhardt's solution, 5% Salmon Sperm DNA). A mix of Digoxigenin-labelled D1 mRNA probe and fluorescein-labelled D2 mRNA probe (diluted in hybridization buffer) was used on the brain sections (20-40 ng of each probe per slide), which were incubated overnight at 60°C. On the second day, slides were washed in 5X SSC and 0.2X SSC at 60°C,

then washed in 0.2X SSC and MABT. Slices were exposed to a blocking solution (by volume: 40% distilled water, 20% 5X MAB, 20% head inactivated fetal bovine serum, 20% 5X blocking reagent) for 20 minutes, followed by incubation with anti-fluorescein-POD diluted at 1/1000 in the blocking buffer for 1 hour. Tissues were washed with MABT and PBST, incubated with the TSA Plus Biotin kit (1/50 in the amplification buffer) for 10 minutes, and washed again with PBST. They were subsequently incubated with NeutrAvidin Oregon Green (1/500 in PBST) for 10 minutes and washed in PBST. On the third day, sections were washed in glycine buffer (0.1M, pH 2.1) for 10 minutes, rinsed in PBST, washed 3% hydrogen peroxide, PBST and MABT, and then blocked with the blocking buffer for 20 minutes. Striatum slices were incubated with anti-digoxigenin-POD (1/2500 in the blocking buffer) for 1 hour, washed with MABT and PBST, and then incubated with the TSA Plus Cyanine 3 kit (1/100 in the amplification buffer) for 10 minutes. Slides were washed in PBST before using the Hoechst solution to stain the nuclei. Finally, the samples were washed with PBST and PBS and subsequently coverslipped with Fluoromount.

2.2 Quantification

Following the labeling of the D1R and D2R mRNA by double *in-situ* hybridization, we generated images of the striatum using a VS120 Virtual Slide Microscope (Olympus) in both WT and KO mice. After the acquisition of the samples, we analyzed the images using Fiji software (Schindelin et al., 2012) coupled with BIOP plug-in. First, we generated virtual grids and extracted equally-sized sub-sections (n=6-8 per group) of 2048 x 2048 pixels, of the striatum (AP: 0.74-0.86; L: 1.75; DV: 2.75-4.00). We then created a mask, based on the DAPI-stained nuclei and preselected criterions such as the size and shape of the nuclei (Matamales et al., 2009). Clusters of cells were not included in the analysis to avoid false positives regarding co-

localization of D1R and D2R mRNA. Next, we converted both red (D1R mRNA) and green (D2R mRNA) signals using the processing tool "find maxima" to generate a map of single points, where each point represented a labeled mRNA. The selected threshold for the signal's detection was identical across all samples. Finally, we merged the mask from DAPI staining and the maps of dots to obtain the profile of the cells. The total of MSNs was established by the sum of all cells expressing either D1R or D2R.

3. Dendritic spine analysis

$D1R^{lox}A2a^{cre}$ KO and WT animals that have undergone chronic saline administration for 6 days were used. Two hours after the last administration, mice were intracardially perfused with 1.5% paraformaldehyde (PFA) and brains were collected in 1.5% PFA overnight and kept in 30% sucrose solution. Striatum slices of 100 µm thickness were collected using the vibratome. A tube containing DiI fluorescence dye was connected to a compressed air bottle. DiI was sprayed on striatum sections. Slices were then immersed in PBS and incubated for at least 2 hours for DiI to absorb into the dendrites. After the incubation, the slices were mounted coverslipped with a Mowiol mounting medium.

The staining was revealed via excitation by 561-nm laser on a Confocal Laser Scanning Microscope. Deconvolution of the images was necessary for precise quantification of dendritic spines. NeuronStudio software was used to quantify and analyze dendritic spines.

4. Behavioural tests

4.1 Acute psychostimulant-induced locomotor activity.

KO and WT animals were injected either with cocaine, amphetamine or vehicle (0.9% NaCl solution). The cocaine dosages groups (in addition to vehicle) include 1, 5, 10 and 20 mg/kg respectively, while the amphetamine dosage groups (in addition to vehicle) include 1 and 3 mg/kg respectively. All drug and vehicle treatment were administered by intraperitoneal injection at the volume of 10 ml/kg.

Immediately after the injection, the locomotor activity of each animal was recorded for 2 hours using the VersaMax Animal Activity Monitoring System. The total distance travelled during the first hour of recording was analysed across dosage and genotype groups.

4.2 Cocaine sensitization

Baseline locomotor activity of KO and WT animals were recorded with the VersaMax Animal Activity Monitoring System for 15 minutes. Animals were then injected with cocaine, after which their locomotor activity was assessed for 2 hours. This process is repeated daily for 6 days. After a 7-day break, the animals undergo the same process on day 14 to assess conditioned activity levels. For this experiment, the chosen cocaine doses were 5 and 10 mg/kg.

The data were analyzed across the 6 experiment days and genotype groups, for each of the dosages used. The conditioned activity measured on Day 14 was assessed across genotype groups for each of the cocaine dosages. This protocol was adapted from Flores et al., 2005.

4.3 Conditioned place preference (CPP)

This experiment is divided across 3 phases: pre-conditioning (Day 1), conditioning (Day 2-4), and post-conditioning (Day 5). During pre-conditioning, animals could explore two separate and distinct chambers connected by a smaller transitory compartment for 15 minutes. Times spent in each chamber and in the transitory compartment were measured separately. During each conditioning day, animals were injected with cocaine followed by free exploration

of one the chambers (drug-paired chamber) for 20 minutes, and they were also injected with vehicle followed by free exploration of the other chamber (vehicle-paired chamber) for 20 minutes. The exits are closed on each chamber during conditioning to ensure that the drug is always consistently and exclusively associated with one chamber. The order of the injections on each condition day (cocaine/vehicle or vehicle/cocaine) was randomly chosen for each animal. The test during the post-conditioning day is the same as during the pre-conditioning day, whereby animals were allowed to explore both chambers freely for 15 minutes and time spent in each chamber were recorded. This experiment was run at the cocaine dosages of 1, 5 and 10 mg/kg.

The relevant measure is the difference of time spent in the drug-paired chamber between the post-conditioning phase and the pre-conditioning phase. This is analyzed across genotype groups at each of the cocaine dosages.

5. c-Fos immunohistochemistry

For the c-Fos experiments, $D1R^{lox}A2a^{cre}$ KO and WT animals were injected acutely with cocaine or vehicle (saline solution). Two hours after the last injection, mice were intracardially perfused with a cold saline solution for 2 minutes followed by a cold 4% paraformaldehyde (PFA) solution for 5 minutes. Brains were collected and placed in a 4% PFA solution overnight and were subsequently kept in 30% sucrose solution. Striatum slices were collected at 35 μm thickness in PBS for free-floating c-Fos immunohistochemistry. Slices were washed in PBS, exposed to a 3% hydrogen peroxide solution for 20 minutes, washed in PBS, and subsequently incubated overnight in primary goat anti-c-Fos antibody (diluted 1/500 in PBS, with 2% normal horse serum and 0.3% triton by PBS volume). The following day, slices were washed in PBS

and incubated with the biotinylated horse anti-goat secondary antibody for 2 hours. The amplification of the signal was accomplished with the avidin/biotin complex (ABC) kit and visualized with a DAB substrate kit. The slices were then mounted on slides and coverslipped with Permount.

The slices were imaged with the Olympus VS120 Virtual Slide Microscope at 10X magnification. The striatum was segmented by hand into CPu and NAc regions using ImageJ software. C-Fos positive cells within each subsection of the striatum were quantified starting from bregma +1.70 mm to bregma +0.50 mm, such that the same number of sections was used for each brain region. The measurement is expressed as a density of c-Fos activated cells per μm^2.

6. Statistics

D1R/D2R fluorescent *in-situ* hybridization sections were analyzed with Mann-Whitney U tests. Dendritic spine data was analyzed with a two-way ANOVA. Acute psychostimulant-induced locomotor activity data were analyzed with two-way ANOVAs. Cocaine locomotor sensitization results were analyzed with a mixed ANOVAs, with the challenge days (day 14) data analyzed by Mann-Whitney U tests. Conditioned place preference data were analyzed with t-tests and Mann-Whitney U tests. The data from c-Fos immunohistochemistry studies were analyzed with two-way ANOVAs. Statistics were computed using the software Statistica (Statsoft).

RESULTS

1. Characterize the D1R/D2R co-expression in the striatal MSNs.

1.1 D1R/D2R fluorescent *in-situ* hybridization

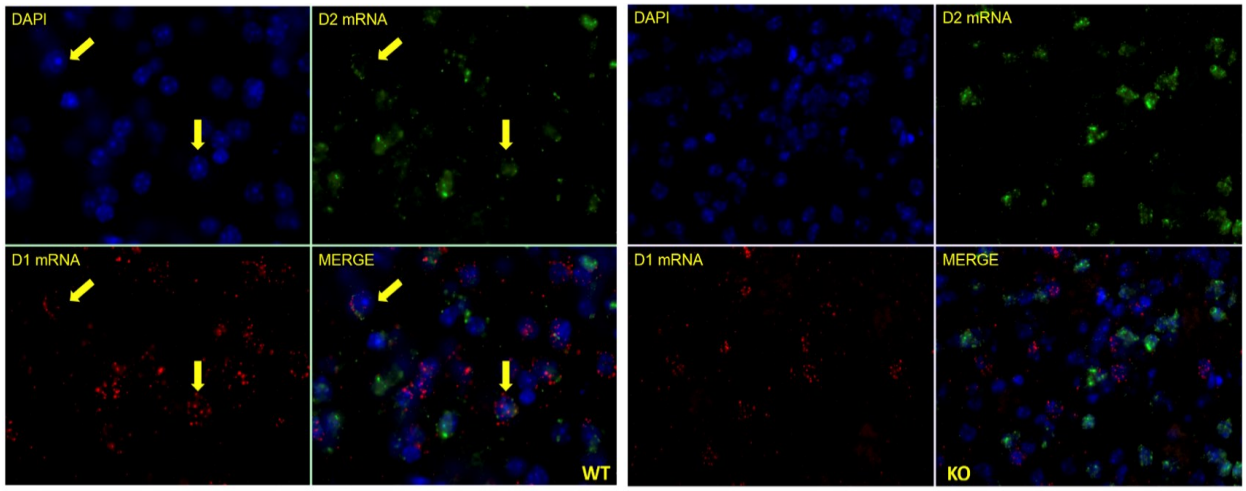

Figure 2. Double fluorescent *in situ* hybridization for D1R and D2R mRNAs in the nucleus accumbens of $D1R^{lox}A2a^{cre}$ WT mouse (left panel, $D1R^{lox-/-}/A2a^{cre/+}$) and KO mouse (right panel, $D1R^{lox+/+}/A2a^{cre/+}$). Blue labelling represents cell nuclei; red labelling represents D1R mRNA; Green labelling represents D2R mRNA. Yellow arrows show cells where red and green staining are present in the same cell (D1R/D2R mRNA co-expression). 20X magnification.

Figure 2 from a sample of the D1R/D2R fluorescent *in-situ* hybridization qualitatively shows the co-expression of D1R mRNA and D2R mRNA in some DAPI positive cells in the nucleus accumbens of a WT mouse. In the KO sample, no cells co-expressing D1R mRNA and D2R mRNA are visible.

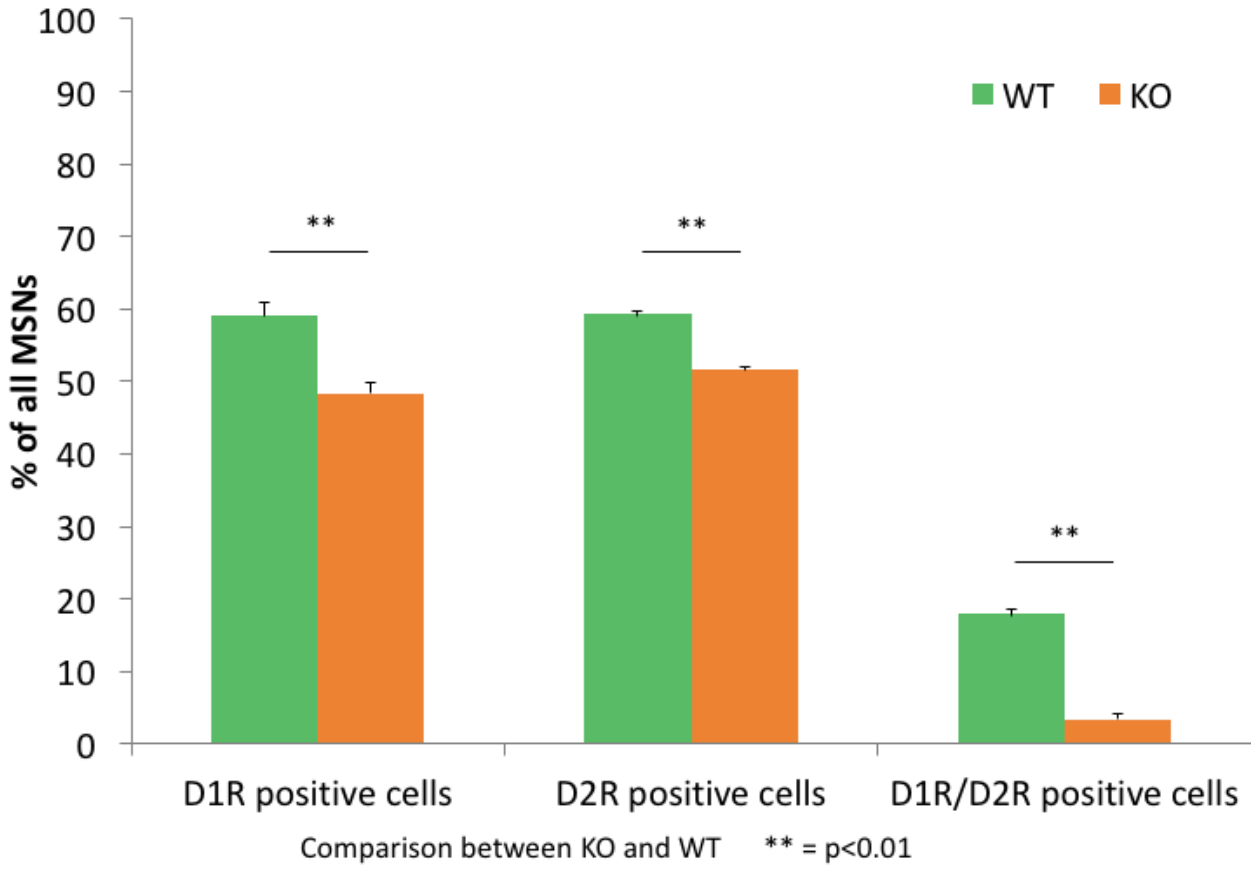

Figure 3. Striatal MSNs expressing D1R mRNA, D2R mRNA, or co-expressing D1R and D2R mRNA in $D1R^{lox}A2a^{cre}$. Values are indicated as percentage of each types positive cells over the total number of mRNA positive MSNs (n=6-8 per genotype condition).

Figure 3 shows quantification of striatal MSNs that are either expressing D1R mRNA or D2R mRNA, as well as those that co-express both types of mRNA, as a percentage of the mRNA positive MSNs in the striatum. Comparisons between the WT and KO conditions were made using Mann-Whitney U tests, which revealed a reduction of D1R-expressing MSNs and D2R-expressing MSNs in the KO as compared to the WT (U=0, p=0.002 and U=0, p=0.002, respectively). In addition, the analysis showed significantly less D1R/D2R co-expressing MSNs in the KO as compared to the WT (U=0, p=0.002).

1.2 Dendritic spine analysis

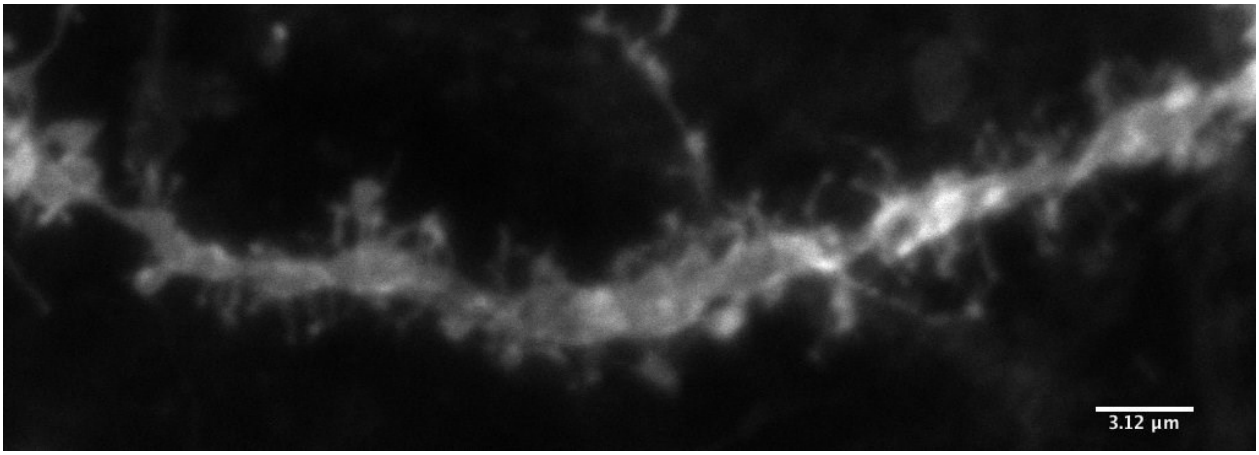

Figure 4. DiI signal in a D1R^{lox}A2a^{cre} WT mouse striatal MSN dendrite revealed with 561-nm laser activation via confocal microscopy. The original image was at 10x magnification. The original image resolution is 0.039 µm/pixel (resized for presentation).

Figure 4 shows a dendrite from a MSN in the NAc, with the dendritic spines visible for quantification. The genotype and treatment condition of each animal from the experiment is coded during the data collection process to control for experimenter bias.

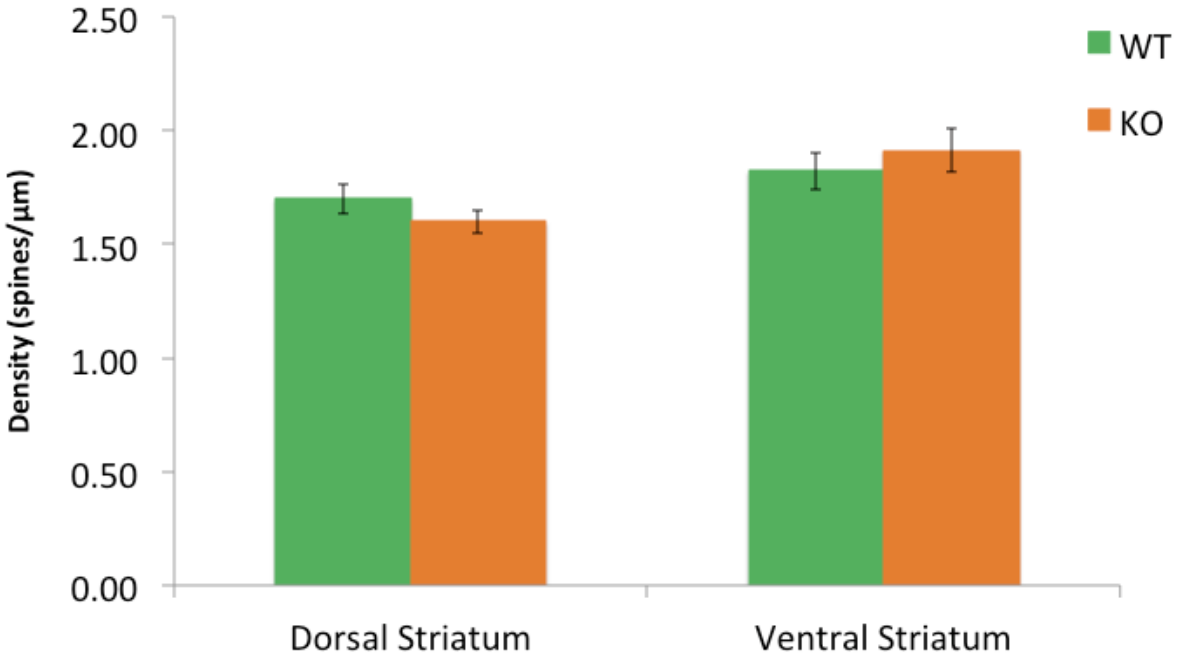

Figure 5. Dendritic spine quantification in the dorsal and the ventral striatum of $D1R^{lox}$-$A2a^{cre}$ mice in baseline conditions. Values are indicated as the mean density (number of spines per µm of dendrite) ± SEM in WT and KO mice dendrite samples (n=11-20 per group).

To assess baseline conditions of the current mouse model, dendritic spine density was assessed in WT and KO mice, with dorsal and ventral striatum areas quantified separately (Fig. 5). There was no evidence of difference between the WT and KO dendritic samples.

2. Evaluate the role of D1R/D2R co-expressing MSNs in regulating behavioural response to psychostimulants.

2.1 Acute psychostimulant-induced locomotor activity

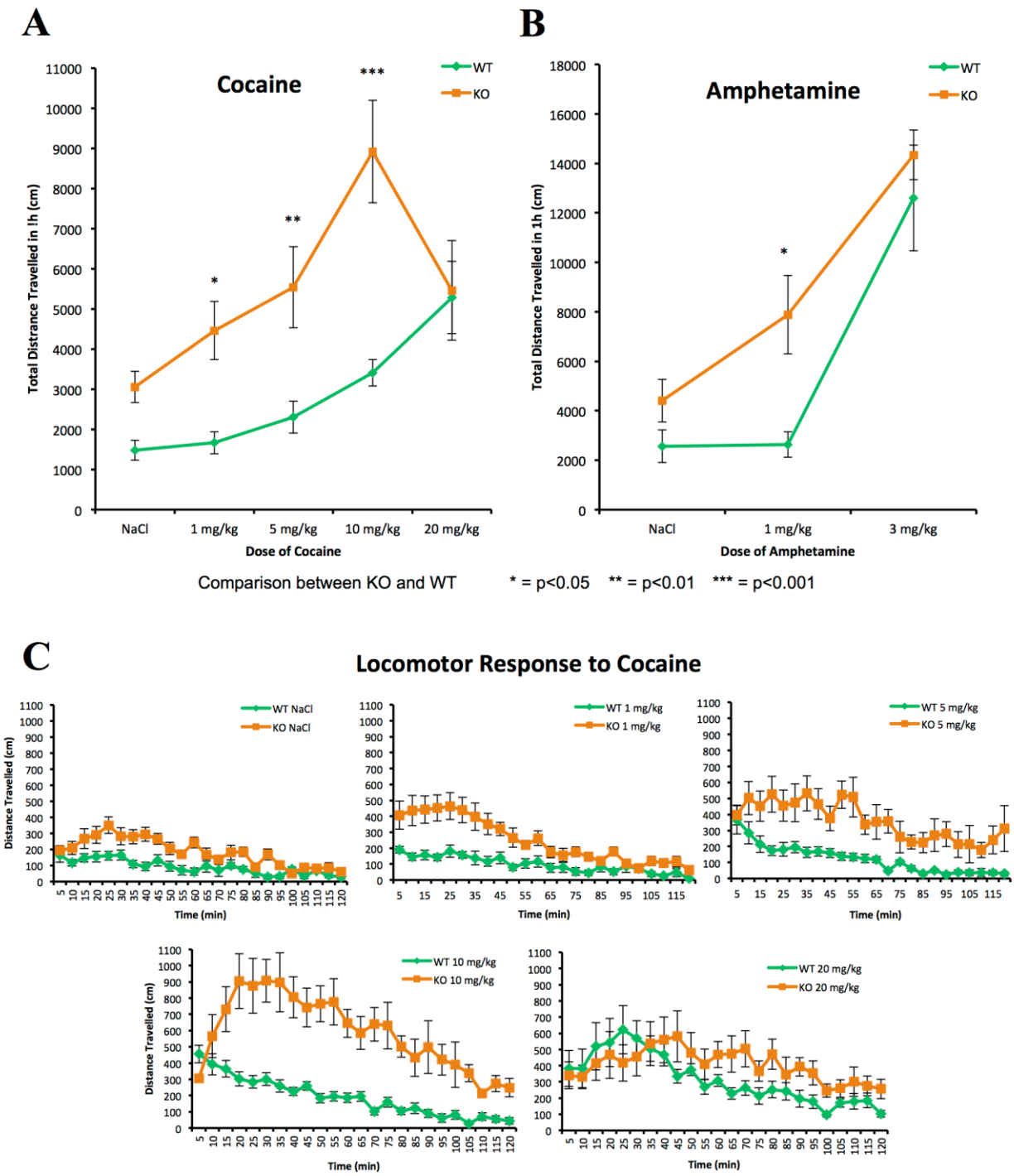

Figure 6. Cocaine (A, C) and amphetamine (B) acute dose response in $D1R^{lox}/A2a^{cre}$. For A and B, values are indicated as the mean of total distance travelled (cm) ± SEM by WT and KO animals during the 1 hour after saline, cocaine or amphetamine acute i.p. injection (n=7-10 per group). For C, values are indicated as the mean of distance travelled (cm) ± SEM at each time interval of 5 minutes after saline or cocaine injection (n=7-10 per group).

To assess the acute locomotor response to psychostimulants, the effects of cocaine (Fig. 6A, 6C) and amphetamine (Fig. 6B) were studied on WT and KO at different dosages in loco boxes. A two-way ANOVA on the total distance travelled over the course of one hour after cocaine administration revealed a significant effect of the genotype [$F(1,72)=25.72$, $p<0.001$], the dose of cocaine [$F(1,72)=7.59$, $p<0.001$] and the genotype x dose interaction [$F(4,72)=2.86$, $p=0.029$]. After amphetamine administration, the two-way ANOVA on the total distance travelled over the course of one hour revealed a significant effect of the genotype [$F(1,34)=6.82$, $p=0.013$] and the dose [$F(1,34)=31.12$, $p<0.001$]. The KO demonstrated higher locomotor activity compared to their WT littermates at 1 mg/kg ($p<0.05$), 5 mg/kg ($p<0.01$) and 10 mg/kg ($p<0.001$) cocaine doses and at the 1 mg/kg ($p<0.05$) amphetamine doses. After saline treatment, KO showed slightly higher activity than their WT littermates for both experiments, though this effect is not significant.

2.2. Cocaine sensitization

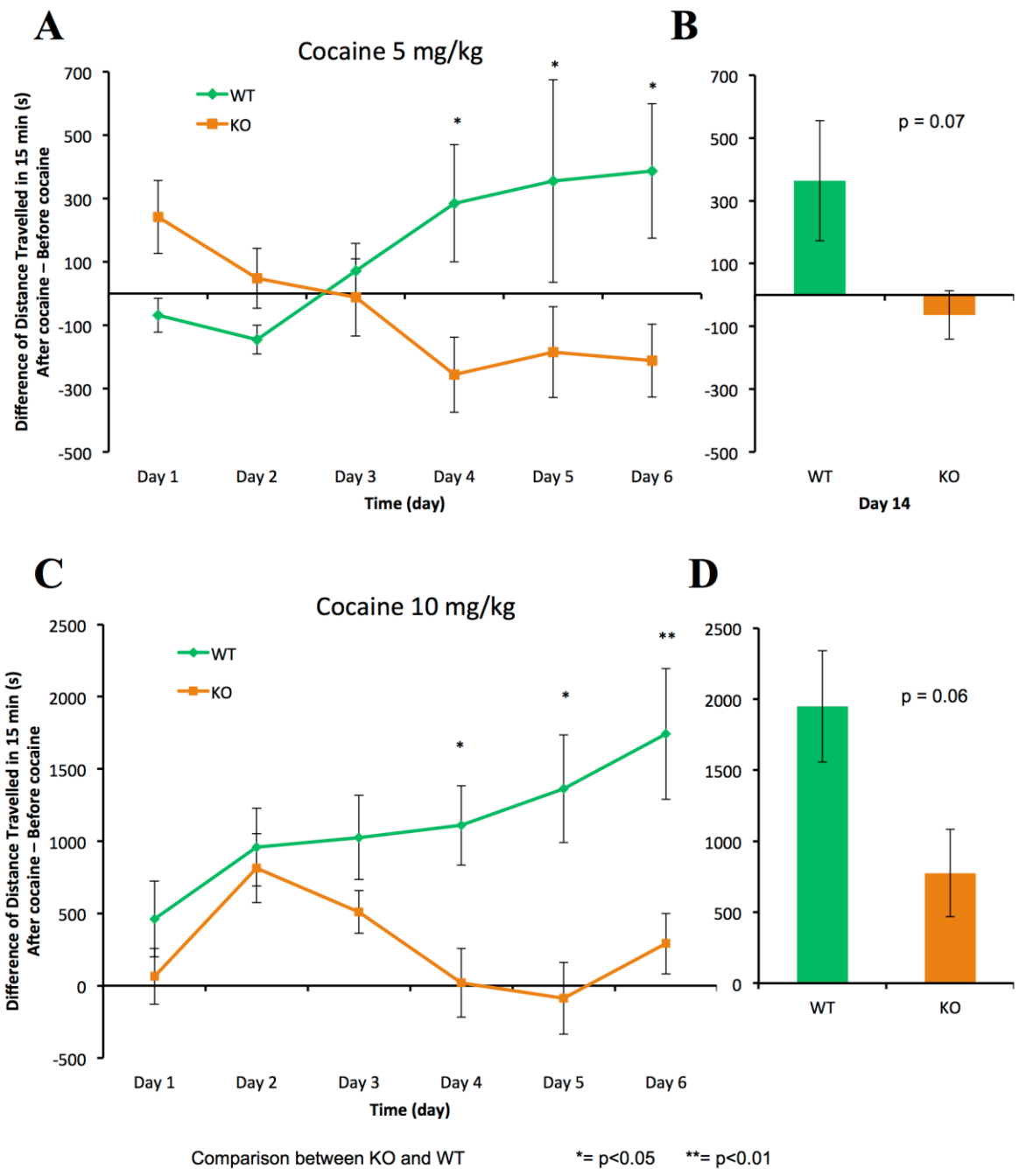

Figure 7. Locomotor sensitization in D1R^{lox}-A2a^{cre} at cocaine doses of 5 mg/kg (A, B) and 10 mg/kg (C, D). For all graphs, values are indicated as the mean of the difference between distances travelled (cm) (during 15 min

after cocaine i.p. injection – during 15 min immediately prior to injection) ± SEM in WT and KO animals (n=8-9 per group).

The cocaine sensitization in WT and KO animals was assessed via a 6-day chronic injection paradigm followed by a drug challenge on day 14, for cocaine doses of 5 mg/kg (Fig. 7A, B) and 10 mg/kg (Fig. 7C, D). At the 5 mg/kg dose, a mixed ANOVA revealed a significant genotype x time interaction [$F(5,75)=4.47$, $p=0.001$]. Post-hoc analysis with LSD test revealed that WT showed significantly more activity during sensitization days 4, 5, and 6 compared to KO (Figure 7A). For the challenge day (day 14), a Mann-Whitney U test revealed a trend between WT and KO ($U=17.0$, $p=0.074$) where WT showed higher activity compared to KO (Figure 7B). At the 10 mg/kg dose, a mixed ANOVA revealed a significant effect of the genotype [$F(1,75)=16.57$, $p=0.001$], time factor [$F(1,75)=6.17$, $p=0.025$] and genotype x time interaction [$F(5,75)=4.47$, $p=0.004$]. Post-hoc analysis with LSD test revealed that WT showed significantly more activity during sensitization days 4, 5, and 6 compared to KO (Figure 7C). For day 14, Mann-Whitney U test revealed a trend between WT and KO ($U=16.0$, $p=0.059$) where WT showed higher activity compared to KO (Figure 7D).

2.3 Conditioned place preference (CPP)

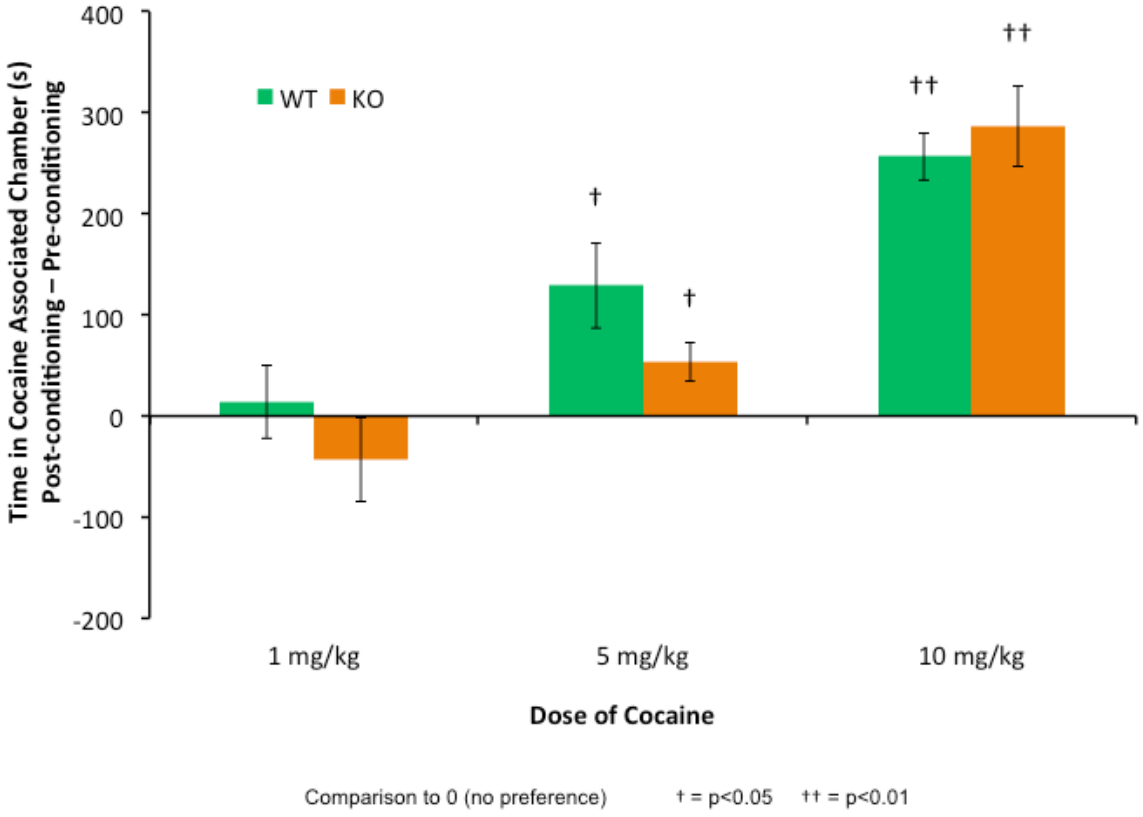

Figure 8. Conditioned place preference in D1R^{lox}-A2a^{cre} at cocaine doses of 1mg/kg, 5 mg/kg, and 10 mg/kg. Values are indicated as the mean of the difference between time spent (s) in the cocaine-paired compartment (post-conditioning session - the pre-condition session) ± SEM in WT and KO animals (n=5-8 per group).

We assessed cocaine-induced CPP in WT and KO at doses of 1 mg/kg, 5 mg/kg, and 10 mg/kg (Fig. 8). Preference for the cocaine-associated compartment was assessed using t-tests. No significant CPP was found at the 1 mg/kg cocaine dose in the WT and KO. A significant CPP towards the cocaine-associated compartment at the 5 mg/kg cocaine exists in both WT and KO groups, $t(6)=2.85$, $p=0.029$ and $t(7)=2.57$, $p=0.037$, respectively. At the 10 mg/kg cocaine dose, a CPP was found in both WT and KO, $t(4)=9.845$, $p<0.001$ and $t(4)=6.37$, $p=0.003$ respectively.

The Mann-Whitney U tests did not reveal any significant difference between WT and KO at all examined doses: 1 mg/kg ($U=15.0$, $p=0.445$), 5 mg/kg ($U=54.0$, $p=0.281$) and 10 mg/kg ($U=30.0$, $p=0.690$). There is no difference between KO and WT in terms of CPP at each dose.

3. Evaluate the role of D1R/D2R co-expressing MSNs in generating psychostimulant-induced neuronal changes.

3.1 c-Fos immunohistochemistry

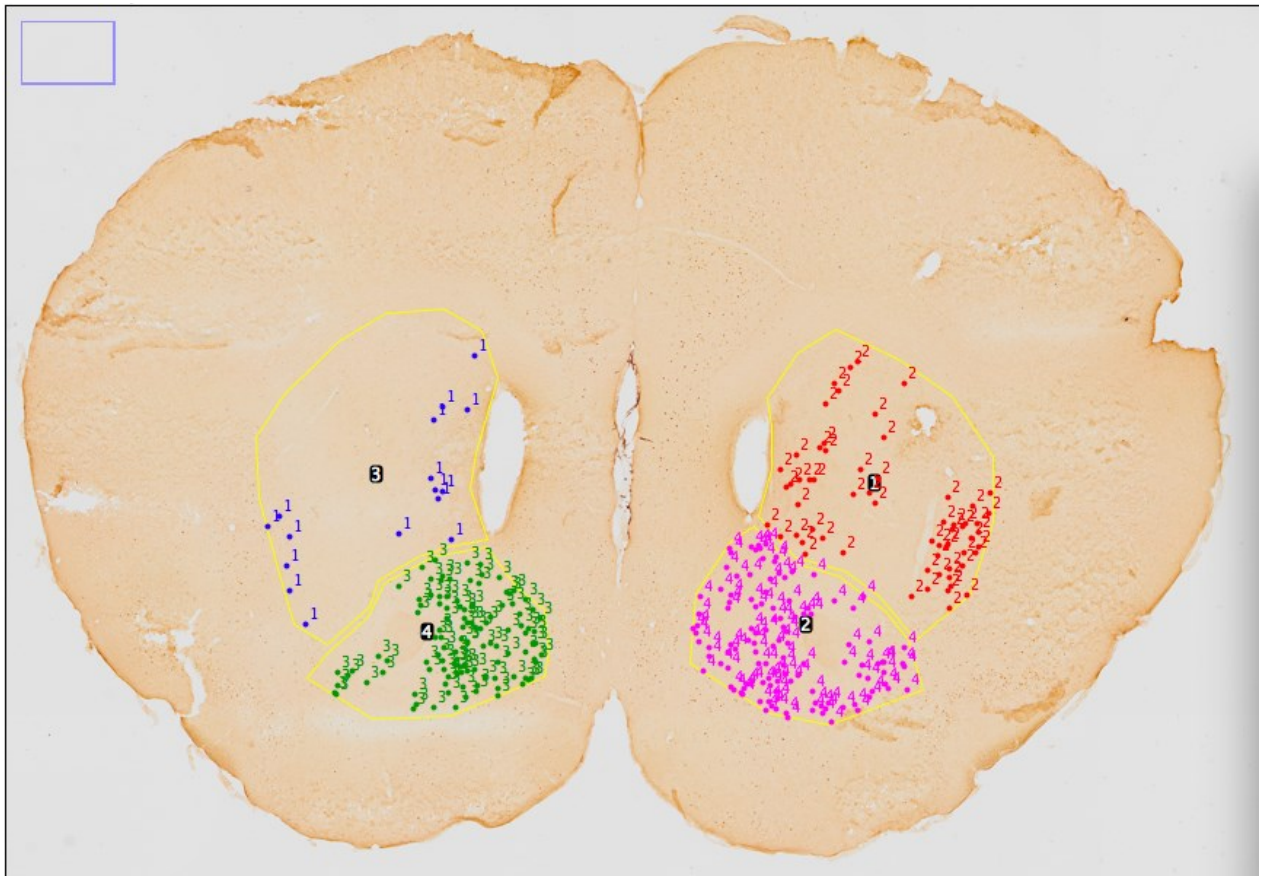

Figure 9. C-Fos immunohistochemistry signals on a bregma +1.5 brain section of a KO mouse ($(D1R^{lox+/+}/A2a^{cre/+})$ after acute saline administration (control injection). Yellow outlines denote the segmentation of the CPu and the NAc; blue and red labelling represents c-Fos positive cells in the CPu; green and pink labelling represents c-Fos positive cells in the NAc. The original image was scanned at 10x magnification (resolution at 0.6934 µm/pixel, resized for presentation).

Figure 9 shows the manual c-Fos quantification via ImageJ. The genotype and treatment condition of each animal is coded during the quantification process, such that the experimenter is blinded during the counting process to control for possible bias.

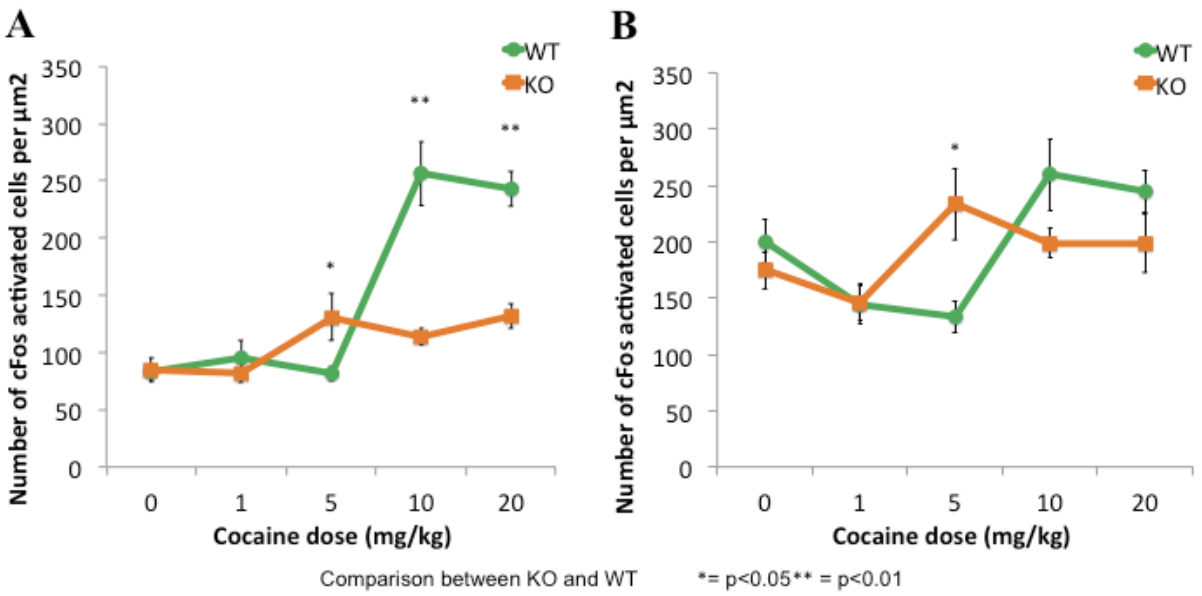

Figure 10. Cocaine-induced c-Fos activation in the dorsal striatum (A) and the ventral striatum (B) of D1R^{lox}-A2a^{cre} mice. Values are indicated as the mean density (number of activated cells per µm^2) ± SEM in WT and KO mice striatum area samples (n=12-30 per group).

The changes in striatal c-Fos activation to cocaine were studied on WT and KO mice. Dorsal and ventral striatal areas were analyzed separately (Fig. 10A, B). A two-way ANOVA on the number of c-Fos activated cells in the dorsal striatum revealed a significant main effect of genotype [$F(1,206)=19.47$, $p<0.001$] and cocaine dose [$F(4,206)=23.11$, $p<0.001$]. This is qualified by a genotype x dose interaction [$F(4,206)=14.39$, $p<0.001$]. The KO demonstrated higher dorsal striatum c-Fos activation compared to their WT littermates at the 5 mg/kg cocaine dose ($p<0.05$). Conversely, the KO showed lower activation in the same region compared to their WT littermates at the 10 mg/kg ($p<0.01$) and 20 mg/kg ($p<0.01$) cocaine doses. In the analysis of the ventral striatum c-Fos activation, a two-way ANOVA revealed a significant effect of cocaine dose [$F(4,206)=5.03$, $p=0.001$] as well as a genotype x dose interaction [$F(4,206)=4.41$,

p=0.002]. The KO demonstrated higher ventral striatum c-Fos activation compared to littermate WT specifically at the 5 mg/kg cocaine dose ($p<0.01$).

DISCUSSION

1. Characterize the D1R/D2R co-expression in the striatal MSNs.

1.1 D1R/D2R co-expression in the striatal MSNs

Through using the $D1R^{lox}A2a^{cre}$ mouse model combined with *in-situ* hybridization, the present study assessed D1R and D2R expression. In the WT, 59% of the striatal MSNs expressed D1R mRNA, and 59% of the same population expressed D2R mRNA. These results were in the same range as estimated from established literature. Importantly, this analysis assessed the degree of D1R/D2R mRNA co-expression directly, and its application yielded as estimate of 18% D1R/D2R co-expression among the striatal MSN population in the current mouse model. This agreed with the estimates established by Bertran-Gonzalez et al. (2008) which indicated that there were up to 17% D1R/D2R co-expression in the striatum. In addition, the method of using *in-situ* hybridization measured mRNA expression directly (as opposed to using a reporter-linked genetic model). However, it should be noted that the automated method used for this quantification has its limitations. The estimated percentage of D1R/D2R+ MSNs in KO was estimated between 2-3% when it should be 0% theoretically.

Relative to the WT animals, the KO showed significant reduction of D1R and D2R expression (18% and 13% respectively). As such, D1R expression showed a higher reduction between the two types of mRNAs in the KO. Considering that the model is designed to inhibit D1R expression in the D1R/D2R co-expressing MSNs, it is expected that the proportion of D1R-expressing MSNs should decrease in the KO, in accordance to these results. Interestingly, the proportion of D2R-expressing MSNs also decreased in the KO, which was not expected. A theory that would explain this phenomenon would suggest there are regulatory pathways maintaining the balance of D1R versus D2R expression, particularly in the cases of non-majority

shifts (such as the 18% reduction in D1R expression seen in the KO animals). Such a pathway would account for the reduction of D2R expression given the experimentally inhibited D1R expression. In fact, the reduction percentages were at similar rates between the two types of mRNA, which supports this theory. The balance between D1R and D2R in MSNs is important in regulating neurological function and behaviour, since they have activating and inhibiting properties respectively, as discussed previously (Kreitzer & Malenka, 2008). It is possible that there is an active feedback system that adjusts the expression level of one receptor when the other's level is changed. Further studies could elucidate this effect further.

Most importantly, the goal of this model is to produce a reduction of D1R/D2R co-expression in the striatal MSNs. In terms of this population of MSNs, the KO showed an 81% reduction relative to the WT. Overall, the present study demonstrates that the $D1R^{lox}A2a^{cre}$ system is a viable mouse model to impair the D1R/D2R co-expressing MSNs. The *in-situ* hybridization method was reliable in characterizing the D1R and D2R expression in the striatal MSNs. It was also effective for the study of the D1R/D2R co-expressing MSNs as it measured mRNA expression directly and corroborated estimates from previous studies. The $D1R^{lox}A2a^{cre}$ system successfully removed most D1R/D2R co-expressing MSNs in the KO mice, which is main intended goal of this animal model. This made it possible to proceed to our other specific aims in the study.

1.2 Dendritic spine analysis

The $D1R^{lox}A2a^{cre}$ WT and KO were assessed for their dendritic spine densities in the dorsal and ventral regions of the striatum at baseline. In both regions, there were no differences between the WT and KO in terms of the number of spines per μm of dendrite, indicating that the

removal of D1R/D2R co-expressing MSN did not affect the dendritic connectivity of the striatum.

Future work could investigate could investigate dendritic spine changes in the $D1R^{lox}A2a^{cre}$ WT and KO after chronic administration of psychostimulants. Previous work has shown that dendritic spine proliferation occurred in both D1R- and D2R-expressing MSNs after chronic administration of cocaine (Lee et al., 2005), and it is important to note that this work used a 28-day period and a 2-day withdrawal as the chronic paradigm. With the current mouse model, the effect of the D1R/D2R co-expressing neurons on dendritic spine density could be studied using same methodology.

2. The role of D1R/D2R co-expressing MSNs in regulating behavioural response to psychostimulants.

2.1 Acute psychostimulant-induced locomotor activity

In the present study, the $D1R^{lox}A2a^{cre}$ were injected with cocaine and amphetamine and put through the acute locomotor activity paradigm. The WT showed a dose-response curve as expected, where higher doses of psychostimulants induced a higher level of locomotor activity. The WT animals has a hypermotility response upon acute exposure to psychostimulants (Di Chiara, 1995)

Compared to the WT animals, the KO displayed an increased level of locomotor activity after being administered psychostimulants acutely, specifically at low to moderate doses (1 to 10 mg/kg for cocaine, 1 mg/kg for amphetamine). These results may suggest that the KO are more sensitive to dopamine extracellular levels increase, particularly if the signal was presented at low to moderate levels. D1R/D2R co-expressing MSNs, therefore, could be essential at regulating the locomotor responses that acute DA increase produces. In the presence of acute minor levels of

extracellular DA increases in the striatum, the D1R/D2R co-expressing MSNs may be acting as an inhibitory buffer to prevent hypermotility, whereas removing this populations of MSNs led to disinhibition and resulted in a heightened hypermotility response. Based on the present findings, this inhibitory role of the D1R/D2R co-expression MSNs could be limited to the situations where this acute DA increase is small or moderate.

Recent evidence showed that some D1R-expressing MSNs, contrary to traditional understanding, projected into the inhibitory indirect pathway (Kupchik et al., 2015). This inhibitory group of D1R-expressing MSNs could be co-expressing D2R as well. These D1R/D2R co-expressing MSNs has been observed to play a role in regulating acute psychostimulant response in the present study, particularly at lower doses.

2.2 Cocaine sensitization

In the cocaine sensitization paradigm, the $D1R^{lox}A2a^{cre}$ WT showed a day-to-day increase of locomotor activity to the same dose of cocaine over the first 6 days of conditioning, and this effect was consistent between the 5 mg/kg and the 10 mg/kg dose, which were doses chosen based on the maximum effect in the KO observed from the acute locomotor activity studies. For both dosage groups on cocaine challenge day (day 14), the heightened locomotor response was preserved (similar to the level found on day 6), indicating that the sensitization effect was maintained across one week (from Day 7 to 13) of no psychostimulant administration. As expected, the WT animals show a normal day-to day increase of locomotor activity typical of cocaine sensitization.

Compared to the WT, the KO showed no day-by-day increasing trend of locomotor activity during cocaine sensitization, in both dosage groups (5 and 10 mg/kg). For the dose of 5 mg/kg, there was a small decreasing trend of activity over days, potentially indicating a

desensitization process. For the dose of 10 mg/kg, there was initially an increase of activity on day 2, but this was not sustained and locomotor activity remained similar to the initial activity level (day 1) in the subsequent days. Due to these patterns, for both dosage groups, the latter days (day 4. 5, and 6) revealed that the KO had significantly lower levels of activity compared to the WT. In addition, on the cocaine challenge day (day 14), both KO dosage groups revealed a trend showing lower activity compared to their WT controls, despite receiving the same chronic treatment of cocaine. This demonstrated that cocaine sensitization was disrupted in the KO animals.

Sensitization is a phenomenon where the response to a stimulus is enhanced after chronic exposure to the same stimulus. The expression of sensitized responses has been thought to involve the mesolimbic DA pathway of VTA to NAc (Steketee, 2005). A later study has shown that the nigrostriatal pathway and thus the inputs to the CPu were more critical at maintaining cocaine locomotor sensitization effects (Beeler et al., 2008). As such, the striatum is a critical structure in maintaining cocaine sensitization. Previous literature demonstrated that both D1R and D2R required activation for the expression of psychostimulant sensitization (Capper-Loup et al., 2002), suggesting that D1R and D2R integration may be essential in this phenomenon. D1R/D2R disruption in the KO of the present study may therefore be preventing cocaine sensization.

2.3 Conditioned place preference (CPP)

The $D1R^{lox}A2a^{cre}$ WT and KO went through the CPP paradigm with cocaine at 1, 5, and 10 mg/kg doses. Analysis for each group demonstrated that 1 mg/kg was not sufficient to induce a CPP, while the 5 and 10 mg/kg doses elicited CPP in both the KO and WT animals. This demonstrated that a sufficient level of extracellular DA concentration is necessary for CPP to

occur. The lack of CPP at the low dose of 1 mg/kg dose is expected for the WT. Since the KO showed a hypersensitivity to the 1 mg/kg dose, it was expected that these mice also might be overly responsive to the CPP test. However, the results indicate that this hypersensitivity in the KO did not generalize to the CPP test.

Comparing the KO to the WT, there were no differences to the level of CPP at all three doses. This suggests that the D1R/D2R co-expressing MSN do not play a significant role in producing the reward-seeking behaviours that underlie this phenomenon. This is in accordance with previous literature, which showed that neither D1R or D2R are critical for CPP (Miner et al., 1995, Welter et al., 2007; Hikida et al., 2010). Given these findings, CPP does not appear to be exclusively dependent on either D1R- or D2R-mediated pathways, indicating that it is a complex behaviour that may involve other neurochemical systems in addition to the DA pathways.

3. Evaluate the role of D1R/D2R co-expressing MSNs in generating psychostimulant-induced neuronal changes.

3.1 c-Fos immunohistochemistry

The $D1R^{lox}A2a^{cre}$ WT and KO were subjected to acute cocaine (1, 5, 10, and 20 mg/kg), and then their brains were assessed for the density of c-Fos activation in the striatum (divided into dorsal and ventral sections) through immunochemistry. In the WT, in both parts of the striatum, the c-Fos activation showed a dose-response relationship where higher doses of cocaine generated more density of positive c-Fos cells. This pattern is accordance with previous research (Graybiel et al., 1990) indicating that the current method was reliable for quantifying c-Fos activation. In the WT animals, there was a large c-Fos density increase starting at 10 mg/kg for

both the dorsal and ventral striatum regions. This could be the critical dose where acute cocaine-induced c-Fos expression increases exponentially, leading to significant neurochemical changes. Previous work has examined the contributions of c-Fos to psychostimulant response, which includes coordinating gene expression in the striatum (NAc and CPu), changing MSN morphology, and producing cocaine sensitization, and the 10 mg/kg was used in this work as a relevant standard dose (Zhang et al., 2006).

In the dorsal striatum, the KO demonstrated no evidence of a dose-response curve, such that c-Fos activation did not increase with the higher doses of cocaine administration. As a result, compared to the WT (which showed the dose-response relationship), the KO had significantly lower c-Fos levels in the dorsal striatum at higher doses of cocaine (10 and 20 mg/kg). The removal of the D1R/D2R co-expressing MSNs ablated the increase of c-Fos activation that high dose of cocaine typically induces. The lack of c-Fos activation involved in the dorsal striatum in the KO could account for why cocaine sensitization is not possible; previous work that implicates c-Fos activation and the CPu as critical elements in generating cocaine sensitization (Zhang et al., 2006; Beeler et al., 2008). Interestingly, at the 5 mg/kg dose, the dorsal striatum of the KOs showed a minor increase in c-Fos activation, which rendered the activation higher compared to the WT controls. The KO's hypersensitivity to acute DA increase at low doses (but not high doses) could be applicable in this situation, which was an effect observed and discussed previously in the acute psychostimulant studies of the present work (see Discussion section 2, Acute psychostimulant-induced locomotor activity).

In the ventral striatum, the KO does not demonstrate a cocaine-induced dose response curve, indicating that cocaine dose increase did not generate an increase in the density of c-Fos activation in this brain region. Similar to the ventral striatum results, the KO showed an increase

of c-Fos activation only at the 5 mg/kg dose, which made it higher compared to the WT. It is possible that the selective hypersensitivity of the KO to lower dose acute psychostimulants, in addition to manifesting in the dorsal striatum c-Fos activation, also manifests in the ventral striatum. This could indicate that both regions of the striatum were involved in removal of D1R/D2R co-expressing MSNs, hence the entire striatum showed evidence of increased cellular activity associated with the hypermotility at lower extracellular DA concentrations. At other doses, there were no significant differences in the ventral striatum between the KO and WT c-Fos levels.

CONCLUSION AND FUTURE DIRECTIONS

The current work presents the $D1R^{lox}A2a^{cre}$ genetic mouse model, which removed the D1R/D2R co-expressing MSNs from the striatum. The *in-situ* hybridization procedure for D1R and D2R mRNA as a method to study direct expression of DA receptors in the striatal MSNs was validated. This procedure was also capable of assessing the degree of D1R/D2R co-expression amongst striatal MSNs. Dendritic spine density was confirmed to be not modified in this genetic model. Compared to the WT in the model, there were changes in the locomotor-related psychostimulant response in the KO animals. KO animals exhibited hypersensitivity of acute administration psychostimulants only at lower doses, while their cocaine sensitization was abolished. Reward-based learning, as demonstrated by conditioned place preference, was not altered in the KO of this model. The level of cocaine mediated c-Fos increase in the striatum was also hypersensitive in the KO compared to the WT, and this effect was only observed in one lower dose.

Given the KO's hypersensitivity to low-dose acute psychostimulants in locomotor activity and c-Fos experiments, it is possible that D1R activation on the D1R/D2R co-expressing MSN typically serve an inhibitory role at these specific levels of extracellular DA, similar to that of the indirect pathway. Recent evidence reveals significant D1R-expressing MSN involvement in the indirect pathway at the NAc level (Kupchik et al., 2015), contrary to the traditional notion that D1R inputs is primarily limited to the direct pathway. The current study strengthens the possibility that such an D1R-expressing inhibitory connection to the indirect pathway may involve the D1R/D2R co-expressing MSNs. In addition, D1R/D2R co-expressing striatal MSNs may interfere with the nigrostriatal pathway, which is involved in sensitization and motor function, given the deficits seen the KO of the present study.

To better understand the specific alterations in the $D1R^{lox}$-$A2a^{cre}$ KO model in the present study, the anatomical characterization of D1R and D2R distribution via autoradiography experiments could be completed. Further behavioural examinations of these mice through cocaine self-administration experiments would be valuable to further assess the reward-seeking component. The characterization of signalling pathway changes following DA receptor activation could be studied in this model to elucidate the biochemical changes that underlie the behavioural and cellular effects observed in the present work. Novel optogenetic models could be developed with the aim to directly activate the D1R/D2R co-expressing MSNs to further understand the function of this subpopulation.

The present study marks an innovative avenue to understand the specific function of the D1R/D2R co-expressing MSNs in the striatum, particularly as related to psychostimulant response. As such, this work represents a novel opportunity of DA research that will enrich the traditional understanding of striatal DA signalling.

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