Role of DAR/NMDAR heteromers and downstream nuclear calcium-dependent signaling in cocaine-induced adaptations

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DOCTORAL THESIS

Role of DAR/NMDAR heteromers and downstream nuclear calcium-dependent signaling in cocaine-induced adaptations

Estefani Aylin Saint-Jour Cubillos

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Thesis defense September 2021
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Abstract

Drugs of abuse hijack reward processing by increasing dopamine (DA) in the mesolimbic system, especially in the striatum, which persistently alters glutamate (Glu) transmission and triggers long-lasting behavioral adaptations. The integration of DA and Glu signals in the striatum is achieved by medium-size spiny neurons (MSN), which are subdivided in two distinct populations: the MSN expressing the DA D1 receptors (D1R), the D1R-MSN, which activation promotes motivation to obtain a reward; and the MSN expressing DA D2 receptors (D2R), the D2R-MSN, which activation inhibits reinforcement. Our laboratory has previously shown that the physical interaction between D1R and GluN1 subunit of glutamate NMDA receptors (NMDAR) was necessary for the facilitation of NMDAR functions by DA in D1R-MSN. In turn, others have shown that the interaction of D2R with GluN2B NMDAR subunit was required for the inhibitory effect of DA on NMDAR in D2R-MSN. However, the function and regulation of these heteromers in vivo in response to drugs of abuse are still unknown. Using the proximity ligation assay technique, we found that repeated cocaine exposure is associated with the formation of D1R/GluN1 and D2R/GluN2B heteromers in the nucleus accumbens (NAc). To identify the roles of the DAR/NMDAR heteromers at different stages of cocaine-induced adaptations in vivo we designed a viral-based approach to disrupt the DAR/NMDAR heteromers in a time-controlled manner owing to a doxycycline-dependent promoter. We found that disrupting D1R/GluN1 interaction in the NAc blocked the development of cocaine-induced psychomotor sensitization and conditioned place preference, as well as long-term synaptic plasticity in D1R-MSN. By contrast, blocking D2R/GluN2B interaction altered the maintenance of sensitizing and rewarding effects of cocaine. Of note, disruption of either heteromer subtype did not affect natural reward processing. Furthermore, D2R/GluN2B heteromers were detected in human caudate putamen samples from individuals with a history of psychostimulant dependence and healthy subjects. This allowed us to show that, despite a marked decrease in D2R expression levels, the proportion of D2R/GluN2B heteromers was three-fold higher in subjects with psychostimulant use disorder compared to healthy controls. This study identifies D2R/GluN2B heteromers as molecular targets with therapeutic potential.

Previous work from the laboratory showed that disrupting of D1R/GluN1 heteromers, in addition to inhibiting long-lasting behavioral adaptations to cocaine, also alters the facilitation of NMDAR-dependent Ca\textsuperscript{2+} influx by DA in D1R-MSN. Nuclear calcium (nucl-Ca\textsuperscript{2+}) signaling is a key route linking neuronal activity changes to gene transcription in multiple models of long-lasting neuroadaptations, but its role in response to drugs of abuse is unknown. Using a genetically-encoded nuclear calcium sensor, GCaMP3-NLS, we first studied nucl-Ca\textsuperscript{2+} dynamics in MSN. We stimulated cultured MSN with a D1R agonist combined with a low dose of Glu, mimicking acute cocaine exposure, and found that this co-stimulation triggered a fast NMDAR-dependent increase in nucl-Ca\textsuperscript{2+}. We also highlighted a synergistic effect of D1R and NMDAR stimulation on nucl-Ca\textsuperscript{2+} signals, which requires D1R/GluN1 heteromerization. Using fiber photometry, we performed nucl-Ca\textsuperscript{2+} imaging in freely-moving mice expressing GCaMP3-NLS specifically in NAc D1R-MSN. Interestingly, we observed a sustained increase in nucl-Ca\textsuperscript{2+} concentration in response to acute cocaine exposure. We then studied the role of nucl-Ca\textsuperscript{2+}-dependent signaling in response to cocaine by expressing, in D1R-MSN or D2R-MSN, the CaMBP4 protein that blocks nuclear Ca\textsuperscript{2+}/CaM-dependent signaling. We found that blocking nucl-Ca\textsuperscript{2+}-dependent signaling in D1R-MSN, but not in D2R-MSN, alters the rewarding effects of cocaine. Finally, we established that nucl-Ca\textsuperscript{2+}-signaling in D1R-MSN controls cocaine-induced morphological changes, gene expression and gates activation of ERK pathway. This study reveals a central role of nucl-Ca\textsuperscript{2+}-dependent signaling in D1R-MSN in cocaine-induced molecular, cellular and behavioral adaptations, therefore contributing to a better understanding of the molecular basis of addiction.
Résumé

Les drogues d’abus détourne le système de récompense en augmentant la dopamine (DA) dans le système mésolimbique, en particulier dans le striatum, entraînant des altérations durables de la transmission dépendante du glutamate (Glu) et des adaptations comportementales persistantes. L’intégration des signaux DA et Glu dans le striatum est réalisée par des neurones épineux de taille moyenne (MSN), qui sont subdivisés en deux populations distinctes : les MSN exprimant les récepteurs DA D1 (D1R), les D1R-MSN, dont l’activation favorise le renforcement; et les MSN exprimant les récepteurs DA D2 (D2R), les D2R-MSN, dont la stimulation inhibent le renforcement. Notre laboratoire a précédemment montré que l’interaction physique entre D1R avec la sous-unité GluN1 des récepteurs NMDA (NMDAR) était nécessaire à facilitation des fonctions des NMDAR par la DA dans les D1R-MSN alors qu’une autre équipe a montré que l’interaction du D2R avec la sous-unité GluN2B de NMDAR est requise pour l’effet inhibiteur de la DA sur la signalisation NMDAR dans D2R-MSN. Cependant, la fonction et la régulation de ces hétéromères in vivo en réponse à la cocaïne sont encore inconnues. En utilisant la technique « proximity ligation assay », nous avons montré que l’exposition répétée à la cocaïne est associée à la formation d’hétéromères D1R/GluN1 et D2R/GluN2B dans le noyau accumbens (NAc). Pour identifier les rôles des hétéromères DAR/NMDAR à différents stades des adaptations induites par la cocaïne in vivo, nous avons conçu une approche virale pour perturber les hétéromères DAR/NMDAR de manière contrôlée dans le temps grâce à l’utilisation d’un promoteur dépendant de la doxycycline. Nous avons constaté que la perturbation de l’interaction D1R/GluN1 dans le NAc bloquait le développement de la sensibilisation locomotrice et de la préférence de place conditionnée (CPP), ainsi que la plasticité synaptique à long terme évoquée par la cocaïne dans le D1R-MSN. En revanche, le blocage de l’interaction D2R/GluN2B interfère avec le maintien de la sensibilisation locomotrice et de la CPP induites par la cocaïne. Il est à noter que la perturbation de l’un ou l’autre sous-type de ces hétéromères n’affecte pas le traitement d’une récompense naturelle. De plus, des hétéromères D2R/GluN2B ont été détectés dans des échantillons de putamen caudé humain provenant d’individus avec des antécédents de dépendance aux psychostimulants et des sujets sains. Cela nous a permis de montrer que, malgré une diminution marquée des niveaux d’expression du D2R, la proportion d’hétéromères D2R/GluN2B est trois fois plus élevée chez les sujets toxicomanes que chez les sujets sains. Cette étude identifie les hétéromères D2R/GluN2B comme cibles moléculaires avec un potentiel thérapeutique.

Des travaux antérieurs de l’équipe montrent également que la perturbation des hétéromères D1R/GluN1, en plus d’inhiber les réponses aux drogues, altère la facilitation des flux calciques dépendants des NMDAR par la DA dans les D1R-MSN. La signalisation du calcium nucléaire (nucl-Ca²⁺) est une voie clé reliant les changements d'activité neuronale à la transcription des gènes dans plusieurs modèles d’adaptations neuronales à long terme, mais son rôle en réponse aux drogues d’abus est inconnu. À l’aide d’une sonde détectant le calcium nucléaire, la GCaMP3-NLS, nous avons d’abord étudié la dynamique du nucl-Ca²⁺ dans les MSN en culture avec un agoniste D1R combiné à une faible dose de Glu, imitant une exposition aiguë à la cocaïne. Nous avons constaté que cette co-stimulation déclenchait une augmentation du nucl-Ca²⁺. Nous montrons également un effet synergique de la stimulation D1R et NMDAR sur les taux de nucl-Ca²⁺ qui dépend de l’hétéromérisation D1R/GluN1. À l’aide de la photométrie de fibre in vivo, nous avons imagé le nucl-Ca²⁺ chez la souris vigile exprimant GCaMP3-NLS spécifiquement dans les NAc D1R-MSN. Nous avons ainsi observé une augmentation soutenue de la concentration de nucl-Ca²⁺ en réponse à une exposition aiguë à la cocaïne. Grâce à l’utilisation d’un bloqueur de la signalisation dépendante du nucl-Ca²⁺, nous avons établi que le nucl-Ca²⁺ dans les D1R-MSN, mais pas dans les D2R-MSN, sous-tend les effets plaisants de la cocaïne. Enfin, nous avons montré que la signalisation nucl-Ca²⁺ dans les D1R-MSN est nécessaire pour les changements morphologiques, l’expression génique et l’activation d'ERK induite par la cocaïne dans les D1R-MSN. Cette étude révèle un rôle central de la signalisation dépendante du nucl-Ca²⁺ dans les D1R-MSN dans les adaptations moléculaires, cellulaires et comportementales induites par la cocaïne et contribue ainsi à une meilleure compréhension des bases moléculaires de l’addiction.
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Abbreviations

AID: Activity-regulated Inhibitor of Death
AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors
AMPC: Cyclic Adenosine Monophosphate
AMPK: AMP-activated kinase
BG: Basal Ganglia
BDNF: Brain-Derived Neurotrophic Factor
Ca\textsuperscript{2+}/CaM: Calcium-Calmodulin complex
CaBPs: Calcium-Binding Proteins
CaM: Calmodulin
CaMK: Ca\textsuperscript{2+}/CaM-dependent protein kinase
CaMBP4: Calmodulin Binding-Peptide 4
CBP: CREB-binding protein
CB-28k: Calbindin28k
CB-D9k: Calbindin-D9k
CDT: C-Terminal Domain
CNS: Central Nervous System
Co-IP: Co-immunoprecipitation
COMPT: Catechol-O-methyltransferase
CP-AMPAR: Ca\textsuperscript{2+} permeable AMPAR
CPP: Conditioned Place Preference
CR: Calretinin
CRE: cAMP-responsive element
CREB: cAMP-response element binding
CS: Conditioned Stimulus
D1R-MSN: MSNs expressing DR1
D1R: Dopamine type 1 Receptor
D1R/GluN1: Heteromers formed by D1R and GluN1-NMDAR subunit
D1R/NMDAR: Heteromers formed by D1Rs and NMDAR subunits
D2R-MSN: MSNs expressing DR2
D2R: Dopamine type 2 Receptor
D2R/GluNB: Heteromers formed by D2Rs and GluN2B subunit
DA: Dopamine
DAG: Diacyl Glycerol
DAR: DA receptor
DAR/NMDAR: Heteromers formed by DARs and NMDAR subunits
DARPP-32: Dopamine- and cAMP-regulated phosphoprotein, 32 kDa
DAT: Dopamine Transporter
DBH: DA Beta-hydroxylase
DLS: Dorsolateral Striatum
DMS: Dorsomedial Striatum
EPSP: Excitatory Post-Synaptic Potential
DRE: Downstream transcription Regulatory Element
DREAM: DREAM Antagonist Modulator
DREADD: Designer Receptors Exclusively Activated by Designer Drugs
DSM-5: Diagnostic and Statistical Manual of Mental Disorders
EMRE: essential MCU regulator
ER: Endoplasmic Reticulum
ERK: Extracellular Signal-Regulated Kinase
ESA: Epidemiological Survey on Addiction
GABA: γ-Aminobutyric Acid
GECIs: Genetically Encoded Calcium Indicators
GFP: Green Fluorescent Protein
GluR: Glutamate Receptor
GPe: External Globus Pallidus
GPi: Internal Globus Pallidus
GPCRs: G Protein-Coupled Receptors
HDACs: Histone Deacetylases
HD: Huntington’s disease
HVA: High Voltage-Activated
ICD-10: International Classification of Diseases and Health Problems
iGluRs: ionotropic Glutamate Receptors
IEGs: Immediate Early Genes
IP3: Inositol 1,4,5-trisphosphate
IP3R: IP3 Receptor
KARs: Kainic Acid Receptors
LBD: Ligand Binding Domain
LTD: Long-Term Depression
LTP: Long-Term Potentiation
LVA: Low Voltage-Activated
MAO: Monoamine Oxidase
MAPK: Mitogen-Activated Protein Kinase
MCU: Mitochondrial Calcium Uniporter
MCUR1: mitochondrial Ca\textsuperscript{2+} uniporter regulator 1
MeCP2: Methyl CpG binding Protein 2
mGluRs: Metabotropic Glutamate Receptors
MNK-1: Mitogen-activated protein kinase interacting protein-1
MSN: Medium-sized Spiny Neurons (striatal neurons)
NA: Noradrenaline
NAC: Nucleus Accumbens
NAcc: NAc core
nAChRs: nicotinic Acetylcholine Receptors
NAcsh: NAc shell
NCX: Sodium-Calcium exchanger
NET: NA transporter
NLS: Nuclear Localization Signal
NMDAR: N-methyl-D-aspartate receptors
OFDT: « Observatoire français des drogues et des toxicomanies »
PD: Parkinson Disease
PFC: Prefrontal Cortex
PIP2: Phosphatidylinositol 4,5-bisphosphate
PKA: Proteine Kinase A
PKC: Proteine Kinase C
PLA: Proximity Ligation Assay
PLC: Phospholipase C
PMCA: Plasma Membrane Ca\textsuperscript{2+} transport ATPases
PNMT: Phenylethanolamine-N-methyl Transferase
PP1: Protein Phosphatase 1
PPTA: Preprotakinin
PS: Phosphatidylserine
PSD: Post-synaptic density fractions
PSD-95: Post-synaptic density protein 95
PV: Parvalbumin
RasGap: RAS GTPase-activating protein
RAS-GRF1: RAS-specific guanine nucleotide-releasing factor
RFP: Red Fluorescent Protein
ROCs: Receptor-Operated (ligand-gated) channels
RSK2: Ribosomal protein S6 kinase
RyRs: Ryanodine Receptors
SERCA: Sarco/Endoplasmic Reticulum type Ca\textsuperscript{2+} ATPases
SERT: Serotonin transporter
SNc: Substantia Nigra Pars Compacta
SNr: Substantia Nigra Pars Reticulata
SPNs: Spiny Projection Neurons
SRE: Serum Response Element
STEP: Striatal-Enriched protein Phosphatase
STN: Subthalamic Nucleus
TCSPC: Time-Correlated Single-Photon Counting
TH: Tyrosine Hydroxylase
TMD: Transmembrane Domain
TRP: Transient Receptor Potential Canonical channels
US: Unconditioned Stimulus
VGCC: Voltage-Gated Ca\textsuperscript{2+} Channels
VEGF: Vascular Endothelial Growth Factor
VMAT2: Vesicular Monoamine Transporter 2
VP: Ventral Pallidum
VTA: Ventral Tegmental Area
Introduction

Chapter I: Reward-based learning in health and disease

1. Dopamine and Reward

Learning is a key process by which skills, knowledge and behavior are acquired or modified. From an evolutionary point of view, animals constantly adapt their behavior in order to make the best choices to keep themselves alive and healthy. Accordingly, decision making is made by repeating pleasant or rewarding experiences and avoiding dangerous or unpleasant experiences. It is generally agreed that reward-dependent learning is processed in specific regions of the central nervous system (CNS), which form the so-called reward circuitry. In order to understand the concept of reward-dependent learning, one has to consider three psychological components: “liking”, “wanting”, and “learning”. From a very general point of view, “liking” refers to the hedonistic properties of a stimuli, in other words, the pleasure and positive feelings generated by the exposure to a given stimuli. However, sometimes the rewarding effect is not always processed consciously. “Wanting” is often a consequence of “liking”. If the reward evoked by an experience is relevant, it will reflect in high motivation to be re-exposed to that experience. “Wanting” is better known as “incentive salience” and can be separated physiologically and neuroanatomically from processes underlying “liking”. Moreover, “learning” refers to behavioral changes driven by the reward itself and/or cues associated to the reward, which will be discussed later. Understanding the molecular and cellular mechanisms underlying these three facets of reward-based learning is important as it can contribute to the development of innovative therapeutic strategies to alleviate disorders associated with dysfunctions of reward-dependent leaning, such as addiction (Berridge et al., 2009; S. Robinson et al., 2005).

1.1 Dopamine and the reward circuit

Physiology of reward is intimately related to Dopamine (DA). DA is part of the catecholamine neuromodulator family together with noradrenaline (NA) and adrenaline. This molecule is produced mostly in dopaminergic neurons starting from the amino acid Tyrosine, which is hydroxylated by the tyrosine hydroxylase (TH) to form the intermediate L-DOPA (limiting rate step in DA formation). L-DOPA is then transformed by the enzyme dopamine decarboxylase (DDC) to finally produce DA, which can be accumulated in vesicles through the vesicular monoamine transporter 2 (VMAT2), or metabolized by DA Beta-hydroxylase (DBH) and phenylethanolamine-N-methyl transferase (PNMT) to form NA and adrenaline, respectively. In response to several stimuli, vesicles full of DA are released into the synapse to stimulate multiple post-synaptic DA receptors of distinct subtypes (see chapter 2 section 2.4). DA levels are rapidly controlled by the DA transporters (DAT) located at the pre-synapse. The DAT mediate DA reuptake, which permits the recycling of DA, which is then degraded into homovanillic acid via monoamine oxidase (MAO)/Catechol-O-methyltransferase (COMT) or will refill again the vesicles to complete the cycle (See fig. 1).
Chapter I: Reward-based learning in health and disease

The soma of DA neurons are mainly located at the ventral mesencephalon in the Substantia Nigra Pars compacta (SNc) and ventral tegmental area (VTA). DA neurons form a heterogeneous group of neurons projecting onto different brain structures including the prefrontal cortex (PFC), striatum, hippocampus, amygdala. Through these multiple projections, the activity of DA neurons modulates a vast panel of complex processes, including selection and initiation of movements, decision making, attention, cognition and motivation (see fig. 2) (da Silva et al., 2018).

The activity pattern of DA neurons can be divided in three different types of firing: fast phasic response (<500 msec, 15 Hz), which is triggered by detection of salient stimuli; secondly, there is a slow phasic response that modulates movement, behavior, stress, attention and reward, and finally a tonic activity (1-8 Hz), which controls movements, cognition, attention and motivation (Klein et al., 2019).

In the past, DA was only associated to the hedonic feature of stimuli, which is one of the components of reward. Nonetheless, later studies showed that DA deficient mice (generated by selective inactivation of the tyrosine hydroxylase gene, which inhibit DA and NA production) exhibited a lack of motivation to engage in goal-directed behaviors, while restoring DA by adding L-DOPA rescued motivation, therefore confirming that DA rather encodes motivation values (Szczypka et al., 2001; Zhou & Palmiter, 1995).
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Animals can detect salient stimuli. Each stimulus elicits dopaminergic phasic activity, including rewarding, neutral and aversive type. Then, a valuation process occurs that identifies rewarding stimuli from the rest. The value of a particular stimulus is subjective and it can change depending on many environmental and physiological factors. A more positive valuation will be transduced in more motivation to pursue exposure to the specific stimulus. DA is fundamental to signal the motivational value and to motivate behaviors, but also to predict a reward and to facilitate memory consolidation of salient events. If the behavior or stimulus is rewarding (positive value), that behavior will be reinforced and, on the other hand, if the experience is undesirable it will not be reinforced. This is the basis of reward-based learning. If this substance or behavior is natural, like water, sugar, social interaction, sex, etc., it will be a natural reinforcer, or else it will be an artificial reinforcer, like drugs of abuse or optogenetic stimulations (Berridge & Robinson, 1998; S. Robinson et al., 2005; Schultz, 1986; Schultz et al., 1993).

1.2 Dopamine and reward-based learning

Appetitive or rewarding stimuli are known to promote reinforcement, which means that the animal will repeat a given behavior associated with a rewarding outcome. However, animals require an anticipatory capacity in order to predict which are the best behavioral choices to pursue in order to survive. This implies that, across trials, the behavioral outcomes will feed back learning, therefore increasing the predictive capacity of the animal. Furthermore, the ability to predict a reward is necessary to prepare behavioral reaction. But, how reward is linked to this predictive capacity? One famous experiment is the Pavlov’s dog conditioning performed in 1927, where a bell was presented to the dog right before food presentation. At the beginning, the dog was salivating at the moment of food presentation, but after several repetitions the dog learned to associate the sound of the bell with the food, and started to salivate at the moment of the sound presentation. Here, a neutral non-rewarding stimulus, the bell, become predictive of the rewarding stimulus. This is known as classical conditioning (Pavlov (1927), 2010). Another type of associative learning is illustrated by the operant conditioning implemented by Skinner in 1937.
The experimental set up consists in a cage where the animal can press a lever in order to get access to certain stimulus. In this case, the rewarding stimulus is associated to an energy consuming task, allowing the observer to evaluate the motivation to obtain a reward (Skinner, 1937).

Do dopamine neurons report an error in the prediction of reward?

![Diagram of reward prediction error encoded by DA neurons activity](image)

Figure 3. Reward prediction error encoded by DA neurons activity. (Top) In absence of prediction, the sweet juice reward triggers DA signal, hence there is a positive prediction error; (Middle) After learning, the conditioned stimulus predicts reward evoking DA signals at cue and reward presentation. No error in the prediction; (Bottom) If the reward is not given after cue presentation, there is a depression in DA signals at the moment when the reward should have occurred, representing a negative prediction error. Each panel shows the peri-event time histogram and raster of impulses from the same neuron. Horizontal distances of dots correspond to real-time intervals. Each line of dots shows one trial. Original sequence of trials is plotted from top to bottom. From (Schultz et al., 1997).

Later, Schulz and collaborators performed a task mixing both types of learning. A non-human primate was trained to press a lever in response to an image (visual stimulus) in order to receive sweet juice (rewarding stimulus), while DA neurons activity was recorded across trials (Schultz, 1986; Schultz et al., 1993). At first, the visual stimulus represents an unconditioned stimulus (US) and the phasic activity in DA neurons was observed only at the moment of juice delivery. When animals learned to associate the visual stimulus to the rewarding stimulus, the cue become a conditioned stimulus (CS). After learning, DA neurons’ phasic activity was observed at the presentation of the predictive cue and not at the moment of reward delivery (fig. 3). Moreover, if no reward was delivered as expected, a decrease in DA neurons activity was observed (Schultz et al., 1993, 1997). These results support the idea that DA release increases when a rewarding event is better than predicted, whereas DA levels do not significantly change in activity if the reward corresponds to what was predicted. By contrast, the activity of DA neurons, and thus DA
release, decreases when the behavioral outcome is less rewarding than predicted. This has led to the conclusion that DA signals encode the reward prediction error, represented in the following equation:

\[ \text{Dopamine response} = \text{Reward received} - \text{Reward predicted} \]

The more unexpected the rewarding event, or the more rewarding the event is, the bigger the value that will be associated to that event, which will transduce in more motivation to repeat it (Schultz, 2006).

1.3 The reward circuit

The pioneers who began to unravel the reward circuit were Olds and Milner in 1954. They discovered that some of the rats implanted with electrodes in a certain brain region, presented a compulsive behavior of electrical self-stimulation in a skinner cage. The location of the electrode was the septal area. Later, several studies confirmed the hypothesis that this compulsive behavior associated with pleasure was controlled by specific regions in the brain, including the midline bundle of the telencephalon, the ventral thalamus, the ventral pallidum, the ventral striatum (nucleus accumbens, NAc), and the median prefrontal cortex, all of which receive DA projections from the VTA (Olds & Milner, 1954). Animals preferred electrical stimulation over natural rewards. Moreover, self-administration of dopaminergic agonist in the same brain regions evoked the same effect as electrical stimulations, while co-administration with DA receptor antagonists prevented this behavior, therefore suggesting a central role of dopaminergic activity (McBride et al., 1999).

Many years of research contributed to the refinement of our knowledge of the reward circuit as it is currently described (see fig. 4).

![Figure 4. Mesolimbic dopamine system regions and their involvement in reward processing. Adapted from (Koob & Volkow, 2010).](image)

Nowadays, what we know from studies in animal models and human imaging, is that reward processing is achieved by multiple interconnected cortical and subcortical brain regions forming the reward circuitry including the ventral striatum (Nucleus Accumbens, NAc), amygdala, prefrontal cortex and, ventral hippocampus. Dopaminergic neurons project from the VTA to the
distinct brain areas of the reward circuit as depicted in figure 4. Each structure has been associated to a specific function: the prefrontal cortex controls decision making and planification, the amygdala provides the emotional component, the ventral hippocampus contributes with context, and the NAc regulates motivation, pleasure and action selection. Particularly, the NAc is a central target structure of drug of abuse since it receives glutamatergic afferents from different brain regions that encode reward-associated stimuli and dopaminergic inputs that are responsible for reward prediction error and incentive values (see chapter II) (Hyman et al., 2006).

1.4 Addiction

Drug addiction is a chronic psychiatric disorder characterized by repeated cycles of uncontrolled drug consumption despite negative consequences, followed by recurrent periods of abstinence, and relapse to use. The Tenth Revision of the International Classification of Diseases and Health Problems (ICD-10) published by the World Health organization defines the dependence syndrome as “being a cluster of physiological, behavioral, and cognitive phenomena in which the use of a substance or a class of substances takes on a much higher priority for a given individual than other behaviors that once had greater value. A central descriptive characteristic of the dependence syndrome is the desire (often strong, sometimes overpowering) to take the psychoactive drugs (which may or not have been medically prescribed), alcohol, or tobacco. There may be evidence that return to substance use after a period of abstinence leads to a more rapid reappearance of other features of the syndrome than occurs with nondependent individuals”. On the other hand, the American society of addiction medicine defines addiction as follows: “Addiction is a treatable, chronic medical disease involving complex interactions among brain circuits, genetics, the environment, and an individual’s life experiences. People with addiction use substances or engage in behaviors that become compulsive and often continue despite harmful consequences”. The criteria to properly diagnose substance use disorder depends on the number of symptoms. Table 1 lists the 11 symptoms considered by the DSM-5 (Diagnostic and Statistical Manual of Mental Disorders, Fifth edition) to define drug addiction. If an individual present two or three symptoms, this indicates a mild use; four or five symptoms corresponds to a moderate substance use, and six or more symptoms reflect a severe substance use disorder classified as addiction (O’Brien, 2011).
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<table>
<thead>
<tr>
<th>DSM-5: Clinical diagnosis of addiction</th>
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<tbody>
<tr>
<td>1. Taking the substance in larger amounts or for longer than you are meant to.</td>
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<tr>
<td>2. Wanting to cut down or stop using the substance but not managing to.</td>
</tr>
<tr>
<td>3. Spending a lot of time getting, using, or recovering from use of the substance.</td>
</tr>
<tr>
<td>4. Cravings and urges to use the substance.</td>
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<tr>
<td>5. Not managing to do what you should at work, home, or school because of substance use.</td>
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<tr>
<td>6. Continuing to use, even when it causes problems in relationships.</td>
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<tr>
<td>7. Giving up important social, occupational, or recreational activities because of substance use.</td>
</tr>
<tr>
<td>8. Using substances again and again, even when it puts you in danger.</td>
</tr>
<tr>
<td>9. Continuing to use, even when you know you have a physical or psychological problem that could have been caused or made worse by the substance.</td>
</tr>
<tr>
<td>10. Needing more of the substance to get the effect you want (tolerance).</td>
</tr>
<tr>
<td>11. Development of withdrawal symptoms, which can be relieved by taking more of the substance.</td>
</tr>
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- Loss of control over the amount of drug use.
- Consumption despite negative consequences (health, work, family, money, social life...).
- Negative effects of tolerance & withdrawal.

Table 1. Diagnostic criteria according to the American Psychiatric Association. (APA - Diagnostic and Statistical Manual of Mental Disorders DSM-5 Fifth Edition, n.d.).

The different stages of addiction development are illustrated in figure 5 (Le Moal & Koob, 2007). First, the individual is exposed to a particular stimulus or substance (acute exposure/social drug taking). There is an uncertain period of time where the consumer can use the substance recreationally, to pass later to a stage of compulsive consumption. Since this uncontrolled phase alters the normal daily life routines, it is rapidly addressed as a problem by the close environment (dependence). This will trigger efforts to stop drug consumption for a while (abstinence) until the compulsive intake takes place again completing a vicious cycle. This pathological state has devastating consequences for vulnerable individuals and for the society as a whole. Not all individuals reunite enough risk factors to be vulnerable to develop addiction in the future. Risks comprise biological vulnerability factors (genetics, developmental attributes, epigenetics, neurocircuitry) and environmental factors (stress, trauma, exposure, social and cultural systems) (Potenza, 2013).
Substance use disorder represent a considerable cost for society. In Europe, addiction affected more than 15 million individuals in 2011, which represent the 4th most prevalent disease with a 65.7 billion euros cost (DiLuca & Olesen, 2014). Of note, multiple comorbidities are associated with addiction, including other types of mental illness like anxiety, depression, stress, cognitive disfunctions, among others. (Gawin & Ellinwood, 1988; N. S. Miller et al., 1993; Weddington et al., 1990). Some studies on national population surveys show that about fifty percent of those experiencing mental illnesses will also experience substance use disorder and vice versa. Moreover, mental illnesses and addiction share many of the same risk factors. These observations suggest that brain dysfunctions underlying drug addiction and other psychiatric disorders may share partially common mechanisms at the level of brain regions, neuronal networks and/or molecular substrates (NIDA: National Institute on Drugs of Abuse, 2020).
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Unfortunately, the existing approaches are insufficient to treat addiction. Medication available is only to treat comorbidities associated to drug use and addictive patients are usually more resistant to it. Similarly, psychosocial treatments like cognitive-behavioral therapy or contingency management interventions present limited effectiveness being different for each patient. It is necessary to increase our knowledge of addiction neurobiology to provide more selective and efficient treatments.

1.5 Artificial increase of Dopamine by drugs of abuse: hijack of reward circuits by cocaine

Many chemicals can be classified as drugs of abuse, and from time to time there are new drugs or variations from old ones that appear on the market. Nonetheless, even though they can differ largely in their chemical structure, accumulating evidence points to DA increase as the main effect and common feature between them (Di Chiara & Imperato, 1988).

First signs regarding DA involvement in acute rewarding effects by drugs of abuse comes from a study showing that cocaine self-administration was blocked when dopaminergic neurons were damaged in rats (Roberts et al., 1977). Later, in order to characterize brain responses to distinct drugs, Di Chiara & Imperato measured DA levels using microdialysis after administrating morphine, methadone, nicotine, ethanol, amphetamine, and cocaine. They found that all these addictive substances increased DA concentration within the reward circuit, particularly in the NAc (Di Chiara & Imperato, 1988). More recently, it has been shown that optogenetic self-stimulation of VTA DA neurons was sufficient to induce a simple form of addiction in rodents, inducing reinforcement and cue-associated stimulation seeking. Importantly, this paradigm of optogenetically-mediated self-stimulation of VTA DA neurons was also able to recapitulate an important aspect of drug abuse, which is the perseverance to consume the drug despite negative
consequence, since a fraction of mice (65–75%) continued to self-stimulate DA neurons even in the face of a predictive subsequent punishment corresponding to an electrical foot shock (Pascoli et al., 2015, 2018).

While DA increase is a shared feature for all drugs of abuse, the molecular mechanism by which this augmentation is achieved is specific and different for each type of drug (see Fig. 7).

![Diagram of reward-based learning in health and disease](image)

**Figure 7. General mechanisms of action of the most common addictive drugs on the CNS.** From (Lüscher, 2016).

In particular, cocaine induce DA increase by interfering with DA reuptake. One single injection of cocaine induces an increase in DA concentration in the NAc, but it does not alter directly glutamate transmission. Of note, cocaine also increases DA in the VTA because these neurons release this neurotransmitter from their dendrites. Furthermore, repeated cocaine exposure increase both, DA and glutamate levels in the NAc (Kalivas, 2004). Notably, this imbalance of DA and glutamate transmission will trigger molecular, transcriptional, epigenetic, synaptic and morphological changes within the reward circuit, which underly long-term behavioral adaptations resulting from alterations of reward processing (Hyman et al., 2006; Lüscher & Malenka, 2011; Robison & Nestler, 2011).

To better understand how the artificial increase of DA levels triggered by drugs of abuse alters reward-dependent learning, it is important to go back to the importance of reward prediction error signals encoded by DA in reinforcement learning. In fact, as it was discussed above (see section 1.2), in a model of natural reward learning where an animal learns to press a lever in order to obtain a reward (sweet juice) DA release in the NAc takes place at the moment of reward presentation. As the animal learn to predict the reward availability, DA release occurs at the cue presentation and induces the behavioral response (see fig. 8.A) (Keiflin & Janak, 2015; Roitman et al., 2004; Schultz et al., 1997). In the case of an animal trained to associate a cue to a cocaine injection, DA release in the NAc occurs at both cue presentation and drug receipt (Phillips et al., 2003). The significant difference between natural and drug of abuse-induced DA release is that, at the moment of reward delivery, cocaine artificially induces a massive DA augmentation, which
persists even after the animal learned the task and the reward can be predicted (Heien et al., 2005; Stuber et al., 2005). Despite the decrease in phasic release of DA during chronic cocaine exposure, DA is still released in large amounts whether cocaine is given by the experimenter or not (see fig. 8.B) (Willuhn et al., 2014).

Figure 8. Differences between natural (food) and artificial (drug) DA-evoked signals in the NAc.
A) Unexpected food reward delivery results in phasic DA signals. As individuals learn that cue presentation indicate food delivery, DA responses are transferred from the reward to the cue. B) If cocaine is the reward, each exposure produces, with some delay, a burst of phasic DA events as an effect of the pharmacological actions of the drug. Also, phasic DA responses progressively emerge to the cue but unlike food, drug induced DA signals persist throughout learning. And C) is a proposed consequence of DA signals on learning. DA signals through reward prediction errors promote learning until the prediction matches the actual outcome (food). In contrast, persistent cocaine-evoked DA signals continue to increase the value of cocaine predictive cues with each trial. Eventually, the value of cocaine cues surpasses the value of the food cues and can bias decision-making toward cocaine. From (Keiflin & Janak, 2015).

Cocaine therefore induces a positive reward prediction-error value, which promotes the reinforcement of behavioral adaptations associated to its pathological consumption, promoting decision-making choices that will be biased towards drugs (see fig. 8.C) (Keiflin & Janak, 2015; Redish, 2004).

1.6 Mechanism of action of cocaine

Cocaine (benzoylmethylecgonine) is a tropane alkaloid extracted from coca leaves (Erythroxylum Coca), originally from South America (fig. 9). It was long used by native Americans and later commercialized by different companies before fully understanding the consequences of its consumption. Cocaine is a psychostimulant like nicotine, caffeine and amphetamines. Users describe increased energy, self-confidence, focus, a sense of well-being and euphoria. In addition, food appetite is diminished while sex drive is increased along with strong fatigue resistance (Fischman & Schuster, 1982).

The prevalence of cocaine addiction has been increased in recent years in Europe. The 2014 report from the OFDT “Observatoire Français des Drogues et des Toxicomanies” in France (Cocaïne données essentielles OFDT; 2014) and the 2012 nationwide Epidemiological Survey on Addiction in Germany (ESA; 2012) (400.000 and 600.000 users over the last 12 months in France and Germany, respectively), revealed that cocaine is the second illicit drug most commonly used after marijuana (3.8 and 3.6 million in France and Germany, respectively). Over a half of the
patients treated for cocaine addiction show signs of psychiatric comorbidities, which is correlated with cocaine consumption level. The global cost of illicit drugs for French society in 2005 has been estimated at 2.83 billion Euros per year (OFDT; Kopp P and Fenoglio 2005).

Figure 9. Cocaine chemical structure. From Karch 2005.

Cocaine effects are different depending on the route of administration. When smoked or injected intravenously, cocaine provokes faster and more intense effects but shorter compared to oral or intranasal methods. In rodents, an acute cocaine injection induces hyperlocomotor effect and even generates stereotypies at high doses (higher than 30 mg/kg) (Thomsen & Caine, 2011; J. Woods, 1977). In humans, chronic cocaine intake induces tolerance, meaning that in order to reach the same rewarding effects in intensity and duration there is a need to increase the dose (Fischman & Schuster, 1982). Dysphoria quickly follows euphoria, along with signs of anxiety and restlessness, and in some cases with panic attacks. At this point, some vulnerable users lose control over drug intake and increase their consumption (Neurobiology of Addiction - 1st Edition, n.d.). The longer the use, the less cocaine will generate euphoric response and is often accompanied by stereotypical movements and impairment of speech fluency. At higher doses, paranoia and hallucinations signs are observed. Regular users may also exhibit psychotic symptoms (Withers et al., 1995). Abstinence following repeated use of cocaine (withdrawal period, Fig. 5) is associated with symptoms of dysphoria, anhedonia, depression, anxiety, anergy, insomnia, and continuous concern about the drug, which increases relapse probability (Gawin & Ellinwood, 1988).

The primary target of cocaine is the membrane transporters of monoamines: the membrane transporter of dopamine or DAT, norepinephrine or NET and serotonin or SERT. However, the rewarding effect of cocaine depends on its action on DAT (R. Chen et al., 2006; Ritz et al., 1987). Cocaine, by binding to DAT, blocks the reuptake of DA after its synaptic release, therefore increasing its extracellular concentration (Yeh & De Souza, 1991). DA concentration rapidly increases (within a few minutes), and then gradually falls back to baseline levels in about one hour after the injection. (Di Chiara & Imperato, 1988). In addition, cocaine activates sigma-1 (σ1R) and sigma-2 (σ2R) receptors (Matsumoto et al., 2002; Sharkey et al., 1988). These receptors are expressed in the majority of reward circuit structures (Alonso et al., 2000), but the endogenous ligands of these receptors and their physiological roles remain poorly understood (Aguinaga et al., 2019). Some studies suggest that these receptors are located in the endoplasmic reticulum (ER) membrane and they could possibly modulate intracellular calcium release (Hayashi & Su, 2001). Others show that sigma receptors modulate glutamate and DA receptor expression at the plasma membrane, but further investigation is required to better understand the role of cocaine binding to sigma receptors on long-lasting adaptations induced by this drug in vivo (Kourrich et al., 2012; Lever et al., 2014; Matsumoto et al., 2002; Navarro et al., 2010, 2013; Romieu et al., 2002).
1.7 Modeling addiction in experimentation animals

Addiction is a complex human pathology that is not easy to model in simpler organisms or cell cultures. Working with dependent subjects is very delicate because they hardly engage to participate in a study or to pursue a treatment. Moreover, human in-depth neurobiological analysis is not possible because of their invasiveness. These ethical issues led researchers to model some aspects of drug addiction using rodents, which are highly social animals sharing a brain structure close to humans, especially the reward circuitry that is conserved from mice to humans (Ahmed, 2012). In rodents, one single injection of cocaine triggers hyperlocomotion and they respond in a dose-dependent manner as humans. The effect will last longer or less depending on the administration route and/or rate (Thomsen & Caine, 2011; J. Woods, 1977).

One of the most used behavioral tests to study the effects of drugs of abuse in animal models is the behavioral sensitization paradigm (fig. 10.A). The principle is that repeated administrations of a fixed dose of drug generates a progressive enhancement of the locomotor response (development), which is persistent since it is observed after a long withdrawal (1 week to 1 month) followed by a re-exposure to the same dose of drug (maintenance of the behavioral sensitization) (Anderson & Pierce, 2005; Paulson et al., 1991). Following the first administration, the initial molecular and cellular changes triggered will drive long-term adaptations in the reward circuit that will prime the locomotor response to subsequent drug exposures (Anderson & Pierce, 2005). It has been proposed that behavioral sensitization would somehow correspond to a motor expression of pathological adaptations of the reward circuit leading to an abnormally increasing level of motivational value attributed to the drug and to its associated context (T. E. Robinson & Berridge, 1993, 2000, 2008). Locomotor sensitization is not a model of addiction itself, but it is instrumental to study molecular and cellular adaptations induced by repeated exposure to a substance, and to unravel whether those mechanisms are preferentially involved in the control of the development and/or maintenance phases of this behavioral response (Vanderschuren & Kalivas, 2000).

Another test used is the Conditioned Place Preference test (CPP) that is based on Pavlovian conditioning to evaluate the rewarding properties of a particular substance or stimulus (fig. 10.B). The animal is trained to associate a reward to a specific context. The CPP test can be divided in three main phases: pre-conditioning (pre-test), conditioning, post-conditioning (test). During the initial phase the animal can freely explore the two or more environments that differ in visual or texture features. The animal must not show any preference for a given environment at this initial phase. In the conditioning phase, the animal is first confined in one of the compartments following the administration of a substance, then confined in the other compartment after receiving the vehicle (neutral substance). This conditioning phase is repeated few times. The animal preference is then evaluated during the test, where the animal has a free access to all compartments. The rewarding effect of the substance is translated into an increase in the time spent in the context previously associated with the substance, which reflects the pleasant effects of this drug for the animal (Bardo & Bevins, 2000). The number of conditioning sessions is correlated to the magnitude of the CPP achieved as well as the dose of the substance, which at high doses can also be aversive (Tzschentke, 2007). Furthermore, since the preference can be maintained after the test, is possible to study memory processes of its retention. Animal can indeed be subjected to an extinction phase during which it has again free access to all compartments without the substance. Once the place preference is extinguished, it is possible to re-expose the animal to
the substance, which will reinstate CPP, and allow the investigation of the mechanisms specifically involved in this reinstatement phase (McKendrick & Graziane, 2020). Although CPP is a very instrumental and non-invasive behavioral paradigm to study positive or negative motivational properties of a substance, this behavioral test does not recapitulate an important aspect of addiction in human since it is a non-operant paradigm, where the substance is administered by the experimenter.

Figure 10. Illustrative images of behavioral tests for addiction modelling. 
A) Behavioral sensitization, B) Conditioned place preference and, C) Self-administration. 
From (Olive & Kalivas, 2011).

The most sophisticated behavioral paradigm to model addiction is the self-administration protocol (fig. 10.C). This is an operant conditioning test first used by skinner (Skinner, 1937), where the animals learn to voluntarily press a lever to get access to a certain stimulus (intracranial self-stimulation, intravenous substance administration, access to a different compartment where the stimulus is, etc.) (Pascoli et al., 2015; Weeks, 1962). It has been shown that this test permits to model the different stages of addiction 1) the development: initiation or acquisition of self-administration with escalation of drug intake; 2) the withdrawal: when a lever press is not
associated to drug injection anymore and 3) the relapse, which can be induced by an injection of the drug itself or the reintroduction of an environmental cue previously associated with drug infusion upon lever press. This will lead the animal to actively press the lever previously associated with drug infusions, even though levers presses are not associated to drug infusions anymore during this phase (Panlilio & Goldberg, 2007). Self-administration also allows to evaluate the motivational value assigned to each substance or stimulus by increasing the number of lever presses required to obtain the same dose of the drug (Ahmed & Koob, 1998; Deroche-Gamonet et al., 2004). The persistence of drug use, despite its negative consequences, can also be studied through the model of resistance to punishment, in which the animal continues to self-administer the drug despite the fact that along with the substance it receives a painful electric shock (Ahmed, 2012).

This behavioral test allowed researchers to learn that most of drugs of abuse (cocaine, morphine, nicotine, amphetamine, etc.) have a high addictive potential in the different animal models tested (rats, cats, dogs, monkeys) being thus an important model to predict substance addictive potential (Ahmed, 2012). For some drugs, animals will self-administer until death, ignoring other natural rewarding substances like water or food (Aigner & Balster, 1978). Self-administration today remains the best and most versatile model for replicating the many facets of drug addiction in humans and for testing and predicting the effectiveness of new pharmaceutical approaches.
Chapter II: From signaling to circuit and behavior: cell-type-specific adaptations in the striatum underlying addiction.

2. Basal ganglia and Striatum

2.1 Basal Ganglia: structures, function and circuits.

In order to study the neurobiological basis of addiction it is necessary to review a group of interconnected brain regions that are named basal ganglia (BG). The BG circuit connects multiple cortical areas to several subcortical regions that play a central role in complex processes such as action selection, movement initiation, goal-directed behavior and reward-dependent learning. The BG circuit also plays a crucial role in diverse cognitive functions, such as language, attention, memory and executive function, and other non-motor domains (Liljeholm & O’Doherty, 2012). Dysfunctions of BG circuits result in a combination of sensorimotor, autonomic, cognitive, and psychiatric symptoms. An example is Gilles-de-la-Tourette syndrome, which is both a neuropsychiatric and a movement disorder reflecting the multiple functions of BG in these nonmotor domains. Other examples of well-described movement disorders resulting from BG dysfunctions include Parkinson’s disease (PD), chorea, dystonia, Huntington’s disease (HD), among others, where patients also show cognitive impairments. In addition, several diseases that involve the development of pathological memories, including addiction or stress-related mental disorders, are also associated with BG dysfunctions (Andres & Darbin, 2017).

The spatial organization and connections among the structures forming BG circuit is crucial for its function. BG circuit includes the external segment of globus pallidus (GPe); the internal segment of globus pallidus (GPi); the subthalamic nucleus (STN); the substantia nigra pars reticulata (SNr), and the substantia nigra pars compacta (SNc), and the striatum; which comprises the caudate, the putamen and the nucleus accumbens (NAc) (fig. 11) (Gerfen & Surmeier, 2011).

Figure 11. Representation of basal ganglia circuits. Direct-pathway projections (red) Indirect-pathway projections (blue) Excitatory inputs (green). From (Gerfen & Surmeier, 2011).
Chapter II: From signaling to circuit and behavior

The striatum is the input structure of BG, it receives excitatory inputs from most sensory, motor, and associative cortical areas - and from the thalamus. Striatum is mainly formed by medium-sized spiny neurons (MSNs) also known as spiny projection neurons (SPNs). Based on the striatal neuronal subpopulation (further discussed in section 2.3), the BG circuit can be simplified in two main pathways: the direct and indirect pathway. The direct pathway originates from MSNs expressing dopamine receptors of D1R subtype (D1R-MSN) that directly connect to the GPi and SNr output nuclei, and the indirect pathway that mostly originates from MSNs expressing dopamine D2R-receptors (D2R-MSN) which indirectly controls the activity of subthalamic nucleus (STN) through their projection onto the GPe. The direct and indirect pathways provide opposite regulations of the basal ganglia output interface (Gerfen et al., 1990; Gerfen & Surmeier, 2011; Handbook of Basal Ganglia Structure and Function, Second Edition, 2017).

The massive cortical and thalamic excitatory innervation impinging onto the striatum is mediated by glutamate. These glutamate inputs along with DA inputs coming from SNc onto the dorsal striatum and VTA onto the NAc are integrated by MSNs (fig. 11), therefore transforming neuronal activity changes into directed behavior. The output signal from the striatum is mediated by MSNs using Gamma aminobutyric acid (GABA) as the main neurotransmitter. Since GABA release contributes to neuron hyperpolarization, the activation of MSNs will silence their target structures. As a consequence, activation of direct pathway neurons will inhibit neurons from the GPi and SNr, which are themselves GABAergic. By contrast, the activation of indirect pathway neurons projecting onto GABAergic neurons of the GPe, will lead to the disinhibition of glutamate neurons of the STN, which in turn project onto SNr and Gpi, thus resulting in their activation (fig. 11). As a consequence, the activity of direct and indirect pathway neurons is important to fine-tune the spiking rate of each basal ganglia structure and their targets (Handbook of Basal Ganglia Structure and Function, Second Edition, 2017).

Although this circuit is highly conserved through evolution, there are some differences between rodents and primates/humans. One of the main salient difference is that the primate striatum is subdivided into caudate nucleus and putamen by the internal capsule that provides a structural separation between these two nuclei. Nonetheless, this feature does not provide a clear separation of functional zones, since sensorimotor, associative and limbic regions are found in both the caudate and putamen sub-regions (Kesby et al., 2018). These functional subdivisions will be further discussed in 2.2 (fig. 12).
Another important anatomical distinction involves the globus pallidus. In primates, the GPi is located directly next to the GPe, while in rodents, the GPi is separated from the GPe and is inserted in the fiber tract of the internal capsule. Despite these anatomical differences the organizational principles of the BG are remarkably conserved in rodents and primates (Handbook of Basal Ganglia Structure and Function, Second Edition, 2017).

2.2 Striatum: anatomical organization and neuronal populations

The striatum is the main input structure of BG circuit. It integrates cortical and thalamic inputs plus dopaminergic information coming from the midbrain nigrostriatal pathway (Bolam et al., 2000). In rodents, the striatum is localized under the corpus callosum and can be divided into sub-regions based on the inputs it receives and its outputs projections. The dorsolateral (DLS) region of the striatum (i.e., putamen) is primarily connected to sensory and motor cortical areas. In contrast, a dorsomedial (DMS) region (i.e., caudate) is connected with frontal and parietal associative cortical areas. There is another region surrounding the anterior commissure called ventral striatum, the nucleus accumbens (NAc), which is subdivided into two regions: the Nucleus Accumbens core (NAcc) and the Nucleus Accumbens shell (NAcsh), which are also connected with limbic structures, including the medial orbitofrontal and anterior cingulate cortical areas (fig. 13) and also the amygdala and the hippocampus (Gerfen & Surmeier, 2011; Liljeholm & O’Doherty, 2012).
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Figure 13. Diagram of specific inputs and outputs within functional striatal subdivisions. From (Liljeholm & O’Doherty, 2012).

It has been shown that medial and lateral regions of the dorsal striatum are involved in the initial learning phase and performance of a well-trained task, respectively. Lately, it has been suggested that, while the ventral striatum supports both learning and performance, the dorsal striatum is only necessary for performance. Other studies have proposed a dorsal-ventral distinction based on how rewards affect performance, since ventral striatum encodes motivational variables and transfer their significance to dorsal regions which in turn is important for response implementation (Liljeholm & O’Doherty, 2012).

Accumulating evidence suggest that the NAc is implicated in Pavlovian learning described above (see section 1.1), where DA neurons release DA into the NAc first in response to food rewards, and then, after several trials, at the onset of reward predictive cues, therefore changing neuronal firing patterns in the NAc across learning. Distinctly, several studies show that DLS appear to be involved in habit formation (novel learning) and DMS has a role in goal-directed instrumental conditioning (performance) (Balleine et al., 2007; Liljeholm & O’Doherty, 2012).

The main neuronal population within the striatum are the medium-sized spiny neurons (MSNs), which represent around 95% of the total. From the remaining 5%, 0.5-1% are cholinergic interneurons (Large, 20-35 µm) and the rest correspond to GABAergic interneurons expressing different markers summarized in table 2 (Gerfen & Surmeier, 2011) with both interneurons subtypes playing a key role in controlling the activity of MSNs. At first, it was believed that this local modulation of striatal activity simply relied on feedforward and/or feedback inhibition but
further investigation showed that interneurons connections are highly selective and specific giving a much more intricated striatal neuronal network than originally expected (Table 2) (Tepper et al., 2010, 2018).

<table>
<thead>
<tr>
<th>Type</th>
<th>Marker</th>
<th>Morphology (Size)</th>
<th>RPM (mV)</th>
<th>SA</th>
<th>Connectivity (MSNs)</th>
<th>Connectivity (Interneurons)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSI</td>
<td>PV</td>
<td>Medium to Large 5-8 principal dendrites</td>
<td>~80</td>
<td>No</td>
<td>High ~80%</td>
<td>FSI (electrotonic coupling), NGF synaptic</td>
</tr>
<tr>
<td>LTS</td>
<td>NPY/NOS/SOM</td>
<td>Medium 3-5 principal dendrites</td>
<td>~56</td>
<td>Yes</td>
<td>Low ~20%</td>
<td>CINs</td>
</tr>
<tr>
<td>THIN</td>
<td>TH</td>
<td>Medium 2-4 principal dendrites</td>
<td>~50</td>
<td>Yes</td>
<td>Low ~20% Reciprocal</td>
<td>LTS, CINs</td>
</tr>
<tr>
<td>NFG</td>
<td>NPY</td>
<td>Medium 5-9 principal dendrites</td>
<td>~85</td>
<td>No</td>
<td>High ~80%</td>
<td>NGF (electrotonic coupling), FAI, CINs</td>
</tr>
<tr>
<td>FAI</td>
<td>Htr3a</td>
<td>Medium 3-5 principal dendrites</td>
<td>~65</td>
<td>No</td>
<td>High ~50%</td>
<td>?</td>
</tr>
<tr>
<td>SABI</td>
<td>Htr3a</td>
<td>Medium 3-5 principal dendrites</td>
<td>~50</td>
<td>Yes</td>
<td>No (~4%)</td>
<td>?</td>
</tr>
</tbody>
</table>

Table 2. Main features of striatal GABAergic interneurons. Modified from (Tepper et al., 2018).

2.3 Medium-sized spiny neurons (MSNs) distribution and roles in responses induced by drugs of abuse.

2.3.1 MSNs circuits and main features

MSNs are GABAergic neurons displaying a high density of dendritic spines. MSNs form two mainly segregated sub-populations based on the dopamine receptor (DAR) subtype they express. Initially, MSN expressing dopamine type 1 receptors (D1Rs), dynorphin and substance P, were described as direct pathway neurons considering their projections to SNr and GPi. Consequently, MSN expressing dopamine type 2 receptors (D2Rs) and enkephalin were categorized as indirect pathway neurons considering their specific projection to the GPe (see fig. 14) (C. R. Gerfen et al. 1990).
However, it has been shown that some MSNs expressing D1R also project to the GPe (Kawaguchi et al., 1990), and there is an important group of NAc D1R-MSNs that project in the ventral pallidum (VP) (Baimel et al., 2019; Pardo-Garcia et al., 2019), both considered canonical outputs of D2R-expressing MSNs of the indirect pathway. Moreover, there is a group of MSNs expressing both type of DA receptors. In dorsal striatum these neurons represent 1.9 % of all MSNs and show a uniform distribution. By contrast, in the NAc this group is larger and they are heterogeneously distributed, representing the 14.6% and the 7.3% of the NAc shell and NAc core neurons, respectively. In other studies, this percentage is a little bit higher depending on the specific technique used to visualize them. It is believed that this group of neurons have a special role in neurotransmission, since they project to several areas including GPi, substantia nigra and ventral pallidum (Bertran-Gonzalez et al., 2008; Y.-P. Deng et al., 2006; Kupchik et al., 2015). However, further experiments are required to unravel the functional significance of this particular MSN subpopulation.

Morphologically, D1R-MSNs tend to have more primary arborizations and subsequently longer dendritic length than D2R-MSNs (fig. 15) (Gertler et al., 2008). A direct consequence of this characteristic is that D1R-MSNs present a larger surface area for synapses to occur. Interestingly, D2R-MSNs present greater excitability compared to D1R-MSNs, showing twice action potential
discharge in response to somatic current injection, which is likely due to their fewer dendritic branches (Gertler et al., 2008; Kreitzer & Malenka, 2007).

Figure 15. Representation of striatal neurons morphological and electrophysiological main features. A) D1R-MSNs and B) D2R-MSNs morphology from P35–P45 BAC transgenic mice; C) GABAergic interneurons for morphology an size comparison; D) Several intrasomatic current injection showed that D2R-MSNs were more excitable in a wide range of values. From (Gerfen & Surmeier, 2011).

For all MSNs, the membrane potential in resting conditions, also called downstate is around \(-85\) mV. When there is high glutamatergic activity onto MSNs, they are depolarized into a second preferred membrane potential called up-state at \(-55\) mV (close to spike threshold). A factor that can alter MSNs excitability is the level of striatal DA. It has been shown that DA increase D1R-MSNs and reduces D2R-MSNs activity, respectively (Gerfen & Surmeier, 2011; Nicola et al., 2000). This opposite neuronal excitability is partly due to the distinct signaling pathways activated downstream D1R and D2R stimulation when DA levels increase (see section 2.5).

As already mentioned, the general consensus is that activation of D1R-MSN circuit stops the phasic inhibitory output of the GPi and SNr, whereas activation of D2R-MSN circuit inhibits GPe inhibitory effect on the GPi and SNr via STN (fig. 14). It was believed for several years that the two pathways had opposite roles with D1R-MSN activating/promoting and D2R-MSN inhibiting movement initiation or motivation (Bock et al., 2013; Kravitz et al., 2012). However, some evidence suggest that striatal circuits are much more complex. A recent study showed that stimulation of NAc D2R-MSNs can induce an increase in motivation for natural reward (Soares-Cunha et al., 2016, 2018). This can be partly explained by the existence of reciprocal modulations of D1R- and D2R-MSNs, the implication of other striatal neurons, as well as the network complexity within basal ganglia (see fig. 16) (Salery et al., 2020).

A quantitative electron microscopic analysis showed that MSNs from the direct and indirect pathway are similarly innervated by cortical and thalamic afferents. Moreover, some qualitative experiments showed that MSNs spines are connected to cortical and thalamic terminals in the
same dendritic fragments (Doig et al., 2010). However, there are some specific projections to particular striatal sub-regions that have an implication in circuit functioning. For example, the anterior cortex preferentially project to D1R- and D2R-MSNs of the NAc core, whereas the lateral hypothalamic area preferentially targets D1R-MSNs in the NAc shell (Gangarossa et al., 2013; Li et al., 2018).

Finally, there is higher order of complexity of BG circuits that is brought about local striatal microcircuit, which can regulate, amplify, and filter the information flow generating a non-linear and different output. Key regulators of local neuronal activity are striatal interneurons forming synapses between them and with MSNs. Some of these local contacts are reciprocal, but the great majority of these contacts are unidirectional. This dense local neuronal network participates to the modulation of striatal output signals by directly altering MSNs activity through axo-somatic, axo-dendritic, axo-axonal synapses changing neuronal excitability, cell signaling, dendritic integration, and gene expression. Furthermore, MSNs can inhibit each other via lateral inhibition by recurrent axon collaterals. Some of the proposed modulation within the local striatal circuit are summarized in figure 16 (Guzmán et al., 2003; Taverna et al., 2005; Tecuapetla et al., 2009).

2.3.2 Spiny projection neurons responses to drugs of abuse

In order to better understand the specific roles of MSN subpopulations in drug of abuse-induced behavioral and molecular adaptations, much research has been performed using different tools available to either enhance or inhibit the activity of MSN in a cell-type-specific or input-specific manner. For example, activation of D1R-MSNs in the NAc using optogenetic approaches reinforce cocaine-induced CPP (Lobo et al., 2010). Consistently, D1R-MSNs inhibition in the NAc (Calipari et al., 2016; Chandra et al., 2013), DS (Ferguson et al., 2011) or in entire striatum (Hikida

Figure 16. Illustrative intra-striatal connectivity between interneurons and MSNs. Abbreviations: dSPN, D1R-MSN; iSPN, D2R-MSN; ChAT, choline-acetyl transferase; TH, tyrosine hydroxylase; SOM, somatostatin; NOS, nitric oxide synthase; NPY, neuropeptide Y; NGF, neurogliaform; 5HT3R, serotonin 3 receptor; PV, parvalbumin. From (Burke et al., 2017).
et al., 2010) reduces drugs of abuse-induced locomotor sensitization or CPP. In operant behavioral task, inhibition of D1R-MSN of the DMS decreases cue-induced reinstatement of cocaine seeking but spares escalation, maintenance, and motivation (Bock et al., 2013). By contrast, activating D2R-MSNs using transgenic mice expressing Gs DREADD (Designer Receptors Exclusively Activated by Designer Drugs) in striatal D2R-MSN blocked amphetamine-induced sensitization (Farrell et al., 2013). Moreover, optogenetic stimulation in NAc D2R-MSNs decreases CPP (Lobo et al., 2010) and reduces maintenance of locomotor sensitization after a withdrawal followed by a challenge injection of cocaine (S. S. Song et al., 2014). Furthermore, the inhibition of D2R-MSNs in the NAc (Durieux et al., 2009) or in DS (Ferguson et al., 2011), has been shown to enhance CPP and locomotor sensitization, respectively, in response to amphetamine. Accordingly, chemogenetic inhibition of NAc D2R-MSNs has been shown to increase the motivation of mice that self-administer cocaine, while their activation reduced drug seeking (Bock et al., 2013). Another study, using optogenetic strategies, showed that the excitatory cortical inputs onto D1R-MSNs, but not D2R-MSNs, were potentiated by cocaine exposure. Furthermore, the same group demonstrated that this cocaine-mediated and input-specific potentiation of synaptic transmission was causally linked to the development of cocaine-induced locomotor sensitization (Pascoli et al., 2012).

From these results, one can conclude that D1R-MSNs activation increases, whereas activation of D2R-MSNs decreases psychostimulants-induced behavioral adaptations. Nonetheless, new findings using tools to target more discrete group of cells show that this may be a simplistic way to apprehend the role of MSNs in drug-adaptive behavior. An example of this is that stimulation of NAc shell D1R-MSNs projections to the hypothalamus, increase cocaine self-administration and drug seeking, while total NAc shell activation stimulates its extinction (Larson et al., 2015). Furthermore, and as mentioned above, the local striatal microcircuits are relevant for information processing. For example, lateral inhibition of D2R-MSNs onto D1R-MSNs in the NAc has been proposed to contribute in the increase of D1R-MSNs activity related to locomotor sensitization development induced by psychostimulants (Burke et al., 2017; Dobbs et al., 2016). Finally, another level of complexity is brought about studies suggesting that both MSN populations can modulate pro-rewarding or aversive behavior depending on the strategy used to manipulate neuronal activity, leading to divergent results (Gallo et al., 2018; Natsubori et al., 2017; Poyraz et al., 2016; Soares-Cunha et al., 2016, 2020; Tsutsui-Kimura et al., 2017; Vicente et al., 2016). Further, both MSN populations are stimulated at the same time in DLS during initiation of reward-directed behavior (Cui et al., 2013; Tecuapetla et al., 2016; Vicente et al., 2016). Overall, these results contribute to the idea that D1R- and D2R-MSNs may act in a dynamic and concerted way to generate and fine tune behavioral responses.

After repeated cocaine injections D1R-MSNs synapses onto VP neurons are potentiated, while D2R-MSNs synapses are oppositely regulated. Depotentiation of D1R-MSN-VP transmission suppressed cocaine-induced behavioral sensitization, suggesting that D1R-MSN-VP projections control locomotor sensitization (Creed et al., 2016). In another interesting study, chemogenetic activation of D1R-MSNs or inhibition of D2R-MSNs in the NAc increased cue-induced reinstatement in cocaine self-administration, which was prevented when impairing VP neurons activity (Heinsbroek et al., 2017). Another group also showed that compulsive self-stimulation of VTA DA neurons requires potentiation of excitatory glutamate inputs from the orbitofrontal cortex onto ventro-central striatum D1R-MSNs (Pascoli et al., 2018). In addition, repeated cocaine exposure potentiates basolateral amygdala-D1R-MSNs synapses but not D2R-MSNs (MacAskill
et al., 2014). Interestingly, hippocampal place cells projections onto D2R-MSNs are potentiated in mice that have developed cocaine-induced CPP (Sjulson et al., 2018), but the functional significance of this input-specific adaptation on the rewarding effect of cocaine is yet unknown.

Studies referring to alterations of MSNs input-specific connections in response to drugs of abuse contribute to explore new potential targets using, for example, deep-brain or transcranial magnetic stimulation (Creed et al., 2015; Diana et al., 2017). At the synaptic level, the strengthening or the weakening of specific connections onto MSNs, especially the converging glutamate and DA inputs, is able to trigger long-lasting behavioral adaptations to psychostimulant. Therefore, understanding how DA and glutamate signaling are integrated by MSNs in response to drugs of abuse is key to contribute to innovative strategies with a therapeutic potential (Salery et al., 2020).

2.4 Role of DA and glutamate receptors in MSNs signaling in responses to psychostimulants.

The integration of converging DA and glutamate inputs onto striatal neurons is critical for the multiple physiological roles played by the striatum. By artificially increasing DA release, drugs of abuse trigger a persistent alteration of glutamate transmission in the striatum, which plays a key role in the development of drug-adaptive behavior. In order to understand how this signal integration is accomplished it is necessary to briefly review the different types of receptors expressed within the striatum, which are involved in DA and glutamate signal transduction in normal conditions and in response to psychostimulants.

2.4.1 Dopamine receptors and their implication in responses to psychostimulants.

DAR have diverse functions in the organism, from modulating voluntary movement, sleep, attention, reward, vision, olfaction, hormonal regulation, etc., to influence gastrointestinal, renal, cardiovascular and immune functions. DAR are G protein-coupled receptors (GPCRs) that have the classical seven-transmembrane structure coupled intracellularly to distinct G protein subunits, which in turn will trigger different signaling cascades depending on the DAR subtype. They can signal through the canonical G-protein-dependent pathway but also through G-protein-independent mechanisms (i.e through binding to beta-arrestins or heteromerization (see section 2.6)), expanding the signaling versatility activated downstream DAR stimulation. There are five types of DARs (D1, D2, D3, D4, and D5), but they have been divided into two main subcategories based in their ability to modulate cAMP production: the D1- and the D2-like family, in the table 3 are presented the main features of each receptor (Baik, 2013; Missale et al., 1998).
## 2.4.1.a D1 Receptors

The D1R is expressed post-synaptically in most dopaminergic innervated tissue, cortex (prefrontal, Cingular and, piriform), hippocampus, amygdala, thalamus, olfactory tuberculus and striatum. D1R can be found in perisynaptic/extrasynaptic regions in the head of dendritic spines or in their neck, but D1R expression is not restricted to these areas since it can laterally diffuse at the plasma membrane due to its high mobility (Q. Huang et al., 1992; Ladepeche et al., 2013; Smiley et al., 1994). Multiple evidence showed that it can be found in post synaptic density as well (Fiorentini et al., 2003; Ladepeche et al., 2013; Scott et al., 2006). Interestingly, D1Rs have also been observed in a small number of presynaptic glutamatergic terminals in striatum (Dumartin et al., 2007).

The D1-family is formed by D1R and D5R that are coupled to G\(_{\alpha_s}\) and G\(_{\alpha_{olf}}\), which are positively coupled to adenyl Cyclase (AC) activity, therefore increasing cAMP levels. By contrast, D2-family (D2R,D3R, and D4R) associates to G\(_{\alpha_i}\) and G\(_{\alpha_0}\) proteins regulating negatively cAMP production (Beaulieu et al., 2015; Corvol et al., 2001; Kebabian & Calne, 1979; Spano et al., 1978). The produced cAMP binds to and activates protein kinase A (PKA), which phosphorylates multiple targets, including the Dopamine- and cAMP-regulated phosphoprotein, MW 32 kDa (DARPP-32) and also numerous glutamate receptor subunits, therefore modulating glutamate-dependent signaling. (Beaulieu & Gainetdinov, 2011; Esteban et al., 2003; Svenningsson et al., 2004; Waltereit & Weller, 2003). DARPP-32 is modulated by phosphorylation at two main regulatory sites, Threonine 34 (T34) and Threonine 75 (T75). When DARPP-32 is phosphorylated on T34 by PKA, DARPP-32 become a potent inhibitor of protein phosphatase 1 (PP1) activity, which negatively modulates the phosphorylation state of PKA downstream targets (fig. 17.A). Oppositely, when DARPP-32 is phosphorylated at T75 by cyclin-dependent kinase 5 (CDK5) it results in PKA inhibition. Interestingly, in response to a sustained D1R activation induced by cocaine, DARPP-32 displays an increase on Thr75 phosphorylation and a decrease in T34 phosphorylation. Conversely, in D2R-MSNs AMPc production is downregulated upon activation of the D2R, therefore DARPP-32 phosphatase activity is decreased (Bateup et al., 2008; Girault, 2012; Svenningsson et al., 2004).
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Figure 17. Schema of intracellular DAR signaling pathways.
A) Canonical vs B) non-canonical DAR downstream signaling pathways. From (Tritsch & Sabatini, 2012).

DA activates the cAMP/PKA pathway in D1R-MSNs. Activation of D1R signaling cascade potentiates glutamate-mediated calcium influx through NMDAR. This is achieved by different mechanisms, including receptor phosphorylation, membrane trafficking but also heteromerization which will be further discussed in section 2.6 (Braithwaite et al., 2006; Flores-Hernández et al., 2002; Gao & Wolf, 2008; Hallett et al., 2006; Pascoli et al., 2011; van Huijstee & Mansvelder, 2015).

Psychostimulants, such as cocaine, increase DA within the reward circuit (see section 1.5). An acute cocaine exposure induce a rapid D1R-mediated calcium increase in D1R-MSNs in DS of anesthetized mice (Luo et al., 2011), therefore highlighting the ability of D1R stimulation to modulate calcium-dependent signaling. Moreover, it was shown that an acute cocaine dose increase activity in discrete clusters of D1R-MSNs in the DLS that correlate with hyperlocomotion (Barbera et al., 2016). Interestingly, one single cocaine injection does not alter D1R expression levels in the striatum (Q. Huang et al., 1992). Moreover, D1R knockout animals do not develop locomotor sensitization (Xu et al., 2000) but they develop CPP in response to cocaine (Miner et al., 1995). Of note, these mice perform normal food self-administration but not to cocaine (Caine et al., 2007). The use of pharmacological strategies to block D1R activity to determine their functions in responses to drug of abuse showed inconsistent results. In some cases D1R inhibition blocked CPP in response to cocaine, while in other cases did not have any effect (Adams et al., 2001; S. M. Gu et al., 2020; Kuribara, 1995; Liao et al., 1998). Surprisingly, using a D1R antagonist increased cocaine self-administration and drug seeking behavior (Corrigall & Coen, 1991; Koob et al., 1987, 1996).

2.4.1.1. D2 Receptors

D2 receptors are widely expressed in the brain. They are found in the dorsal and ventral striatum, olfactory bulb, amygdala, hippocampus, substantia nigra, VTA and cortex (prefrontal, cingular
anterior and, entorhinal). They are found at pre and post synaptic levels (Sesack et al., 1994). The pre-synaptic receptors play a central role in modulating DA transmission and DA neurons activity, by controlling synthesis, release and re-uptake of DA (Ford, 2014). The D2 receptor is presented in two isoforms, D2L and D2S, which are produced by alternative splicing of the same gene (Dal Toso et al., 1989; Montmayeur et al., 1991). D2L is larger of 29 amino acids in the third intracellular loop, which is crucial for coupling with G proteins and with different interactors. It has been shown in mice lacking D2L that D2S had preponderant presynaptic autoreceptor function (Radl et al., 2018). Thus, the length disparity elicit distinct physiological properties and subcellular localization resulting in different functions in vivo (Montmayeur et al., 1991).

Since D2R stimulation decrease cAMP through its coupling to Gi, DA release represses the cAMP/PKA pathway in D2R-MSNs (fig. 17). As opposed to what was observed in D1R-MSN, an acute cocaine injection induces a slow calcium decrease in the soma of D2R-MSNs in the DS of anesthetized mice (Luo et al., 2011). Moreover, an acute dose of cocaine decreases the activity of D2R-MSNs in the dorsal striatum of freely moving mice (Barbera et al., 2016; Calipari et al., 2016).

D2R knockout animals present a decreased cocaine-induced CPP and enhanced cocaine self-administration (Caine et al., 2002; Welter et al., 2007). When administering D2R antagonists, the development of locomotor sensitization is prevented (Fontana et al., 1993; Manvich et al., 2019; White et al., 1998), as well as the development of cocaine-induced CPP (Adams et al., 2001; S. M. Gu et al., 2020). By contrast, D2R antagonists have been shown to increase cocaine self-administration (Corrigall & Coen, 1991).

2.4.2 Glutamate receptors and their implication in responses to psychostimulants.

Glutamate is the most common excitatory neurotransmitter in the CNS of vertebrates. This neurotransmitter plays a key role in cocaine-induced adaptations. In response to acute administration, psychostimulants trigger an increase of DA in the striatum but glutamate levels remain unchanged. However, acute DA increase trigger signaling cascades that will affect glutamate transmission by altering glutamate receptors activity (see section 2.5). By contrast, repeated administrations of psychostimulant increase both, DA and glutamate levels in the striatum (Kalivas, 2004; Reid et al., 1997; Y. Zhang et al., 2001). Furthermore, micro dialysis experiments have demonstrated that a unique injection of cocaine performed after several weeks of withdrawal following chronic cocaine treatment induces a release of glutamate in the NAc of sensitized rats, while it does not cause a release of glutamate in naive or non-sensitized animals (Torregrossa & Kalivas, 2008). Glutamate acts on different glutamate receptors (GluRs) which are classified in two main types: ionotropic glutamate receptors (iGluRs) and metabotropic glutamate receptors (mGluRs), represented in figure 18A. In general, GluRs are glutamate sensors and their activation and subsequent downstream signaling occur in a wide range of timescales, from less than milliseconds to hours (fig. 18 B and C). GluRs activity is modulated by a large variety of signals since their function is regulated by cations, anions, many endogenous modulators, membrane voltage and even lipid composition (Kubo et al., 1998; Ohana et al., 2006; Paul et al., 2013; Traynelis et al., 2010; Vergnano et al., 2014).

iGluRs can be organized in three main subtypes according to their structure and pharmacological properties, which are the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors
(AMPARs), N-methyl-D-aspartate receptors (NMDARs) and kainic acid receptors (KARs). These receptors share the same central cation-permeable tetrameric structure and are mainly activated by presynaptic release of L-glutamate (Greger et al., 2017). All subunits share a general structure that comprises an extracellular amino-terminal domain (ATD), a ligand binding domain (LBD), a pore-forming transmembrane domain (TMD), and an intracellular C-terminal domain (CTD). AMPARs and KARs are less sensitive to glutamate showing EC50 (half-maximal effective concentration) values for glutamate that are around hundreds of micromolar to millimolar range (Traynelis et al., 2010), while NMDARs display EC50 values between 0.4–4 mM (Paoletti et al., 2013). This results in AMPARs and KARs being extremely fast receptors, which are activated by high glutamate concentrations and produce rapid excitatory currents that are deactivated rapidly when glutamate concentrations drop back to basal concentrations or when receptors are desensitized as a result of sustained increase of glutamate levels (Fig.18.B and C) (Traynelis et al., 2010). Moreover, NMDARs do not display fast ligand-induced desensitization and are more permeable to calcium than others iGluRs, enhancing to a greater extent the activation of calcium-dependent intracellular signaling cascades (Reiner & Levitz, 2018; Traynelis et al., 2010).

mGluR are part of the GPCR family but structurally differ by the presence of a long extracellular LBD that is fused to the 7-helix TMD through a cysteine-rich domain (CRD). The time scale of activation regarding mGluRs is variable. GPCRs are known to signal over timescales of seconds to minutes, but for some mGluRs signaling can occur much more rapidly (Fig.18.B). Besides, some mGluRs also produce basal glutamate-independent activity that may maintain a key modulatory tone to the synapse (Ango et al., 2001; Vafabakhsh et al., 2015).

Figure 18. Glutamate receptors.
A) Structural organization and subunit-dependent classification, iGluRs (green), mGluRs (blue); B) Synaptically relevant glutamate concentrations for each type of GluR; and C) Timescales of synaptic transmission and plasticity for each GluR and subsequent activation of downstream signaling pathways. From (Reiner & Levitz, 2018).
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2.4.2.a NMDA Receptors

NMDARs are formed by GluN1, GluN2 (GluN2A, GluN2B, GluN2C, GluN2D), and GluN3 (GluN3A, GluN3B) subunits. These receptors are composed of two obligatory GluN1 subunits and two GluN2 regulatory subunits that can be replaced by GluN3 subunits. NMDARs are Na⁺/Ca²⁺ permeable channels that contribute to postsynaptic depolarization and subsequent intracellular calcium-dependent signaling activation. The CTD regions from GluN1 and GluN2 subunits are important for activation of intracellular signaling pathways and for subcellular localization of these receptors, for example their interaction with PSD-95 (P0st-synaptic density protein 95) at the synapse (Traynelis et al., 2010). NMDAR are blocked at resting potential by a Mg²⁺ cation and membrane depolarization induced by AMPARs activation removes this blockade (Nowak et al. 1984). NMDA-dependent Ca²⁺ influx activates cAMP-response element binding (CREB)-dependent gene regulation via the Ras-ERK1/2 and the nuclear Ca²⁺–calmodulin (CaM) kinase pathways, which are key for long-term synaptic plasticity adaptations (learning and memory) (Barco et al., 2002; Hardingham et al., 2001; Hardingham & Bading, 1999; Impey & Goodman, 2001; Wu et al., 2001). Interestingly, activation of extra-synaptic NMDA, has been linked to neuronal death following acute trauma such as stroke, mechanical trauma or seizure activity (Choi & Rothman, 1990; Hardingham & Bading, 2010). In addition, an excessive stimulation of NMDAR, especially the extra-synaptic ones, is believed to originate and/or contribute in the development of many neurodegenerative disorders such as Huntington’s disease, HIV-associated dementia, and Alzheimer’s disease (Chohan & Iqbal, 2006; Fan & Raymond, 2007; Lancelot & Beal, 1998).

Psychostimulants, such as cocaine, affect the expression and composition of NMDA receptors depending on the dose and treatment duration (Q. Huang et al., 1992; Schumann & Yaka, 2009; Smaga et al., 2019; W.-L. Sun et al., 2009). When studying cocaine rewarding properties using the CPP paradigm, Calipari E. and collaborators detected a Ca²⁺ increase in NAc D1R-MSNs right before entrance to the drug-paired compartment, whereas in D2R-MSNs Ca²⁺ was decreased when the animal stayed in the compartment (Calipari et al., 2016). Since NMDARs are one of the major Ca²⁺ gateways, several studies have been made trying to understand the role of NMDAR in response to psychostimulants. There are some controversial results when using MK-801, a non-competitive inhibitor of NMDAR. In some cases, locomotor sensitization and CPP development and maintenance were blocked, while in other cases NMDAR pharmacological blockade failed to block locomotor sensitization (T. E. Brown et al., 2008; Karler et al., 1989; H. S. Kim et al., 1996; Wolf, 1998). Moreover, different NMDAR antagonists have different effects regarding behavioral responses to drugs of abuse (Allen et al., 2005; Hyytia et al., 1999; Schenk et al., 1993). NMDAR knockout in D1R-MSNs block amphetamine-induced sensitization, a phenotype that is rescued by restoring functional NMDAR in the NAc of D1R-MSNs or deleting NMDAR in all MSNs (Beutler et al., 2011). Conversely, NMDAR knockout in D1R-MSNs, D2R-MSNs or both did not alter the development and extinction of cocaine-induce CPP, while CPP reinstatement was impaired in D1R-MSNs lacking NMDAR (Joffe et al., 2017).

2.4.2.b AMPA Receptors

AMPAR are complex signaling assemblies constituted by four core subunits (GluA1, GluA2, GluA3, and GluA4) in homo- or heterotetramers. These receptors are the main actors of fast excitatory transmission at synapses and are critical for brain plasticity. AMPAR subunit
Chapter II: From signaling to circuit and behavior

AMPAR extrasynaptic membrane expression is increased along with its open probability when phosphorylated by PKA. (Shepherd & Huganir, 2007; X. Sun et al., 2005, 2008). D2R activation decreases AMPAR presence at the membrane (X. Sun et al., 2005). In addition, co-stimulation of AMPAR and D2R prevents action potentials and membrane current changes induced by AMPAR agonists alone (André et al., 2010; Hernández-Echeagaray et al., 2004). In some cases AMPAR-induced action potentials, are either unaffected by D1R agonists (Calabresi et al., 1995; Seamans et al., 2001; Zheng et al., 1999) or potentiated (André et al., 2010; Levine et al., 1996). Some studies showed that D1R agonists and D2R antagonists induce PKA-dependent phosphorylation of AMPAR, while D2R agonist diminished it by favoring PP1 activity (Håkansson et al., 2006; Snyder et al., 2000). Overall, these results show that DA facilitates the function of AMPAR in D1R-MSNs and prevents it in D2R-MSNs (Salery et al., 2020). Accordingly, AMPARs expression at the synapse is altered in response to cocaine. After withdrawal from a single or chronic exposure to cocaine (at high doses), as well as after cocaine self-administration, GluA2-lacking AMPARs were found in D1R-MSN and in D2R-MSN, altering AMPAR/NMDA ratio (M. T. C. Brown et al., 2010; Terrier et al., 2016). Interestingly, the incorporation of Ca\(^{2+}\) permeable AMPARs increases the amplitude of excitatory postsynaptic currents of MSN, and blocking them decreases the search for cocaine (Conrad et al., 2008). These studies suggest that, even after a long period of abstinence, the potentiation of glutamatergic transmission induced by cocaine, plays a central role in relapse (Conrad et al., 2008).

2.4.2. c Kainate Receptors

Kainate receptors are formed by GluK subunits (GluK1, GluK2, GluK3, GluK4, and GluK5). GluK1 to GluK 3 are required to constitute functional receptors and can form both homo and heterotretamers alone or with GluK4 and GluK5 subunits. KRs have been less studied in comparison to the others glutamate receptors, but they have been linked to certain forms of memory and learning (Traynelis et al., 2010). They can also trigger LTP and LTD and rapid alterations of their trafficking can alter synaptic transmission during synaptic plasticity and neuronal development, in which they are likely playing a major role (Jane et al., 2009; Ryazantseva et al., 2020). Little is known about kainate receptors involvement in response to
psychostimulants since there is no specific antagonist for this type of receptors. However, a study suggest that GluK5 subunit may negatively modulate psychostimulant and rewarding effects of cocaine, since GluK5 KO mice exhibited enhanced cocaine-induced place preference and sensitization as compared with WT mice (Gregus et al., 2010). Another study also indicated that KARs containing GluK5 subunit are important for the development of morphine tolerance, without altering the morphine-induced increase in locomotor activity, nor its rewarding properties (Bogulavsky et al., 2009). More research is needed in order to clarify the role of kainate receptors in response to drugs of abuse.

2.4.2.d Metabotropic Glutamate Receptors

The other type of glutamate receptors, mGluRs, belong to the GPCRs family. There are 8 subtypes, mGluR1–mGluR8, which can form constitutive dimers. They are divided into three groups depending on their sequence homology and their preferred G protein signaling partners, where group I represents mGluRs coupled to \( G_q \) (mGluR 1 and 5), while group II (mGluR 2 and 3) and III (mGluR 4, 6, 8, and 7) are coupled to \( G_i \). In general, mGluRs are widely expressed throughout the nervous system with the exception of mGluR 6, which is mainly expressed in retinal ON bipolar cells (Ferraguti & Shigemoto, 2006). mGluRs are key for the development of different forms of synaptic plasticity and also to fine-tune neuronal firing and neurotransmitter release at the pre-synapse, where the stimulation of a specific mGluR subtype can increase or reduce glutamate release. For that reason, they have been considered as potential pharmacological targets for the development of treatments to alleviate neurodegenerative and neuropsychiatric disorders (Crupi et al., 2019). Additionally, mGluRs can also be linked to drug-induced adaptations. For instance, in the NAc of mice exposed to cocaine withdrawal, it was found that mGluR2/3 expression at the pre-synapse was reduced in response to decreased glutamate transmission (Kalivas, 2009; Xi et al., 2002). Also, a pre-treatment of a mGluR type III receptor agonist, L-AP4, administered by bilateral dorso-striatal injections, prevented cocaine locomotor sensitization acquisition (Mao & Wang, 2000), and mGluR5 knockout mice did not develop cocaine-induced locomotor sensitization nor self-administration (Chiamulera et al., 2001).

2.5 Convergence of dopamine and glutamate signaling in response to psychostimulants: ERK signaling in MSNs.

DA and glutamate activate several intracellular downstream cascades through their canonical respective receptors, as reviewed in section 2.4. In general, DA will have opposite effects on the two MSN populations by activating the canonical cAMP/PKA pathway in D1R-MSNs and repressing it in D2R-MSNs (Greif et al., 1995; Perez et al., 2006; X. Sun et al., 2005; Surmeier & Kitai, 1993). Accordingly, this leads to opposite regulations of multiple targets downstream the cAMP/PKA signaling pathway. In addition, the co-stimulation of DARs and GluRs triggers reciprocal activity regulation through local signaling crosstalk. Evidence of this crosstalk come from studies analyzing how DA alters MSNs intrinsic excitability through PKA activation and subsequent phosphorylation of voltage gated K\(^+\), Na\(^+\), and Ca\(^{2+}\) channels (Surmeier & Kitai, 1993). Overall, DA increase will induce depolarization in D1R-MSN, while it will hyperpolarize D2R-MSN (Gerfen & Surmeier, 2011; Hernández-López et al., 1997; Surmeier et al., 1995; Wickens & Arbuthnott, 2005).
By inducing DA release, all drugs of abuse induce activation of the extracellular-signal regulated kinase (ERK) pathway in the striatum (Valjent et al., 2004). This activation occurs specifically in D1R-MSNs in response to cocaine (Bertran-Gonzalez et al., 2008), and depends on the concomitant activation of NMDAR (Cahill, Pascoli, et al., 2014; Valjent et al., 2000). Downstream from these receptors, ERK activity and its subsequent nuclear translocation has a central role in chromatin remodeling and gene expression regulation, being necessary for long-term neuronal and behavioral adaptations induced by drugs of abuse. Indeed, pharmacological blockade of ERK activation impaired conditioned place preference (Valjent et al., 2000) and locomotor sensitization (Valjent, Corvol, et al., 2006) induced by cocaine, as well as the reconsolidation of cocaine-associated contextual memories (C. A. Miller & Marshall, 2005; Pascoli, Cahill, et al., 2014; Valjent, Corbillé, et al., 2006).

In a previous study from the lab, Pascoli and collaborators developed an in vitro model of cultured striatal neurons that recapitulates the main effects of an acute cocaine injection in vivo on D1R-MSNs. In order to mimic the effect of endogenous glutamate signals coming from the cortex and other glutamatergic inputs, they used a low dose of glutamate (0.3 µM) combined with a D1R agonist, SKF38393 (3 µM), to mimic the effect of increased DA. They showed that this co-stimulation of D1R and NMDAR induced a D1R-mediated facilitation of calcium influx through NMDAR triggering ERK activation in vitro. The stimulation of D1R was indeed able to induce the phosphorylation, in a cAMP-independent manner, of the tyrosine kinase Fyn, which in turn phosphorylates GluN2B subunit of NMDAR and promotes a Ca\(^{2+}\)-dependent activation of ERK (see fig. 19). They also showed that preventing this local signaling linking D1R stimulation to the facilitation of GluN2B-containing NMDAR in vivo blocked cocaine-induced ERK activation in the striatum as well as locomotor sensitization and CPP (Pascoli et al., 2011).

Figure 19. Intracellular crosstalk between DR1 and NMDAR in response to a single cocaine exposure. From (Pascoli et al., 2011)
Downstream from D1R, the canonical cAMP/PKA pathway targets specific NMDAR subunits contributing to facilitate its functions (Flores-Hernández et al., 2002). The downstream phosphatase DARPP-32 can indirectly amplify ERK activation, through inhibition of PP1. This will prevent MEK1/2 de-activation by PP1 as illustrated in figure 19 (Cahill, Salery, et al., 2014; Pascoli, Cahill, et al., 2014; Valjent et al., 2005). Interestingly, PKA activity also promotes ERK1/2 activation by inhibiting the striatal-enriched protein phosphatase (STEP) which, when active, de-phosphorylates ERK1/2 (fig. 19) (Pascoli, Cahill, et al., 2014; Pulido et al., 1998; Valjent et al., 2005). As mentioned above, DA silence cAMP/PKA pathway downstream DR2 signaling, having the opposite effect in ERK activity (see fig. 20 right).

After local dendritic activation, ERK translocates to the nucleus (Lavaur et al., 2007; Trifilieff et al., 2009) to induce immediate early gene (IEG) expression and morphological changes in response to cocaine, by targeting indirectly the transcription factors CREB (Brami-Cherrier et al., 2005) and directly the transcription factor Elk-1 (Besnard et al., 2011). Of note, the lab showed that a single cocaine injection induce a fast de novo dendritic spines formation in the NAc shell of D1R-MSN, which maintenance over time relies on non-nuclear functions of ERK, through the targeting of the cytoplasmic mitogen-activated protein kinase interacting protein-1 (MNK-1) by ERK (Dos Santos et al., 2017).
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2.6 DA and glutamate receptor heteromers as integrators of DA and glutamate signaling in the striatum: implication in response to cocaine.

DA and glutamate receptor crosstalk is not only based on local intracellular pathways as described in the previous section. It has been shown that DARs and GluRs can physically interact through electrostatic interactions to form heteromers, adding complexity to signal integration processes. By definition, heteromers are higher order entity assemblies of two or more different receptors that activate distinct downstream signaling pathways compared to the non-assembled component receptors. Over time, DAR and NMDAR heteromers have emerged as promising molecular substrates for the spatio-temporal fine-tuning modulation of component receptors functions and downstream signaling pathways, with a particular interest as potential therapeutic targets in numerous neurological and psychiatric diseases, including Parkinson’s disease or addiction (Andrianarivelo et al., 2019; Borroto-Escuela, Carlsson, et al., 2017; Borroto-Escuela, Narváez, et al., 2017; Missale et al., 2006; Wang et al., 2012).

2.6.1 D1R/D2R Heteromers

It has been shown that D1 and D2 receptors are co-expressed in around 15% in MSNs from ventral striatum (NAcsh) (Bertran-Gonzalez et al., 2008; Gangarossa et al., 2013). The team led by Susan R. George has been studying the heteromers formed by D1 and D2 receptor for many years (Y.-P. Deng et al., 2006; Gagnon et al., 2017; Hasbi et al., 2009; Perreault et al., 2010). George and collaborators studied D1R/D2R heteromers using co-immunoprecipitation and proximity ligation assay (PLA) techniques in rodents and non-human primates (Hasbi et al., 2017; Perreault et al., 2016; Rico et al., 2017). Using a peptide able to disrupt D1R/D2R heteromers in the striatum they found increased locomotor sensitization and CPP, as well as reinstatement after self-administration in response to psychostimulants (Hasbi et al., 2014, 2017; Perreault et al., 2014; Shen et al., 2015). However, this work has been questioned by Frederick and collaborators who showed that D1R/D2R heteromers were not detectable by using the same approach in mice ventral striatum (Frederick et al., 2015). Moreover, the interfering peptide used by George’s team designed to disrupt D1R/D2R complexes, called TAT-D1 corresponding to amino acid 396–413 of the D1R CTD, mimic the effect of D1R/GluN1 interaction, inducing NMDAR potentiation and ERK activation by itself without cocaine stimulation (Cahill, Pascoli, et al., 2014). In addition, Tat-D1 peptide alone showed pro-locomotor effects (Shen et al., 2015) and induced place-preference (Hasbi et al., 2017; Perreault et al., 2016). Further research is needed to clarify the existence and function of these heteromers in the context of cocaine-induced behavioral adaptations.

2.6.2 D1R/GluN1 Heteromers

Another heteromer potentially relevant for drugs of abuse-mediated responses is the one formed by D1R and GluN1 subunit of NMDAR. Indeed, D1R stimulation facilitates NMDAR functions through its direct interaction with NMDAR, while NMDAR stimulation induces D1R surface expression and subsequent cAMP production (Flores-Hernández et al., 2002; F. J. S. Lee et al., 2002; Pei et al., 2004; Scott et al., 2006; Wittmann et al., 2005). D1R is mainly localized extrasynaptically and the recruitment of D1R at synaptic sites relies on D1/GluN1 heteromer formation. According to this, upon glutamate release, D1R/GluN1 heteromers are mainly formed within, or adjacent to synapses (Scott et al., 2006). Recently, D1/GluN1 heteromers have started to be positioned as molecular bridges by which synergistic DA and glutamate mediate responses
to drugs of abuse. These D1R/ GluN1 heteromers were found in the mouse striatum (Cahill, Pascoli, et al., 2014), including in post synaptic density (PSD) fractions (Fiorentini et al., 2003), in addition to human post-mortem caudate putamen tissues (see article 1). It has been established that there are two domains of D1R that interact with NMDAR subunits, which are D1Rt2 (L387- L416) and D1Rt3 (S417-T446). They are part of the CTD domain and bind to the CTD of GluN1 (D1Rt2) and GluN2A (D1Rt3) subunits (fig. 21) (F. J. S. Lee et al., 2002). Interestingly, interacting amino acid sequences of D1R (D1Rt2) and GluN1 (GluN1-C1) are conserved across species, suggesting that D1R/NMDAR heteromers might be involved in important physiological processes that have been maintained through evolution (A. S. Woods et al., 2005).

Using the in vitro co-stimulation model of acute cocaine exposure described in the previous section (2.5) a significant increase of D1R/GluN1 heteromers was found in cultured striatal neurons (Cahill, Pascoli, et al., 2014). Remarkably, ex vivo electrophysiological recordings from D1R-MSNs showed that disruption of D1R/GluN1 heteromers blocked D1R-mediated potentiation of NMDAR postsynaptic currents and long term synaptic plasticity in D1R-MSNs, in addition to prevent cocaine-induced ERK activation (Cahill, Pascoli, et al., 2014). Moreover, using Fluo-4 to measure Ca\(^{2+}\) signals, it was shown that NMDAR-dependent Ca\(^{2+}\) influx mediated by D1R agonist was blocked when using the D1R/GluN1 disrupting peptide in cultured striatal neurons. Of note, the disrupting peptide corresponded to cassette C1 which efficiently separated D1R and GluN1 without altering individual functions of each receptor (Cahill, Pascoli, et al., 2014). Disruption of D1R/GluN2A assemble had no effect on ERK1/2 activation downstream of neither D1R or NMDAR (Cahill, Pascoli, et al., 2014). Moreover, disruption of D1R/GluN1 heteromers did not change basal or acute cocaine-induced locomotor response, whereas it interfered with locomotor sensitization in response to a second injection a week later (Cahill, Pascoli, et al., 2014).
2.6.3 D2R/GluN2B Heteromers

D2Rs have also been shown to form heteromers with GluN2B subunits of NMDAR. These heteromers have been found in postsynaptic density fractions of rat hippocampus, prefrontal cortex and striatum (X.-Y. Liu et al., 2006). The critical interaction domain for this heteromerization involves the R220-A234 residues within the third intracellular loop of D2R (D2R-IL3) (fig. 21). Studying D2R/GluN2B heteromers, Liu and collaborators showed that an acute injection of a very high dose of cocaine led to an increase in D2R/GluN2B interaction in the NAc core and DS, along with decrease in phospho-GluN2B-Ser1303 levels (X.-Y. Liu et al., 2006). Furthermore, D2R/GluN2B heteromerization negatively affects NMDAR currents in D2R-MSNs through a mechanism that involves CaMKII. Moreover, using an interfering peptide to disrupt D2R/GluN2B heteromers prevented hyperlocomotion and stereotypies induced by acute cocaine administration in mice (X.-Y. Liu et al., 2006). This study added arguments to postulate that D2R/GluN2B heteromers could mediate the inhibitory effects of psychostimulants on D2R-MSNs but the role of such D2R-NMDAR heteromers in long-lasting molecular, cellular and behavioral adaptations remain unknown.

Certainly, DAR-NMDAR heteromers have great therapeutic potential, however most data regarding their functional role come from *in vitro* and *ex vivo* studies. Therefore, it was necessary to study the modulations and functions of endogenous D1R-GluN1 and D2R-GluN2B heteromers *in vivo* by drugs of abuse and to unravel their potential roles in cocaine-induced long-lasting adaptations. This was addressed by a recent work of the laboratory, in which I participated (article I).
Chapter III: Calcium-dependent signaling and its role in drug of abuse-evoked adaptations

3. Calcium signaling in central nervous system

Calcium (Ca\(^{2+}\)) plays a pivotal role in numerous processes in the central nervous system (CNS) not only in neurons but also in other brain cells. In neurons, it acts as a second messenger activating different signaling pathways that are involved in multiple processes, including cell fate, dendritic development, synaptogenesis, etc. Ca\(^{2+}\) has also been shown to modulate release of neurotransmitters, membrane excitability, and energy production among many others processes. Moreover, Ca\(^{2+}\)-dependent signaling play a key role in regulating neuronal gene expression as well as processes related to information processing, learning, and memory (Islam, 2020). Despite many years of research, how Ca\(^{2+}\) signaling modulate such different processes is still not fully understood. Transitory changes in cytosolic calcium concentrations ([Ca\(^{2+}\)]\(_C\)) in discrete cellular microdomains, with distinct temporal dynamics, and in specific cell populations, is thought to trigger different downstream molecular events, which explains the versatility of this second messenger. Moreover, calcium signals communicate to neighboring neurons, astrocytes and glial cells, opening other possibilities of network regulation (Braet et al., 2004).

Neuronal calcium transient modulation is achieved by regulating the activity of plasma membrane channels and channels localized in the membrane of intracellular organelles, in addition to intracellular proteins buffering Ca\(^{2+}\). The combination of these mechanisms keep cytosolic Ca\(^{2+}\) levels low against extracellular calcium levels, which are 10\(^4\) times higher, therefore generating an electrochemical gradient across the plasma membrane allowing Ca\(^{2+}\) entry in a stimulus-dependent manner (Kawamoto et al., 2012). The large variety of ionotropic and metabotropic Ca\(^{2+}\)-coupled receptors, bearing distinct intrinsic different properties, emphasizes the extreme versatility of Ca\(^{2+}\)-dependent signaling. Additionally, the combined expression of multiple Ca\(^{2+}\)-coupled receptor subtypes, in different subcellular compartments and within distinct neural populations, finely tunes the dynamics of Ca\(^{2+}\) transients in a cell-type-specific manner, which is key to shape specific transcription patterns and long-lasting behavioral adaptations.

3.1 Regulation of intracellular calcium concentrations: calcium channels, pumps and exchangers

Cytosolic [Ca\(^{2+}\)]\(_C\) concentrations can vary widely from a very low concentration, defined as resting concentration of approximately 10\(^{-7}\) M, to concentrations that can reach 10\(^{-3}\) M or higher in subcellular compartments. Accordingly, a similar resting Ca\(^{2+}\) concentration have been found in the nuclear ([Ca\(^{2+}\)]\(_N\)) and mitochondrial matrix ([Ca\(^{2+}\)]\(_M\)). On the other hand, some cellular organelles constitute intracellular [Ca\(^{2+}\)] stores, accumulating Ca\(^{2+}\) up to 10\(^{-4}\) M. The most important intracellular Ca\(^{2+}\) stores are the endoplasmic reticulum, the nuclear envelope and the mitochondria, which can release Ca\(^{2+}\) into the cytoplasmic, nuclear and mitochondrial matrices, respectively, in a stimulus-dependent manner (Islam, 2020). [Ca\(^{2+}\)]\(_C\), [Ca\(^{2+}\)]\(_N\) and [Ca\(^{2+}\)]\(_M\) are kept at resting concentrations to maintain the electrochemical gradient in order to favor Ca\(^{2+}\) increase over the different compartments. This increase will be achieved through the activation of different channels mediated by distinct stimuli (Bagur & Hajnóczky, 2017).
Intracellular Ca\textsuperscript{2+} variations are mediated by a variety of Ca\textsuperscript{2+} channels, exchangers and ATPases, which will be briefly reviewed in the following section. This review will include the voltage-gated Ca\textsuperscript{2+} channels (VGCCs) and receptor-operated (ligand-gated) channels (ROCs), which includes Inositol1,4,5-trisphosphate receptor (IP3R) and the ryanodine receptors (RyRs). Furthermore, the functions of the sodium-calcium exchanger (NCX), the plasma membrane Ca\textsuperscript{2+} transport ATPase (PMCAs), the Sarco/endoplasmic reticulum type Ca\textsuperscript{2+} ATPase (SERCAs) and the mitochondrial calcium uniporter (MCU) will also be briefly presented.

3.1.1 VGCCs

Voltage-dependent Ca\textsuperscript{2+} channels are a group of voltage-gated ion channels located in the plasma membrane. At resting membrane potential VGCCs are normally closed. They open in response to depolarizing membrane potentials, allowing Ca\textsuperscript{2+} influx into the cell, which will trigger distinct responses depending on the cell type. VGCCs can be separated into two major categories: high voltage-activated (HVA) channels that open in response to large membrane depolarizations and low voltage-activated (LVA) channels that are activated by smaller voltage changes near neuronal resting membrane potentials. They are composed by a Cavα1 subunit that forms the Ca\textsuperscript{2+} selective channel pore. The channel subtype is determined by the Cavα1 subunit type. There are ten types of Cavα1 subunits divided into three families: Cav1 and Cav2, that encodes HVA channels and Cav3 for the LVA channels (Catterall, 2011). The Cav1 family encodes four different types of L-type channels, whereas the Cav2 family include P/Q-type, (Cav2.1), N-type (Cav2.2) and R-type (Cav2.3) channels, with P- and Q-type channels being distinguished by alternative splicing and channel subunit composition (Catterall, 2011). HVA channels contain several accessory subunits: Cavo25, Cavβ1–4, and Cavγ involved in anchorage and regulatory functions. The LVA channels, known as T-type, are formed by three types of Cav3 subunits: Cav3.1, Cav3.2 and Cav 3.3 (Catterall 20011, zampone 2014). The cytoplasmic regions of CaVα1 subunits show the highest sequence variations, they are key for the regulation of channel function mediated by distinct second messengers and/or critical protein-protein interactions with other regulatory elements, such as G proteins and protein kinases (Dai et al., 2009; Hall et al., 2013; Simms & Zamponi, 2014; Zamponi et al., 1997)

3.1.2 ROCs

ROCs are activated in response to the binding of specific ligands to the extracellular domain of the receptor. This will cause a structural conformational change in the protein, leading to the opening of the channel pore and subsequent Ca\textsuperscript{2+} influx across the plasma membrane. Examples of ROCs include the glutamate receptors already reviewed in chapter 2 (section 2.4.2) such as NMDAs, AMPAs, and KARs, with NMDAR bearing the highest Ca\textsuperscript{2+} permeability. The other channel receptors that are permeable to Ca\textsuperscript{2+} are nicotinic acetylcholine receptors (nAChRs), Serotonin receptor (5-HT3) and ATP P2X receptors, which are activated extracellularly by different ligands. Finally, transient receptor potential canonical channels (TRPC), which are activated by both extracellular and intracellular ligands, are also permeable to Ca\textsuperscript{2+} (Kawamoto et al., 2012). This information is summarized in table 4.
### 3.1.3 IP3Rs

As mentioned before, intracellular $[\text{Ca}^{2+}]$ can increase as a result of $\text{Ca}^{2+}$ release from intracellular calcium stores mediated by different receptors and channels. One of the main receptors modulating $\text{Ca}^{2+}$ release is Inositol 1,4,5-trisphosphate receptor (IP3R). IP3Rs are tetrameric $\text{Ca}^{2+}$ channels located at the membrane of several organelles. The activation of IP3R is made by four molecules of Inositol 1,4,5-trisphosphate (IP3), which are produced at the plasma membrane downstream from the stimulation of $\text{G}_q$-coupled receptors including metabotropic glutamate receptors ($\text{mGluRs}$). There are three types of IP3R subunits (IP3R1–3) that show different affinities for IP3 ($\text{IP3R2} > \text{IP3R1} > \text{IP3R3}$) and they can be assembled into homo or heterotetramers. $\text{Ca}^{2+}$ acts as a bi-directional modulator of the activity of the receptor, while low $[\text{Ca}^{2+}]$ increases IP3R activity, high $[\text{Ca}^{2+}]$ decreases it (Prole & Taylor, 2019).

### 3.1.4 RyRs

Another very important intracellular receptor, which is the largest ion channel known to date, is the Ryanodine receptor (RyR). RyRs are homotetramers (565 kDa/subunit) $\text{Ca}^{2+}$ permeable channels very similar to IP3Rs. There are three different isoforms (RyR1–3) and each isoform is found in various cell types. RyRs are considered as $\text{Ca}^{2+}$ signal amplifiers because they open in response to $\text{Ca}^{2+}$ through $\text{Ca}^{2+}$ sensors present in their structure, but they can also be blocked by high amounts of $\text{Ca}^{2+}$ (>100uM) (Kawamoto et al., 2012).

### 3.1.5 NCXs

Once the $\text{Ca}^{2+}$ concentration has increased, the initial electrochemical gradient in the system has to be restored by decreasing $[\text{Ca}^{2+}]_c$ back to resting levels. This can be achieved by the sodium-calcium exchanger (NCX), which is a bi-directional membrane ion transporter. Benefiting from the energy of the sodium gradient, the transporter produces an electrogenic exchange of three sodium ions for one calcium ion. In neurons under normal physiological conditions, sodium is transported into the cell, and calcium is extruded from the cytoplasm. When the electrochemical gradient for sodium is reversed, like during membrane depolarization or the opening of gated sodium channels, the exchanger transports sodium out of the cell and calcium into the cell. The
NCX has three isoforms (NCX1-3) which generate at least 17 splice variants, and the three are present in the CNS (Giladi et al., 2016).

### 3.1.6 PMCAs

When $[\text{Ca}^{2+}]_c$ are decreasing, another mechanism starts to lead to fully restore the basal $\text{Ca}^{2+}$ levels in an energy-consuming manner. One key actor involved is the plasma membrane $\text{Ca}^{2+}$ transport ATPase (PMCAs, ATP2B1-4 gene). PMCA is part of the ATPase family and shares some basic structural and catalytic features with them. It is composed of several domains, and one of the most important is the intramembrane domain (M-domain) which consists of 10 helices that provide the coordinated binding for the ligand, the ATP, and one for a cytosolic $\text{Ca}^{2+}$ ion which will be exported to the extracellular medium. The N-domain binds an ATP molecule necessary for hydrolysis; the P-domain participates in stabilizing the high-energy intermediate and the A-domain coordinates the movement of the other domains. The catalytic domains (M, N, and P) are highly conserved, while the A and C-tail domains (regulatory region) present a substantial sequence divergence. PMCA is not only a $\text{Ca}^{2+}$ extrusion pump, it is also responsible for an active modulation of $\text{Ca}^{2+}$ signals in the cytoplasm. This is achieved through different subunit composition and expression patterns in specific cell types, leading to diverse kinetic properties and multiple regulatory-binding partners possible (Islam, 2020).

### 3.1.7 SERCAs

Another mechanism to decrease free $[\text{Ca}^{2+}]_c$ following a stimulation relies on the uptake of free $\text{Ca}^{2+}$ by intracellular organelles. A key $\text{Ca}^{2+}$ ATPase participates in the uptake of $\text{Ca}^{2+}$ by the ER is the Sarco/endoplasmic reticulum type $\text{Ca}^{2+}$ ATPase (SERCAs, ATP2A1-3), which is intimately related to the PMCAs since it displays similar structure among the catalytic domains with an average of 30% sequence homology. SERCAs are located at the membrane of the endoplasmic reticulum (ER) facilitating the influx of $\text{Ca}^{2+}$ into the ER lumen at the expense of ATP hydrolysis. They are widely expressed in the CNS in different isoforms, and it has been shown that SERCA-dependent $\text{Ca}^{2+}$ dysregulations are implicated in the pathophysiology of multiple disorders that affect cognitive functions, including Schizophrenia and Alzheimer’s disease among others (Britzolaki et al., 2018).
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3.1.8 MCUs

The mitochondrial calcium uniporter (MCU) also contribute to decrease \([\mathrm{Ca}^{2+}]_\text{c}\). The MCU has low affinity for \(\mathrm{Ca}^{2+}\) and the uptake of \(\mathrm{Ca}^{2+}\) by the mitochondria is energetically favorable due to the respiratory proton electrochemical gradient. The activity of MCU complex is modulated by transmembrane regulatory subunits: MCUb, the essential MCU regulator (EMRE), mitochondrial \(\mathrm{Ca}^{2+}\) uniporter regulator 1 (MCUR1) and membrane-associated regulatory subunits in the intermembrane space (MICU1-3) (Mishra et al., 2017).

Even if the balance between \(\mathrm{Ca}^{2+}\) entry and efflux is critical to maintain basal levels of \([\mathrm{Ca}^{2+}]_\text{c}\) once \(\mathrm{Ca}^{2+}\) has raised, other proteins that bind to \(\mathrm{Ca}^{2+}\) are mandatory for regulating \(\mathrm{Ca}^{2+}\) intracellular homeostasis.

3.2 Calcium Homeostasis: buffering system

At resting states, levels of free intracellular \(\mathrm{Ca}^{2+}\) are \(10^4\)-\(10^3\) times lower compared to the extracellular space and internal \(\mathrm{Ca}^{2+}\) stores. This electrochemical gradient is fundamental to allow \(\mathrm{Ca}^{2+}\) entry in favor of the gradient and thus permitting calcium signal spreading inside the neuron. As discussed in the previous section, when a stimulus triggers \(\mathrm{Ca}^{2+}\) entry from the extracellular compartment or its release from internal stores, ATPases, exchangers and the MCU will play a major role to restore the balance. But this is not the only mechanism by which a neuron can
decrease and modulate free \([\text{Ca}^{2+}]_c\), there is a whole buffering system that shapes the dynamic of \(\text{Ca}^{2+}\) signals across the cell called calcium-binding proteins (CaBPs). Fast buffering is usually considered to be mediated by CaBPs, while slow buffering is performed by MCU, ATPases and exchangers (Bagur & Hajnóczky, 2017).

Proteins with negatively charged groups in a determined configuration can act as chelators of \(\text{Ca}^{2+}\) due to the formation of electrostatic interactions between CaBPs and \(\text{Ca}^{2+}\) ions. One of the most characterized structural domains that binds to \(\text{Ca}^{2+}\) is the EF hand motif, which gives the name to the family of EF-hand proteins. The EF-hand family includes parvalbumins (PV), calbindin-D9k (CB-D9k), calbindin-28k (CB-28k), and calretinin (CR). In response to an increase in \([\text{Ca}^{2+}]_c\), the buffering effect mediated by these proteins will depend on distinct parameters: their affinity for \(\text{Ca}^{2+}\) and to other ions (metal-binding affinities); their kinetics for \(\text{Ca}^{2+}\) binding and release (metal-binding kinetics); and their capacity to diffuse within the cytoplasm (mobility). Finally, their pattern of subcellular expression is also a key parameter that can vary significantly between different cell types. Some of the parameters are illustrated in table 5 to compare among calcium buffering proteins (Schwaller, 2020).

<table>
<thead>
<tr>
<th>Calcium Buffer Protein Parameters</th>
<th>Parvalbumin α (PV)</th>
<th>Parvalbumin β (PV)</th>
<th>Calbindin-D9k (CB-D9k)</th>
<th>Calbindin-28k (CB-28k)</th>
<th>Calretinin (CR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\text{Ca}^{2+})-binding sites (functional)</td>
<td>3 (2)</td>
<td>3 (2)</td>
<td>2 (2)</td>
<td>6 (4)</td>
<td>6 (5)</td>
</tr>
<tr>
<td>(\text{Ca}^{2+})-specific/ mixed (\text{Ca}^{2+}/\text{Mg}^{2+}) sites</td>
<td>0/2</td>
<td>1/1</td>
<td>2/0</td>
<td>4/0</td>
<td>5/0</td>
</tr>
<tr>
<td>(\text{KD},\text{Ca} \text{ (nM)})</td>
<td>4–9</td>
<td>Mixed: 42–45 (\text{Ca}^{2+})-specific: 590–780</td>
<td>KD1=200–500</td>
<td>high aff. (h) KD1≈ 180–240</td>
<td>KD(T) 28 (\mu\text{M})</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>KD2 = 60–300</td>
<td>medium aff. (m) KD2≈ 410–510</td>
<td>KD(R) 68</td>
</tr>
<tr>
<td>(\text{D}_{\text{Cabuffer}} \text{ (}\mu\text{m}^2\text{s}^{-1}))</td>
<td>12-47</td>
<td>NA</td>
<td>NA</td>
<td>(\approx 25; &gt;100)</td>
<td>(\approx 25)</td>
</tr>
</tbody>
</table>

Table 5. Properties of selected calcium binding proteins. Modified from (Schwaller, 2020).

Calcium signals are mostly initiated by an external stimulus such as neurotransmitter release. The amplitude, frequency, and duration of the signal generated is a result of the nature of the stimulus combined with specific activation of channels and receptors added to \(\text{Ca}^{2+}\) buffering protein actions. As a consequence of the complexity of \(\text{Ca}^{2+}\) homeostasis, different spatio-temporal \(\text{Ca}^{2+}\) signal patterns can occur in the same cell for a given stimuli. Moreover, some studies indicate that \(\text{Ca}^{2+}\) homeostasis is not a fixed parameter since it can be different for each type of neuron and even the same stimulus can produce radically different \(\text{Ca}^{2+}\) signals at different developmental stages (Mills, 1991).

Most of CBPs are calcium sensors. Calcium sensors are proteins that undergo conformational changes upon binding to \(\text{Ca}^{2+}\). Examples of calcium sensors are \(\text{Ca}^{2+}\) buffers mentioned above but also signaling proteins as calmodulin, calcineurin, and annexins, among others. The main role of \(\text{Ca}^{2+}\) sensors is to regulate \(\text{Ca}^{2+}\) concentrations as well as transducing \(\text{Ca}^{2+}\) signals to downstream signaling molecules, thus modulating activity of downstream signaling cascades. Within the brain there is a large number of \(\text{Ca}^{2+}\) sensors that are intermediates in the regulation
of several processes, including exocytosis, gene transcription, synaptic plasticity or dendritic remodeling (Bagur & Hajnóczky, 2017). Another signaling pathway activated by cytosolic Ca\(^{2+}\) rise through NMDAR is the extracellular signal-regulated kinase (ERK)/mitogen-activated protein (MAP) kinase cascade (see section 2.5 in chapter II). Calcium activates the neuronal-specific RAS guanine nucleotide-releasing factor (RAS-GRF1) (Fasano et al., 2009), which in turn activates ERK signaling cascade. Moreover, calcium inhibits a synaptic RAS GTPase-activating protein (RasGap), thus leading to disinhibition of ERK (Rumbaugh et al., 2006). There is a large number of other calcium binding proteins, such as troponin C and annexins, which are intermediate of specific signaling pathways with an important role in tissues outside the CNS (Greer & Greenberg, 2008).

3.3 Calcium-dependent signaling

3.3.1 GPCRs activation and intracellular signaling amplification

At the level of plasma membranes, activation of GPCRs coupled to G\(_{q}\) protein trigger the activation of phospholipase C (PLC). PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2) to diacyl glycerol (DAG) and IP3, while IP3 production and diffusion to ER triggers IP3R opening and Ca\(^{2+}\) release from intracellular stores (Bading, 2013; Prole & Taylor, 2019). DAG, phosphatidylserine (PS) and Ca\(^{2+}\) rise activate protein kinase C (PKC) (Islam, 2020). PKC in turn will phosphorylate and regulate a wide range of proteins playing a role in different signaling pathways including the dopamine transporter, GABA receptors, \(\mu\)-opioid receptors, and mGluR5 receptors, among many others. Moreover, PKC phosphorylates AMPAR GluA2 subunit, which is necessary for AMPAR internalization from the postsynaptic membrane (promoting LTD), and also phosphorylates AMPAR GluA1 subunit, which is implicated in the synaptic incorporation of AMPAR (promoting LTP) (Callender & Newton, 2017).

3.3.2 Calmodulin and CaMKs signaling

A central Ca\(^{2+}\) binding protein regulated by calcium is Calmodulin (CaM), a calcium sensor capable of modulate many intracellular signaling cascades. Calmodulin is a 17 kDa protein with no enzymatic activity, which contains two EF hand motifs with capacity to harbor four Ca\(^{2+}\) ions, forming the Ca\(^{2+}\)/CaM complex. CaM is able to adopt different conformations through its binding to a variety of target proteins in order to transduce calcium signal to distinct downstream enzymes. Key signaling cascades activated by CaM comprise members of the Ser/Thr kinases, CaM-kinase (CaMKs) family. Best studied members of the CaMK family and their activation mechanism are summarized in table 6. In general, they are activated by Ca\(^{2+}\)/CaM complex and induce gene transcription, although they differ both in their specific targets and physiological roles, ranging from apoptosis and vesicle mobilization to synaptic plasticity (Swulius & Waxham, 2008). The fine control of such diverse processes mediated by CaMKs is achieved through distinct and specific targets of each member of the CaMK family (See table 6).
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<table>
<thead>
<tr>
<th>Kinase</th>
<th>Type</th>
<th>Subunit Composition</th>
<th>Mechanism of Activation</th>
<th>Targets</th>
<th>Physiological Role</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaMKK</td>
<td>Multifunctional</td>
<td>Monomer</td>
<td>Ca2+/CaM</td>
<td>CaMKI, CaMKIV</td>
<td>Gene Transcription, Apoptosis</td>
</tr>
<tr>
<td>CaMKI</td>
<td>Multifunctional</td>
<td>Monomer</td>
<td>Ca2+/CaM and CaMKK</td>
<td>Synapsin 1, CREB</td>
<td>Gene Transcription, Vesicle Mobilization</td>
</tr>
<tr>
<td>CaMKII</td>
<td>Multifunctional</td>
<td>Dodecamer</td>
<td>Ca2+/CaM, Autophosphorylation</td>
<td>CaMKII, AMPA/NMDA receptors, L-, T-, P/Q-type Ca2+ channels</td>
<td>Synaptic Plasticity, Regulation of Ion Channels, Gene Transcription</td>
</tr>
<tr>
<td>CaMKIV</td>
<td>Multifunctional</td>
<td>Monomer</td>
<td>Ca2+/CaM, CaMKK, Autophosphorylation</td>
<td>CaMKIV, CREB, CBP, SRF, HDAC4, Oncoprotein 18</td>
<td>Gene Transcription</td>
</tr>
</tbody>
</table>

Table 6. General characteristic of selected CaMK members
Modified from (Swulius & Waxham, 2008)

One of the most studied protein from the CaMK family is CaMKII. There are four different isoforms of CaMKII (alpha, beta, gamma, delta) sharing the same substructure domain organization: N-terminal section that comprises the catalytic domain like any other CaM-kinase added to a C-terminal domain that is required for assembly of the CaMKII holoenzyme into a twelve-subunit complex (Fig. 23). When Ca2+ concentration rises, the barrel-shaped core assembly with six catalytic domains leaves the cytoskeletal fraction and enters the cytosol increasing substrate phosphorylation probability. The CaMKII hyper structure and kinase activity confers a long-lasting period of activation, which outlasts the initial Ca2+ elevation, called the autonomous state, requiring an auto-phosphorylation that is fundamental for certain forms of long-lasting synaptic plasticity, learning and memory (Lisman et al., 2012; Swulius & Waxham, 2008). Notably, CaMKII phosphorylates AMPA/NMDA receptors in GluR1 and GluN2B subunits, respectively, in addition to L-, T-, and P/Q-type VGCCs, thus impacting on downstream signaling cascades, such as the ERK pathway and gene transcription, to shape long-term neuronal adaptations (Lisman et al., 2012; Swulius & Waxham, 2008; Zhu et al., 2002).
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Figure 23. Illustration of CaMKII structure and regulation.
A) Side and top view of the holoenzyme tridimensional reconstruction from cryo-electron micrographs. B) Schema showing sequence of events leading to the activation and autophosphorylation of CaMKII. 1) initially inactive state 2) Ca\(^{2+}\)/CaM-induced activated state. 3) Autophosphorylation 4) Autonomous state (Ca\(^{2+}\)/CaM-independent). From (Swulius & Waxham, 2008).

Although CaMKII is mainly known as a cytosolic protein, some isoforms have been found in the nucleus of several types of cells including cultured hippocampal cells. Their kinase activity has been associated with methyl CpG binding protein 2 (MeCP2) phosphorylation, a methylated DNA-interacting protein. MeCP2 levels correlates with the expression of certain genes including the one encoding Brain-Derived Neurotrophic Factor (BDNF), which is a key modulator of synaptic plasticity (Buchthal et al., 2012; J. V. Deng et al., 2010).

Ca\(^{2+}\)/CaM complexes also activate CaMKK, a monomeric CaM kinase member present in α and β isoforms located at the cytoplasm, whereas the β isoform also can be found in the nucleus (Sakagami 2008). CaMKK primary targets are CaMKI (cytosolic) and CaMKIV (nuclear), which should be previously activated by Ca\(^{2+}\)/CaM complex. Other target proteins are protein kinase B and AMP-activated kinase (AMPK) (see fig. 24) (Swulius & Waxham, 2008; Wayman et al., 2008).
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Figure 24. Signaling mediated by CaMKs. The Ca2+/CaM complex activates CaMKII, CaM KK, CaMKI, and CaMKIV. CaM KK targets Ca MKI (cytosolic) and CaMKIV (nuclear) and secondly targets PKB/Akt and members of the AMP-kinase family, including SAD-B. cAMP/PKA inhibits CaM KK and CaMKI activates MEK/Erk. From (Wayman et al., 2008)

CaMKK activity is regulated by a negative feedback since CaM-dependent adenyl cyclases are activated at the same time increasing levels of cAMP, thus activating PKA which inhibits CaMKK activity (fig. 24) (Davare et al., 2004; Wayman et al., 1997). Moreover, there is a very important crosstalk between the CaMK cascade and the MEK/ERK pathway that appears to be crucial for NMDAR-dependent early-LTP and for activity-dependent dendritic arborization (Schmitt et al., 2005; Wayman et al., 2008). Stimulation of the NMDAR in cultured neurons or hippocampal slices strongly activates ERK signaling pathway. Using specific pharmacological inhibitors and dominant negative forms of CaMKK or CaMKI prevents this activation (Schmitt et al., 2005). Exactly how CaMKK/CaMKI activate MEK/ERK pathway is not known yet (Wayman et al., 2008).

3.4 Nuclear and cytosolic calcium signals and implication in distinct subsequent signaling

Ca2+ signals generated at the membrane, for example through opening of NMDAR and VGCCs, generate Ca2+ rise within the nucleus via different mechanisms. Studies, conducted mainly by Dr. Hilmar Bading’s group, have shown that Ca2+ itself allows synapse-to-nucleus communication. In fact, increases in synaptic activity at excitatory synapses are able to trigger nuclear calcium increases. Importantly, these nuclear calcium transients have been shown to play a major role in transcription-dependent long-term neuronal plasticity, as reviewed in the next sections (Bading,
2013). One of the first study showing that nuclear Ca\textsuperscript{2+} signals were necessary to induce gene expression was performed using a calcium chelator injected directly in the nucleus of AtT20 cells, a pituitary cell line. The Ca\textsuperscript{2+} chelator BAPTA was coupled to a large macromolecule, dextrane (70 kDa), to prevent diffusion outside the nucleus and decrease free Ca\textsuperscript{2+} availability in this compartment without altering cytosolic Ca\textsuperscript{2+} signals. In response to membrane depolarization, the nuclear calcium chelator was able to block the induction of genes bearing a Ca\textsuperscript{2+} and cAMP-responsive element (CRE) on their promoter. By contrast, this manipulation did not impact the transcription of genes displaying a regulatory serum response element (SRE) in their promoter region (Hardingham et al., 1997). Interestingly, it has been shown on hippocampal neurons that cytosolic and nuclear Ca\textsuperscript{2+} transients induce transcription through different molecular mechanisms. On one hand, SRE regulatory sites of transcription function as cytoplasmic Ca\textsuperscript{2+} effector element activated downstream the ERK/Elk-1 signaling pathway. On the other hand, nuclear Ca\textsuperscript{2+} promotes transcription through the activation of CRE sites, which require activation of CREB transcription factor on its Ser 133 by CaMKIV (Bading, 2013; Hardingham et al., 2001).

As already mentioned the spatial and temporal dynamics of Ca\textsuperscript{2+} signaling from the cytoplasm to the nucleus is tightly controlled by synaptic activity. The nucleus is not a barrier to Ca\textsuperscript{2+} which can freely diffuse, through the nuclear pore complex, from the soma towards the nucleus. Two non-mutually exclusive mechanisms have been proposed to yield nuclear Ca\textsuperscript{2+} transients. One mechanism includes synaptic activation of AMPAR and NMDAR leading to a local Ca\textsuperscript{2+} increase, which will generate in some cases an excitatory post-synaptic potential (EPSP) with the main consequence of VGCCs opening and Ca\textsuperscript{2+} entry along the axon finally reaching the nucleus (illustrated in figure 25.a) (Hagenston & Bading, 2011). The other mechanism involves ER receptors. In response to synaptic G\textsubscript{o}-coupled GPCR activation producing IP3 activating IP3 and RyR receptors releasing the main intracellular Ca\textsuperscript{2+} store at the proximity of the nucleus, as showed in figure 25.b (Bading, 2013).

The dialogue between synapses and cell nucleus is key for long-lasting neuronal adaptations. This synapse-to-nucleus communication can thus be achieved by synaptic activation of signaling pathways, including ERK, which will translocate to the nucleus upon activation or by Ca\textsuperscript{2+} ions themselves. It is important to mention that not all cytosolic calcium transients lead to nuclear calcium increases, but independent and autonomous nuclear calcium transients have not been identified yet (Bading, 2013).
Figure 25. Schematic representation of two main pathways underlying nuclear calcium transient following synaptic inputs. A) Intracellular Ca\(^{2+}\) increase is triggered by action potential initiation through NMDA/AMPA receptors and subsequently propagated towards the nucleus by VGCCs. B) Another pathway involves GPCR stimulation which activates PLC generating IP3, stimulating IP3R in the ER inducing release of intracellular Ca\(^{2+}\) stores from the ER. From (Bading, 2013).

3.5 Nuclear calcium sensors

Genetically Encoded Calcium Indicators (GECIs) are key tools to monitor Ca\(^{2+}\) dynamics, especially in a cell-type-specific manner. The most used GECIs are single-wavelength green indicators based on a structurally modified GFP (Green Fluorescent Protein) linked to both a M13 peptide and a calmodulin (GCaMP sensors). M13 peptide, a sequence from the light chain of myosin, has a high affinity for calmodulin when bound to Ca\(^{2+}\) (four Ca\(^{2+}\) ions). When calcium concentration rises in the cell, a conformational change occurs within the sensor inducing an increase in fluorescence emission. Many modifications has been made in order to improve dynamic properties of GCaMP sensors, in addition to include localization sequences to target specific compartments within the cell, such as the nucleus (Akerboom et al., 2013).

Hilmar Bading and colleagues used a GCaMP sensor coupled to a nuclear localization signal (NLS) to perform Ca\(^{2+}\) imaging specifically on the nucleus. They showed that nuclear Ca\(^{2+}\) transients in CA1 pyramidal neurons correlate with the number of spikes evoked by high frequency or theta burst stimulations after activating the Schaffer collaterals (Fig. 26. A). They also observed that inhibiting NMDARs or L-Type VGCCs decreased nuclear Ca\(^{2+}\) signals and action potentials, which was not the case when blocking internal Ca\(^{2+}\) store release or glutamate
metabotropic receptors (Bengtson et al., 2010). Similar experiments were made in mouse spinal cord in a preclinical model of chronic pain and in mushroom bodies of *drosophila melanogaster* during olfactory associative learning (fig 26. B and C, respectively) (Simonetti et al., 2013; Weislogel et al., 2013).

![Diagram of different models](image)

**Figure 26.** Detection of nuclear calcium signals in different models. A) neuronal plasticity in mouse hippocampus; B) chronic pain in mouse spinal cord; and C) olfactory associative learning in *drosophila melanogaster* mushroom bodies. From (Bading, 2013)

### 3.6 Nuclear calcium-dependent signaling

Upon nuclear Ca\(^{2+}\) rise, Ca\(^{2+}/CaM\) complex activates the nuclear serine/threonine kinase CaMKIV (see table 6). CaMKIV phosphorylates the transcription factor CREB on its serine 133, making possible the interaction between CREB and CREB-binding protein (CBP) to promote CRE-dependent transcription. The cAMP/PKA pathway is also sufficient to induce CREB and CBP-dependent transcription. In addition to CaMKIV and PKA, CREB is also indirectly activated downstream from the ERK cascade, via the activation of the CREB kinase ribosomal protein S6 kinase (RSK2) (De Cesare et al., 1998), and Mitogen and Stress-activated Kinases (MSK1-MSK2) in response to growth factor (Wiggin et al., 2002).

Both CREB and CBP need to be activated and interact to promote CRE-driven gene transcription, and it has been shown that CBP is also activated by nuclear Ca\(^{2+}\) transients and CaMKIV (Fig. 25). CBP interacts with multiple transcription factors, modulating the expression of a large number
of genes, especially immediate early genes (Chawla et al., 1998; Goldman et al., 1997; Hardingham et al., 1999). Moreover, the transcription factors CREB and c-Jun are activated by Ca\(^{2+}\) signals in a CBP-dependent manner (Cruzalegui et al., 1999). CBP possesses a histone acetyltransferase activity, and increases histone acetylation that is necessary for chromatin remodeling and DNA unpacking. As such, it exposes target regions for polymerase II to transcribe. Changes in chromatin organization also involve a family of histone deacetylases (HDACs), which promote DNA compacting. Interestingly, nuclear Ca\(^{2+}\)-dependent mechanisms are responsible for the nuclear exportation of HDAC4 and HDAC5, two class II HDACs, favoring histone acetylation and thus, gene transcription (Fig 27) (Chawla et al., 2003; Malvaez et al., 2011).

Another chromatin remodeling process controlled by nuclear Ca\(^{2+}\), which is especially relevant for transcription, is DNA methylation. It has been associated mostly with gene silencing but some recent evidence showed that DNA methylation can promote gene activation as well. Oliveira and collaborators showed that the expression of a DNA methyltransferase, Dnmt3a2, is partly controlled by nuclear Ca\(^{2+}\) signaling (Oliveira et al., 2012). Dnmt3a2 is found in actively transcribed regions (euchromatin) and its activity is necessary to reverse memory impairments in aging (Fig. 27) (Oliveira et al., 2012).

A distinct transcriptional-related event modulated by nuclear Ca\(^{2+}\) involves the downstream transcription regulatory element (DRE) antagonist modulator (DREAM) and the forkhead transcription factor FOXO3A. DREAM is a transcription repressor that contains several EF hand
motifs allowing its direct binding to Ca\(^{2+}\) ions. At low Ca\(^{2+}\) levels, DREAM is bound to DNA at certain regions containing DRE element. When nuclear Ca\(^{2+}\) rises, Ca\(^{2+}\) directly binds to DREAM, which promotes the dissociation of this transcription repressor from DNA, therefore promoting transcription (Carrión et al., 1999; Mellström & Naranjo, 2001). Finally, nuclear Ca\(^{2+}\) has been also linked to neuroprotection and cell survival through a mechanism involving a CaMKIV-dependent nuclear export of the transcription factor FOXO3A preventing pro-apoptotic mediated transcription (Fig 27) (Dick & Bading, 2010).

3.7 Nuclear calcium-dependent forms of long-term adaptations

Nuclear Ca\(^{2+}\)-dependent transcription has been shown to play a key role in diverse models of long-lasting neuronal adaptations, including in long-term synaptic plasticity, neuroprotection, memory and chronic pain. Acquired neuroprotection is defined by an increased ability of neurons to survive in noxious environmental conditions. In several studies, it has been shown that synaptic NMDAR-dependent Ca\(^{2+}\) influx triggers a genetic program favoring neuronal survival (Hardingham & Bading, 2003; S.-J. Zhang et al., 2007), whereas pharmacological blockade of synaptic NMDAR with MK-801 triggers apoptotic neurodegeneration in many brain regions (Ikonomidou et al., 1999). Interestingly, extra-synaptic NMDAR activation triggers a different signaling cascade inducing the rapid nuclear importation of FOXO, among other modulations, promoting apoptotic cell death (Hagenston & Bading, 2011). Zhang and collaborators used a model of neurotoxicity induced by bicuculline, a GABA-A receptor antagonist, combined with 4-amino pyridine, a blocker of voltage-activated K\(^{+}\) channel family and an enhancer of VGCC currents, on hippocampal neurons expressing a nuclear Ca\(^{2+}\)-dependent signaling blocker. They used the calmodulin binding-peptide 4 (CaMBP4) to block nuclear Ca\(^{2+}\)-dependent signaling in order to identify genes regulated by nuclear Ca\(^{2+}\) involved in acquired neuroprotection. CaMBP4 is a synthetic protein that contains four copies of the M13 peptide in addition to a nuclear localization signal. Thus, CaMBP4 binds to and inactivates Ca\(^{2+}\)/CaM complexes within the nucleus. The over expression of this protein induces a competitive blockade of nuclear Ca\(^{2+}\)/CaMKIV-dependent signaling. In cultured hippocampal neurons, CaMBP4 revealed a core set of neuroprotective genes highly dependent on nuclear Ca\(^{2+}\) signaling. These genes are named activity-regulated Inhibitor of Death (AID) and include: Atf3, Btg2, GADD45b, GADD45c, Inhibin b-A, Interferon activated gene 202B, Npas4, Nr4a1, and Serpinb2. AIDs represent a shield against cell death and excitotoxic damage. Some of these nuclear Ca\(^{2+}\)-dependent AID genes are known to promote mitochondrial resistance to cellular stress and toxic particles (S.-J. Zhang et al., 2009).

To dissect the role of nuclear Ca\(^{2+}\) signaling in memory formation, Limbäck-Stokin and collaborators generated a transgenic mouse expressing CaMBP4 in the nuclei of forebrain neurons. These mice showed decreased CREB phosphorylation in response to neuronal activity increases, as well as reduced expression of c-Fos, an IEG. These mice also displayed severe impairment of long-term, but not short-term memory, therefore revealing a role of nuclear Ca\(^{2+}\) in memory consolidation (Limbäck-Stokin et al., 2004). Another study, using a transgenic animal expressing a dominant-negative form of CaMKIV in the forebrain, also showed decreased CREB phosphorylation and c-Fos expression as well as an impairment in memory consolidation (Kang et al., 2001). Among the group of genes modulated by nuclear Ca\(^{2+}\) signaling, the one encoded by vascular endothelial growth factor D (VEGFD) appears to be essential for memory consolidation in rodents. Downstream from nuclear calcium, CaMKIV activity induces the
expression of VEGFD, which controls the length and complexity of dendritic arborization in hippocampal neurons, both in vitro and in vivo. Decreases in VEGFD protein levels using interference RNA generated morphological and behavioral changes, reducing density and size of dendritic spines and causing impairments in long-term memory formation (Mauceri et al., 2011). Another form of neuronal plasticity, in which the implication of nuclear Ca\(^{2+}\)-signaling has been studied, is the maladaptive response to persistent nociceptive stimulus generating hypersensitivity known as chronic pain. It was indeed known that the transition from acute to the pathological chronic pain relies on the transcription of a specific set of genes. Simonetti and colleagues observed that nociceptive-like stimulus evoked nuclear Ca\(^{2+}\) transients in the mouse dorsal horn neurons associated to an increased phosphorylation of both CREB (Serine 133) and MECP2 (serine 421). To study the contribution of nuclear Ca\(^{2+}\)-dependent signaling, Simonetti and collaborators used an AAV virus expressing CaMBP4 protein to block Ca\(^{2+}\)/CaM activity in the nucleus of superficial spinal horn neurons and evaluated the effects in a model of chronic pain. This nuclear Ca\(^{2+}\) blockade was able to prevent the transition from acute to long-term nociceptive sensitization in a mouse model of chronic inflammatory pain. In addition, they performed a microarray-based transcriptome analysis and found that the gene PTGS2 (coding for Cyclooxygenase 2) was key for the development of long-lasting nociceptive hypersensitivity downstream from nuclear Ca\(^{2+}\). A second target gene found in this study was C1QC coding for C1q, a negative modulator of synaptic spines formation, which restoration after silencing, can alleviate morphological (mal)adaptations induced in a chronic pain model (Simonetti et al., 2013).

3.8 Calcium signaling alterations induced by psychostimulants in the striatum.

As reviewed in chapter II, the main neuronal population in the striatum are the MSNs, which are segregated in two subpopulations: the MSNs expressing dopamine receptor type 1 and the type 2. In addition to modulating cAMP levels, stimulation of DARs can modulate calcium fluxes, in particular by modulating calcium fluxes by NMDARs. Accordingly, in response to a single injection of cocaine it has been shown that cocaine triggers a D1R-dependent increase of intracellular Ca\(^{2+}\) concentration in D1R-MSNs while the stimulation of D2R induces a decrease of intracellular Ca\(^{2+}\) levels in D2R-MSNs (see figure 30) (Luo et al., 2011). We previously discussed how the crosstalk between DRs and NMDARs can alter Ca\(^{2+}\) signals (chapter II, section 2.4), however less is known concerning the molecular mechanisms by which DAR stimulation can alter nuclear Ca\(^{2+}\) signaling in D1R-MSN and D2R-MSN in response to drugs of abuse.

3.8.1 Calcium-dependent signaling in striatal neurons evoked by drugs of abuse

Several studies have shown that distinct events controlled by synaptic activity that induce nuclear Ca\(^{2+}\)-dependent transcription, have been causally linked to addictive behaviors (Bading, 2013). Psychostimulants induce MeCP2 phosphorylation at Ser421, which controls the activity-dependent induction of BDNF (J. V. Deng et al., 2010). Some studies showed that BDNF affects cocaine-induced behaviors. BDNF infusion in the NAc increases sensitivity to the psychomotor stimulant effects of cocaine (Horger et al., 1999), and produce a long-lasting augmentation in cocaine self-administration (Graham et al., 2007). Oppositely, disruption of the BDNF gene in the NAc reduced rewarding effects of cocaine assessed with the CPP paradigm (Graham et al., 2009). CREB activity is also induced by drugs of abuse (Carlezon et al., 2005) and enhanced CREB function decreases rewarding effects of psychostimulants and food. Conversely, disruption of CREB activity specifically in NAcsh in mice was associated with antidepressant-like effects and
increased reward, evaluated with force swimming test and CPP, respectively (Barrot et al., 2002; Dinieri et al., 2009). In an interesting study, Bilbao and collaborators generated mice with CAMK4 gene ablation specifically in D1R-expressing neurons (Camk4^{D1Cre} mice). Loss of CaMKIV was observed in much of the striatum, cortex and other brain areas. They performed microarray analysis to evaluate differences in gene expression (IEGs especially) and found that animals lacking CaMKIV exposed to an acute cocaine injection had mild effects in gene transcription in both, saline and cocaine condition. However, after chronic cocaine treatment, they observed significantly higher levels of FosB expression in Camk4^{D1Cre} animals while Fos levels remained unchanged. These mice were tested under the CPP paradigm and Camk4^{D1Cre} animals displayed an augmented preference as well as augmented reinstatement after extinction in response to cocaine. These findings provide evidence of a central role for CaMKIV in the development and persistence of cocaine-induced adaptations (Bilbao et al., 2008).

3.8.2 Cell-type-specific striatal calcium signals in response to rewarding stimuli

As mentioned above, a pioneer study aiming to measure intracellular Ca^{2+} transients in each MSN population of mice dorsal striatum in response to a single cocaine injection was performed by Luo and collaborators in 2011. They performed Ca^{2+} imaging using rhodamine in BAC D1R-eGFP and D2R-eGFP animals to identify each population. Results showed that a fast increase of intracellular Ca^{2+} concentration in D1R-expressing neurons occurred after and acute cocaine dose (8 mg/Kg). By contrast, a progressive decrease of intracellular calcium was observed in D2R-MSNs illustrated in figure 28 (Luo et al., 2011). A pretreatment with a D1R antagonist (SCH23390) prior to an acute cocaine dose (8mg/kg) blocked the rapid increase of Ca^{2+} in D1R-MSNs, while blocking the slow decrease of Ca^{2+} concentration in D2R-MSNs. On the other hand, the use of a D2R antagonist, raclopride, completely blocked slow decrease in [Ca^{2+}] in D2R-MSN, while [Ca^{2+}] rise in D1R-MSNs was only partially blocked. Together, these results give some hints of crosstalk between the two populations (Luo et al., 2011).

![Figure 28. Ca^{2+} dynamic in a D1R-MSN. (a, blue) and a D2R-MSN (b, red). Fitting lines where applied to compare Ca^{2+} dynamics comparing slopes between the first 10 min with the rest of the measurement. A positive or negative slope k implies an increase or decrease in [Ca^{2+}], respectively. From (Luo et al., 2011).]
In a recent work, using fiber photometry Ca\(^{2+}\) imaging in the NAc in combination with cocaine-induced conditioned place preference, changes of neuronal activity of D1R- and D2R-MSNs have been studied during formation of reward-context associations in freely-moving animals (Calipari et al., 2016). D2R-MSNs presented more than five-fold higher transient frequency compared to D1R-MSNs in basal conditions. During pairing in CPP, cocaine increased frequency of calcium transients in D1R-MSNs and lowered it in D2R-MSNs. In the course of the test day in the CPP paradigm they measured robust Ca\(^{2+}\) spikes in D1R-MSNs just before the entry of the mice in the drug-paired chamber. By contrast, Ca\(^{2+}\) levels were decreased in D2R-expressing neurons once the mice had entered in the paired chamber. These results therefore highlight that both, cocaine itself and cocaine-associated cues, are able to modulate Ca\(^{2+}\) signals in a cell-type-specific manner in D1R- and D2R-MSN (Calipari et al., 2016). Furthermore, the selectively blockade of \([\text{Ca}^{2+}]\) increases in NAc D1R-MSN using a cell-type-specific inhibitory DREADD receptor, blocked both cocaine-induced CPP and Ca\(^{2+}\) spike observed before mice entry to drug-paired chamber. Moreover, when animals were tested again for preference 2 weeks later with a functional D1R, preferences scores remained poor, therefore indicating that D1R-MSN inhibition at the context association stage is sufficient to abolish the association between the drug and the context indefinitely (Calipari et al., 2016).

Cui and collaborators used the GECI sensor GCaMP3 expressed either in D1R-MSNs or D2R-MSNs of the dorsal striatum of mice. By using optic fibers and time-correlated single-photon counting (TCSPC) in mice performing an operant task to obtain a natural reward (food), they observed Ca\(^{2+}\) transients in both populations when the animals initiated the action to obtain the reward, while they did not observe Ca\(^{2+}\) signals when they were inactive. They also showed that MSNs from right hemisphere anticipated the initiation of left-directed movements and vice versa, as well as predicted the occurrence of specific movements within 500 ms (Cui et al., 2013). These observations challenge the classical view of D1R-MSNs and D2R-MSNs having opposite functions in motor coordination and reward processing. A later study by Barbera et al., showed that an acute cocaine exposure does not change global neuronal activity in either type of MSN in dorsal striatum. However, they detected an increased activity in discrete clusters of D1R-MSNs, specifically in DLS, which were correlated with cocaine-induced hyperlocomotion (Barbera et al., 2016).

As reviewed above, nuclear calcium has been shown to be important for several complex processes involving gene transcription including, long-term plasticity, acquired neuroprotection, memory consolidation and the development of chronic pain. Addiction is considered as a pathological form of neuronal plasticity that requires structural and functional changes mediated by gene transcription in different brain structures. Nevertheless, a role for nuclear calcium in addiction has not been determined yet. Since, D1R- and D2R-MSNs calcium signaling appears to be involved in drug-induced adaptations as reviewed in chapter II, I studied the dynamics of nuclear calcium in the striatum and its role in cellular, molecular and behavioral adaptations evoked by cocaine (article II).
**Results: Article I**

**Introduction Article I**

The convergence of the dopaminergic and glutamatergic systems within the reward circuit, especially within the striatum, plays a central role in the development of persistent alterations induced by psychostimulants. By increasing DA concentration, addictive substances durably modulate glutamate transmission in the striatum, especially in the nucleus accumbens (NAc) which leads to a vast repertoire of molecular, morphological, synaptic and behavioral adaptations. Previous work from the laboratory established that the synergy between D1R and NMDAR-dependent signaling mediates the activation of the ERK pathway and plays a central role in long-lasting adaptations induced by cocaine (Pascoli et al., 2011). More recently, the team also showed that the physical interaction between D1R and the GluN1 subunit of NMDAR was necessary for the facilitation of NMDAR functions by DA in D1R-MSN, and for cocaine-induced ERK activation in the striatum *in vivo* (Cahill, Pascoli, et al., 2014). Conversely, another team showed that the binding of D2R to GluN2B subunits of NMDAR was responsible for the inhibition of NMDAR by DA in D2R-MSN (X.-Y. Liu et al., 2006). A currently prevailing hypothesis is that drugs of abuse exert their potent reinforcing effects by modulating the activity of D1R-MSN and D2R-MSN in opposite ways. In this context, it is critical to identify the molecular mechanisms by which drugs of abuse alter the integration of dopamine and glutamate signals by D1R-MSN and D2R-MSN to induce long-term behavioral alterations. Characterization of such molecular events could indeed contribute to the identification of potential targets for the development of innovative therapeutic strategies. Accordingly, the objective of the present work was to test whether D1R-GluN1 and D2R-GluN2B heteromers, by activating and inhibiting the activity of D1R-MSN and D2R-MSN, respectively, could play a role in cocaine-induced responses.

First, we studied the effects of cocaine on D1R-GluN1 and D2R-GluN2B heteromerization in the mice striatum *in vivo*. We found that cocaine increased D1R-GluN1 and D2R-GluN2B heteromerization, with a more pronounced effect in the NAc core and shell sub-divisions. We then explored the role of these heteromers in the different phases of long-term responses induced by cocaine. We developed an approach to block heteromer formation in a temporally-controlled manner, using a virus expressing an interfering peptide (specific for each interaction). We found that disrupting D1R interaction with GluN1 preferentially blocks the development of the sensitizing and rewarding effects of cocaine, while preventing D2R-GluN2B interaction blocks the maintenance of cocaine-induced sensitizing and rewarding effects. Interestingly, disrupting either heteromer subtype, spared food reward processing. Finally, we established such heteromers exist in human post-mortem caudate putamen samples. Strikingly, we found that D2R-GluN2B complexes are detectable in human samples and showed that, despite a decreased D2R protein expression in the NAc, individuals with psychostimulant misuse display a higher proportion of D2R forming heteromers with NMDAR. These findings contribute to a better understanding of molecular mechanisms underlying addiction and uncover D2R-GluN2B heteromers as targets with potential therapeutic value.
Article I

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Full title:
Disrupting D2-NMDA receptor heteromerization blocks cocaine’s rewarding effects but preserves natural reward processing

Short title: Dopamine-glutamate receptor heteromers in addiction

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Abstract

Addictive drugs increase dopamine in the nucleus accumbens (NAc), where it persistently shapes excitatory glutamate transmission and hijacks natural reward processing. Herein, we provide evidence, from mice to human, that an underlying mechanism relies on drug-evoked heteromerization of glutamate NMDA receptors (NMDAR) with dopamine receptor 1 (D1R) or 2 (D2R). Using temporally-controlled inhibition of D1R-NMDAR heteromerization, we unraveled their selective implication in early developmental phases of cocaine-mediated synaptic, morphological and behavioral responses. In contrast, preventing D2R-NMDAR heteromerization blocked the persistence of these adaptations. Importantly, interfering with these heteromers spared natural reward processing. Strikingly, we established that D2R-NMDAR complexes exist in human samples and showed that, despite a decreased D2R protein expression in the NAc, psychostimulant-addicts display a higher proportion of D2R forming heteromers with NMDAR. These findings contribute to a better understanding of molecular mechanisms underlying addiction and uncover D2R-NMDAR heteromers as targets with potential therapeutic value.

Teaser

From mice to humans: evidence that D2-NMDAR receptor heteromers are potential therapeutic targets in drug addiction.
Introduction

Drug addiction is characterized by compulsive patterns of drug-seeking and drug-taking behavior in spite of detrimental consequences and a high rate of relapse after withdrawal. A hallmark of addictive drugs is their ability to increase dopamine concentration in discrete brain regions, which persistently shapes excitatory glutamate transmission within the reward circuit, thereby hijacking natural reward processing (1, 2). This calls for a better understanding of the precise molecular events underlying the detrimental interplay between dopamine and glutamate signaling triggered by drugs of abuse.

The enduring behavioral alterations induced by protracted drug exposure are largely believed to result from persistent drug-evoked neuronal adaptations within the striatum, especially in its ventral part, the nucleus accumbens (NAc) (1, 3). The striatum is indeed a key target structure of drugs of abuse that integrates convergent glutamate inputs from limbic, thalamic and cortical regions, encoding components of drug-associated stimuli and environment, and dopamine signals that mediate reward prediction error and incentive values (4). Integration of dopamine and glutamate signals is achieved by the two segregated subpopulations of GABAergic medium-sized spiny neurons (MSN) expressing either the dopamine receptor (DAR) type 1 (D1R) or type 2 (D2R), although a fraction of MSN in the NAc expresses both receptors (5). Cell-type-specific manipulations of neuronal activity showed that inhibiting and activating D1R-MSN respectively dampens and potentiates long-term drug-evoked responses, in line with their “pro-reward” action (2, 6–9). By contrast, the majority of studies supports an inhibitory role of D2R-MSN activation on drug-mediated adaptations (6, 7, 10–12). These studies, based on direct manipulations of MSN activity, were extremely instrumental to highlight the role of MSNs as putative players in drug-related behavioral adaptations. However, they do not establish how drugs of abuse persistently impact the functionality of each MSN subpopulation or the underlying cellular and molecular mechanisms. In this context, increasing evidence suggests that such a central role of MSN subpopulations in drug-induced behavioral adaptations originates, at least in part, from dopamine-dependent long-lasting changes at excitatory striatal synapses. Indeed, long-term potentiation of specific glutamatergic afferences impinging onto D1R-MSN induced by dopamine is responsible for both the induction and maintenance of long-lasting behavioral adaptations to repeated cocaine exposure (13–15). Interestingly, glutamate transmission onto D2R-MSN seems to be spared by cocaine exposure, but selectively altered during cocaine craving after long access to high doses of cocaine (16).

It is therefore timely to identify molecular mechanisms by which drug-evoked increases in dopamine can permanently hijack glutamate transmission onto MSN. Although a number of studies have described the crosstalk between D1R and glutamate receptor of the NMDA (NMDAR) subtype as a key player in the behavioral effects of psychostimulants (2, 17–21, 21, 22), the underlying molecular mechanisms remain elusive. Moreover, the processes by which dopamine impairs D2R-MSN activity to promote long-lasting drug-induced reinforcement is yet unknown.

Heteromeric complexes formed between dopamine receptors (DAR) and glutamate NMDAR have been proposed as integrators of dopamine and glutamate signals in both MSN populations (23). Receptor heteromers are of particular interest, not only because of their ability to dynamically modulate the component receptor’s functions in time and space, but also because they exhibit
functional properties distinct from the component receptors, making them attractive targets for the development of more selective pharmacological strategies (24–27). Most evidence generated to date regarding dopamine-NMDA receptor heteromer functions come from in vitro and ex vivo studies and their potential role in long-term drug-induced adaptations has been overlooked. D1R form heteromers with GluN1 subunits of NMDAR in vitro (28, 29) and in vivo in the striatum (30, 31) and have been shown to allow the facilitation of NMDAR signaling by dopamine in D1R-MSN ex vitro on striatal slices (31). By contrast, the binding of D2R to GluN2B subunits of NMDAR mediates the inhibition of NMDA currents by dopamine in D2R-MSN and controls acute stereotypic locomotor responses to high cocaine doses (32). DAR-NMDAR heteromers therefore appear as putative molecular platforms that mediate crosstalk between dopamine and glutamate transmission onto MSNs. However, whether such receptor heteromers might constitute molecular substrates by which drugs of abuse enduringly alter glutamate transmission and trigger long-lasting behavioral alterations has not been studied.

We therefore investigated D1R-GluN1 and D2R-GluN2B heteromerization in the striatum in vivo in response to repeated cocaine exposure. We found that cocaine triggers a transient increase of D1R-GluN1 heteromerization in the entire striatum, which returns to baseline level upon withdrawal from the drug. By contrast, cocaine induces a stable heteromerization of D2R-Glu2NB that is mostly restricted to the NAc and persists over a withdrawal period. Using a temporally-controlled disruption these receptor heteromers, combined with electrophysiological recordings, imaging and behavioral assessments, we showed that D1R-GluN1 heteromerization controls the development of cocaine-evoked long-term synaptic plasticity and morphological changes in D1R-MSNs, as well as behavioral adaptations. In contrast, D2R-GluN2B heteromerization mediates the persistence of these adaptations after a withdrawal period followed by a re-exposure to the drug. Importantly, the targeting of either type of heteromers preserves natural reward processing. Strikingly, we found that such receptor complexes also exist in human post-mortem brain samples and showed that, despite a substantial decrease of D2R protein expression, psychostimulant-addicts display a significantly higher proportion of D2R that form heteromers with GluN2B in the NAc. Our results support a model by which the heteromerization of dopamine and glutamate receptors induced by drugs of abuse in D1R- and D2R-MSNs is a key endogenous molecular event underlying the detrimental interplay between these two neurotransmitter systems in drug addiction. The role of D2R-GluN2B heteromers in the persistence of the sensitizing and rewarding effects of drugs makes them potential targets not only for addiction in humans, but also more broadly in multiple neuropsychiatric disorders.
Results

Behavioral sensitization to cocaine is associated with transient D1R-NMDAR heteromerization and prolonged D2R-GluN2B heteromerization in the NAc

Before studying the role of DAR-NMDAR heteromers in cocaine-evoked adaptations, we investigated whether a cocaine regimen that triggers persistent behavioral adaptations can modulate the formation of these receptor complexes in vivo in the striatum. Mice were subjected to five daily injections of cocaine (15 mg/kg), which elicits a progressive locomotor sensitization (Fig. 1A) that is known to persist for several weeks after cocaine withdrawal. This behavioral paradigm is a straightforward model to study the mechanisms involved in drug-induced behavioral adaptations (2, 33). Mice were sacrificed one day after the last injection to detect endogenous DAR-NMDAR proximity in distinct striatal sub-regions through Proximity Ligation Assay (PLA) (34). The brightfield PLA assay yielded a brown punctate signal for D1R-GluN1 and D2R-GluN2B complexes that was absent when one of the two primary antibodies was omitted (Fig. 1B), similar to what was previously found when PLA was performed in DA receptor KO mice (31, 34, 35). Using this approach, we found that cocaine-treated mice displayed increased D1R-GluN1 heteromerization in the dorso-lateral (DL Str) and dorso-medial (DM Str) striatum, as well as in the nucleus accumbens core (NAc core) and shell (NAc shell) sub-divisions (Fig. 1C). Cocaine also increased D2R-GluN2B heteromerization, but primarily in the NAc, with a smaller effect in the DL Str (Fig. 1D). This increased heteromerization occurred in the absence of changes in global expression levels of the component receptors (Fig. S1A,B). Of note, and as previously observed (36), repeated cocaine exposure decreases expression levels of the synaptic scaffold protein PSD-95 (Fig. S1C), which could partly explain our results as the interaction of NMDAR and D1R with PSD-95, through partly overlapping domains, has been described to prevent D1R-GluN1 interaction (37).

To study the kinetics of receptor heteromerization, mice were subjected to a cocaine-induced locomotor sensitization paradigm followed by one-week withdrawal and a challenge injection of saline or cocaine. We found that cocaine-mediated D1R-GluN1 heteromerization returned to baseline levels upon withdrawal but increased again, in all striatal sub-regions except in the DL Str, after the challenge injection of cocaine (Fig. 1E). Strikingly, D2R-GluN2B heteromerization appeared to be sustained over the withdrawal period specifically in the NAc core. The challenge injection of cocaine further increased D2R-Glu2NB heteromers in the NAc, but not in dorsal parts of the striatum (Fig. 1E). To assess the role of D1R or D2R stimulation for receptor heteromerization, mice underwent a cocaine-locomotor sensitization followed by a withdrawal. Before a cocaine challenge, mice received an intraperitoneal injection of D1R or D2R antagonist that blunted the expression of behavioral sensitization. This allowed us to show that the stimulation of D1R or D2R was mandatory for cocaine-induced D1R-GluN1 and D2R-GluN2B heteromerization, respectively (Fig. 1F). Altogether, these data show that behavioral sensitization to cocaine is associated with a dopamine receptor-dependent transient heteromerization of D1R-GluN1 in the whole striatum, whereas D2R-GluN2B heteromerization occurs primarily in the NAc and is maintained during cocaine withdrawal in the core subdivision.
Cocaine-evoked potentiation of glutamate transmission onto D1R-MSNs requires D1R-GluN1 heteromerization.

To study the function of the D1R-GluN1 heteromers in cocaine-induced adaptations, we designed an adeno-associated virus (AAV)-based strategy to disrupt heteromers in a spatially- and temporally-controlled manner (Fig. 2A). The AAV Tet-On-GluN1C1 allows a doxycycline (dox)-inducible bicistronic expression of the RFP reporter protein together with a peptide corresponding to the C1 cassette (D864-T900) of GluN1 that binds to D1R (28). This peptide blocks D1R-GluN1 interaction in vitro, while preserving the functions of individual D1R and NMDAR independently of their heteromerization (31). The control virus, Tet-On-GluN1C1Δ, encodes a C1 cassette deleted of 9 amino acids that are required for electrostatic interactions between D1R and GluN1 (38). This mutated cassette does not interfere with D1R-GluN1 interaction in vitro (31). After stereotaxic injections in the NAc, the treatment with dox triggered a rapid and sustained expression of RFP (Fig. 2A). In naive mice, analysis of D1R-GluN1 proximity in RFP-positive neurons showed that Tet-On-GluN1C1 significantly reduced D1R-GluN1 PLA puncta when compared to the control AAV (Fig. 2B,C), indicating an efficient disruption of these heteromers in vivo. We also verified that blocking D1R-GluN1 heteromerization altered downstream cocaine-mediated signaling events (20, 31), including GluN2B phosphorylation and extracellular-signal regulated kinase (ERK) pathway activation (Fig. S2), without compromising neuronal survival (Fig. 2D).

Long-lasting changes of glutamate transmission at cortical projections onto D1R-MSN of the NAc have been causally implicated in the development of cocaine-induced locomotor sensitization (13). To study the contribution of D1R-GluN1 heteromerization in drug-induced plasticity at these synapses, we injected mice with Tet-On-GluN1C1 together with a mixture of AAV-PPTA-Cre – driving the expression of the Cre recombinase under the control of the D1R-MSN-specific prepro-tachykinin promoter - and AAV-DIO-eGFP to tag D1R-MSN (7, 39, 40) (Fig. S3A). These mice were supplemented with dox before and during daily injections of saline or cocaine for 5 days (5 d) followed by 10 d of withdrawal. As previously shown (16), cocaine triggered an increase of AMPA/NMDA (A/N) ratio – an index of synaptic plasticity - in D1R-MSN of mice injected with the control virus in the NAc. By contrast, while preserving basal synaptic transmission in the saline-treated group, the inhibition of D1R-GluN1 heteromerization blunted the cocaine-evoked increase in A/N ratio (Fig. 2E,F), without modifying the amplitude or the kinetics of NMDAR EPSCs (Fig. 2G,H). These data show that D1R-GluN1 heteromerization controls cocaine-evoked changes in glutamate transmission in D1R-MSNs.

D1R-GluN1 heteromerization controls the development of cocaine-induced locomotor sensitization

We next evaluated the role of D1R-GluN1 heteromerization in the behavioral sensitizing properties of cocaine by supplementing Tet-On-AAV-injected mice with dox prior to and during saline or cocaine administration. Uncoupling D1R from GluN1 did not affect basal locomotion (Fig. 2I), nor the acute hyperlocomotor response triggered by the first cocaine injection, but fully blocked the development of the behavioral sensitization induced by subsequent injections (Fig. 2J). Of note, dox supplementation did not alter body weight, basal locomotion or behavioral sensitization to cocaine in mice that were not injected with Tet-On viruses (Fig. S4A-C).
By temporally controlling expression of the interfering peptides, we assessed the contribution of D1R-GluN1 heteromerization to the maintenance phase of locomotor sensitization. Mice injected with Tet-On viruses were treated with saline or cocaine for 5 d in the absence of dox. As expected, these mice displayed a similar cocaine-induced locomotor sensitization regardless of the virus injected. To switch off D1R-GluN1 heteromerization after behavioral sensitization, dox was given after the last saline or cocaine injection and during a 7 d withdrawal followed by a challenge injection of saline or cocaine (Fig. 2K-top). We found that mice displayed the same level of sensitization in response to the cocaine challenge regardless of the AAV used, demonstrating that D1R-GluN1 heteromerization is not required for the maintenance of locomotor sensitization (Fig. 2K-bottom). Since cocaine also enhanced D1R-GluN1 heteromerization in the dorsal striatum (Fig. 1C), we also targeted heteromers in this striatal sub-region and obtained the same results (Fig. S4D,E). Overall, these data show that D1R-GluN1 heteromerization in the striatum controls the development, but not the maintenance, of cocaine's sensitizing effects.

D2R-GluN2B heteromerization selectively controls the maintenance of cocaine sensitizing effects

In light of the significant and persistent impact of cocaine on D2R-GluN2B heteromerization in the NAc (see Fig. 1D,E), we generated AAV Tet-On-D2R-IL3 to achieve a dox-inducible expression of a peptide corresponding to a small fragment (T225-A234) located within the 3rd intracellular loop (IL3) of D2R. This IL3 domain is known to play a key role for D2R-GluN2B interaction (32). Since critical amino acids responsible for D2R-GluN2B interaction have not been yet identified within this D2R-IL3 fragment, we used a control virus (Tet-On-D2R-IL3-scr) driving the expression of a scrambled peptide (Fig. 3A). The Tet-On-D2R-IL3 efficiently reduced D2R-GluN2B PLA puncta and preserved neuronal survival (Fig. 3B-D). Importantly, this peptide altered the interaction of D2R with GluN2B while sparing the functions of individual component receptors, as shown by its lack of effect on D2R-mediated inhibition of cAMP production (Fig. 3E) and NMDA currents (see below).

Since repeated cocaine exposure does not modify A/N ratio in D2R-MSN (16), the consequences of uncoupling D2R from GluN2B was studied in saline-treated animals with virally-tagged D2R-MSN owing to the co-injection of AAV-PPE-Cre – driving the expression of the Cre recombinase under the control of the D2R-MSN-specific prepro-enkephalin promoter (7) and AAV-DIO-eGFP (Fig. S3B). We found that the inhibition of D2R-GluN2B heteromerization did not alter, by itself, A/N ratio in D2R-MSN when compared to D2R-MSN transduced with the control virus (Fig. 3F,G). Of note, Tet-On-D2R-IL3 also left unchanged the amplitude and kinetics of NMDA currents (Fig. 3H,I), indicating a lack of non-specific effect on individual NMDAR functions, thereby validating our interfering viral strategy.

At the behavioral level, interfering with D2R-GluN2B heteromerization preserved both basal locomotion (Fig. 3J-left) and the development of cocaine-induced locomotor sensitization (Fig. 3J-right). Strikingly, we found that the inhibition of D2R-GluN2B heteromerization during cocaine withdrawal reduced the maintenance of the behavioral sensitization compared to cocaine-treated mice injected with the control virus (Fig. 3K). These mice were sacrificed to analyze ΔFosB expression levels, used here as a proxy for neuronal activity. We observed a significant increase of D2R-MSN expressing ΔFosB after the cocaine challenge upon inhibition of D2R-GluN2B interaction (Fig. 3L,M). Since D2R-Glu2NB heteromerization has been shown to mediate the D2R...
agonist-induced inhibition of NMDAR ex vivo (32), our data suggest that the impaired maintenance of the sensitizing effects of cocaine observed upon D2R-GluN2B uncoupling may result from increased D2R-MSN activity. Altogether, these data demonstrate that D1R-GluN1 and D2R-GluN2B heteromerization controls the development and maintenance of cocaine sensitizing effects, respectively.

**Differential roles of D1R-GluN1 and D2R-GluN2B heteromers in controlling the rewarding effects of cocaine.**

We next investigated the role of these heteromers in the rewarding effects of cocaine using a conditioned place preference (CPP) paradigm. Mice injected in the NAc with a control virus and supplemented with dox developed a significant cocaine-induced CPP, which was blunted when D1R and GluN1 were uncoupled (Fig. 4A). Although single or repeated cocaine administration has been shown to trigger dendritic spine formation in D1R-MSN (39, 41), there is no study showing such morphological changes in the NAc in the context of CPP. Mice were thus sacrificed the day after the behavioral test to perform a 3D morphological analysis of GFP-tagged D1R-MSN. Control mice, which developed CPP to cocaine, displayed a significant increase of dendritic spine density in D1R-MSN, which was inhibited when D1R and GluN1 were uncoupled (Fig. 4B). To study the implication of D1R-GluN1 heteromerization on relapse to CPP, AAV-injected mice were initially trained for CPP in the absence of dox. Once mice developed CPP, dox was added to alter D1R-GluN1 interaction during an extinction period followed by a cocaine-induced relapse. We observed that the inhibition of D1R-GluN1 interaction did not alter the kinetics of extinction, nor the relapse to CPP (Fig. 4C), thus supporting a critical role for D1R-GluN1 heteromers in the development of the rewarding effects of cocaine, as observed for locomotor sensitization, but not in the propensity to relapse.

The uncoupling of D2R from GluN2B also blocked the development of cocaine CPP (Fig. 4D) but this was not correlated to morphological changes in D2R-MSNs since the CPP paradigm did not trigger any modification of dendritic spine density in D2R-MSN regardless of the AAV used (Fig. 4E). Inhibiting D2R-GluN2B heteromerization once the mice have developed CPP did not impact the extinction of CPP but significantly reduced cocaine-induced relapse (Fig. 4F). This indicates that D2R-GluN2B heteromerization is required for both the development and relapse of cocaine-induced CPP, independently of morphological changes in GFP-tagged D2R-MSNs.

**Inhibiting D1R-GluN1 or D2R-GluN2B heteromerization does not alter conditioned place preference for food.**

Manipulating D1R-GluN1 or D2R-GluN2B heteromerization in vivo allowed us to reveal their selective implication in controlling distinct phases of long-term cocaine-evoked adaptations. We next examined whether receptor heteromerization also controls non-drug reward processing. We found that disrupting either heteromer subtype, using comparable conditions as for our studies with cocaine, failed to alter the rewarding properties of food (Fig. 5), supporting a role of these heteromers in controlling the rewarding effects of cocaine but not a non-drug reward.
D2R-GluN2B heteromerization is increased in post-mortem brain samples from addict subjects despite decreased D2R expression.

As evidenced above, D2R-GluN2B heteromerization plays a cardinal role in the maintenance of cocaine’s effects without affecting natural reward processing in mice, which positions this heteromer subtype as a potential therapeutic target for drug addiction. We therefore investigated whether D2R-GluN2B heteromerization could be detected in human brain tissues and modulated in subjects with a history of psychostimulant dependence.

PLA has recently been shown as a suitable approach to detect single proteins or receptor heteromers, including D2R-A2AR, in human brain samples (42, 43). We therefore performed single detection of D2R, GluN2B and D2R-GluN2B heteromers in post-mortem human samples from control subjects and matched individuals with a history of dependence. Although often poly-addicts, these individuals were selected for their main dependence to psychostimulants and the presence of traces of psychostimulants in their blood at the time of death (Table S1). From whole-slide images of caudate putamen samples, automated detection of PLA signal was performed from 25 high-magnification images per subjects randomly selected within the ventral part of the samples, which corresponds to the mouse NAc (Fig. S5).

D2R single detection produced a dense punctate pattern in control subjects (Fig. 6A), as already reported (42). As expected, this signal was absent when PLA was performed in the absence of the primary antibody. Interestingly, we detected a significant decrease of relative D2R protein expression in sample from addicts compared to control subjects (Fig. 6B), consistent with the well-established decrease of striatal D2R availability reported in psychostimulant abusers by PET imaging (44–48). By contrast, GluN2B single detection produced a dense GluN2B signal that was not different between samples from addicts and controls (Fig. 6C,D). The double recognition of D2R-GluN2B proximity yielded a punctate signal in control subjects, which was undetectable when one of the two primary antibodies was omitted (Fig. 6E). In samples from addicts, there was a trend towards an increase of D2R-GluN2B heteromers in samples from addicts (Fig. 6F), despite the drastic decrease of D2R levels. We therefore analyzed whether a correlation between D2R levels and D2R-GluN2B heteromerization could exist within each addict sample. We found a significant inverse correlation between these two parameters (Fig. 6G), suggesting that despite the lower levels of D2R expression in addicts, at least a remaining pool of D2R was preferentially involved in D2R-GluN2B heteromerization. By contrast, there was no correlation between GluN2B expression and D2R-GluN2B heteromerization (Fig. 6F). Considering the decreased D2R levels in samples from addicts, we normalized the D2R-GluN2B PLA signal to D2R levels for each individual and found a significant increase in relative D2R heteromerization with GluN2B in samples from addicts when compared to controls (Fig. 6I). To the best of our knowledge these results provide the first evidence of a decreased D2R protein expression in the striatum of psychostimulant addicts, which is associated with an increase of D2R-GluN2B heteromerization. Together with our interventional approach in mice, our data support D2R-GluN2B heteromers as therapeutic targets of potential interest in addiction.
Discussion

Optogenetic studies undeniably showed that distinct phases of drug-induced behavioral adaptations rely on DA-evoked synaptic adaptations at specific glutamate inputs onto MSN subpopulations (13-15, 49, 50). Nonetheless, the underlying molecular mechanisms remain poorly understood (2). This is an important issue because the identification of events responsible for such a detrimental interplay between dopamine and glutamate signaling may help in the development of innovative strategies with therapeutic potential. Herein, we provide multiple lines of evidence, from mice to humans, that the heteromerization of glutamate NMDAR with D1R or D2R is enhanced by psychostimulants and preferentially controls the development and maintenance phases of cocaine-evoked long-term adaptations, respectively.

The focus on dopamine and NMDA receptor heteromers as potential integrators of dopamine and glutamate inputs that may control drug-mediated adaptations stems from in vitro and ex vivo studies showing that such a direct physical interaction allows a reciprocal fine-tuning of the component receptors’ functions (23, 27). In particular, patch-clamp recording from striatal slices showed that D1R-GluN1 and D2R-GluN2B interactions respectively facilitate and inhibit NMDAR-mediated signaling upon DA increase (31, 32). An appealing hypothesis would therefore be that, by linking dopamine to glutamate signaling in opposite ways, these heteromers could constitute molecular substrates for drugs of abuse to exert their differential effects on the activity of MSN subtypes, which has been proposed to underlie the switch from recreational drug consumption to addiction (3, 51).

In agreement with this model, our PLA analysis showed that locomotor sensitization induced by repeated cocaine injections was associated with an increase of both heteromers in the NAc. We also found that this increased heteromerization requires dopamine receptor stimulation. While the PLA method cannot establish the direct physical contact of the two proteins or the stoichiometry of the complex, it does indicate that the proteins are in close molecular proximity (34). Nonetheless, previous studies have provided evidence for direct interactions between the D1R-GluN1 and D2R-GluN2B (28, 32) and we find that our viral minigenes selectively decrease the PLA signals; we therefore interpret these PLA data as support for receptor heteromerization. While in-depth characterization of the molecular events responsible for this increased receptor interaction upon cocaine exposure is beyond the scope of this study, a possible explanation may lie in the observation that repeated cocaine exposure decreases PSD-95 expression in the NAc (36). In fact, PSD-95 is a known endogenous inhibitor of D1R-GluN1 interaction (37) that also binds to D2R (52) and the GluN2B c-terminal end (53). Even though the PLA approach is able to provide a snapshot of the impact of cocaine on receptor heteromerization in situ in their native environment (31, 34, 35), future work is needed to investigate whether heteromerization of DA and NMDA receptors is an input-specific process and whether it relies on the modulation of receptor surface expression and/or dynamics.

The development of the sensitizing effects of cocaine has been previously causally linked to the potentiation of glutamate transmission at cortical projections onto D1R-MSN of the NAc (13). Since we observed that preventing D1R-GluN1 heteromerization reversed both alterations in A/N ratio and the development of behavioral sensitization, while sparing the function of individual component receptors (see (31)), our results suggest that D1R-GluN1 heteromers are key molecular platforms for the development of cocaine-induced long-term adaptations. In contrast,
disrupting D1R-GluN1 interaction during a withdrawal from cocaine did not impact maintenance of the sensitized state, supporting a preferential role of this heteromer subtype in the initial phases of cocaine-mediated adaptations. In agreement with this hypothesis, we observed that preventing D1R-GluN1 heteromerization during CPP conditioning also blocked the development of the rewarding effects of cocaine, but failed to alter the extinction and relapse phases. This critical time window of D1R-GluN1 heteromer function restricted to the early developmental phase of cocaine-evoked adaptations agrees with our observation that cocaine-induced D1R-GluN1 heteromerization is a transient mechanism that does not outlast a 7 d withdrawal period. Instead, the temporally-controlled disruption of D2R-GluN2B heteromers revealed their preferential role in the maintenance of the sensitizing and rewarding effects of cocaine. In agreement with these findings, we found that D2R-GluN2B heteromerization persisted through withdrawal from cocaine. Moreover, this persistent heteromerization was specifically observed in the NAc core, which has been identified as a common output structure of neuronal circuits involved in both cue- and drug-induced relapse (54). Since the optogenetic activation of D2R-MSNs in the NAc has been shown to preserve cocaine-induced locomotor sensitization but to blunt its expression after withdrawal (55), our results support a model by which inhibiting endogenous D2R-GluN2B interaction during withdrawal hinders the persistence of cocaine-evoked responses by potentiating D2R-MSN activity. In support of this hypothesis, we found that the alteration of the maintenance phase of locomotor sensitization observed upon D2R-GluN2B heteromer disruption was associated with an increase of D2R-MSN activity, as revealed by an increased expression of FosB in D2R-MSN. These observations therefore suggest that D2R-GluN2B heteromerization is a key molecular mechanism triggered by cocaine that dampens D2R-MSN activity and contributes to the persistence of cocaine-evoked adaptations.

Direct manipulations of MSN activity have clearly revealed that D1R-MSN and D2R-MSN activation, respectively, facilitates and blunts the development and maintenance phases of psychostimulant-induced behavioral adaptations (2). Strikingly, our findings that D1R-GluN1 and D2R-GluN2B heteromerization are involved in the induction and maintenance of cocaine-induced locomotor sensitization, respectively, highlight that these receptor complexes mediate discrete properties of MSN subpopulations and play complementary roles to mediate the full panel of cocaine-induced adaptations. Importantly, in further support of specific functions of dopamine and glutamate receptor heteromers, we established that their roles in shaping reward processing depends on the nature of the reward, since the disruption of either receptor heteromer blocked the development of cocaine-induced CPP but spared food-mediated CPP. Although the mechanisms underlying such selectivity to drug reward remain to be established, our findings suggest that targeting dopamine-glutamate receptor heteromers has the potential to preferentially alleviate pathological adaptations induced by drugs of abuse. In particular, the role for D2R-Glu2NB heteromerization in maintaining cocaine-induced adaptations combined with its lack of implication in reward processing to a natural reinforcer suggest that D2R-GluN2B heteromers are targets of choice from a translational standpoint. This led us to investigate whether this heteromer subtype could be detected in post-mortem human samples and modulated in subjects with a history of psychostimulant addiction.

Our PLA analysis revealed a strong reduction of D2R protein levels in the NAc of drug abusers. With the “single PLA” approach, the polyclonal secondary antibodies can bind to either a single primary antibody – therefore detecting a single antigen on the D2R – or to two different primary antibodies bound to proximal antigens – potentially revealing D2R homodimers. However, the
latter is likely to be much less efficient and we assume that the single PLA signal in our study mainly reflects the density of single D2R (42). This first observation of a decreased D2R protein expression in post-mortem brain samples from addicts is consistent with the downregulation of D2R mRNA levels that has been described after long-term cocaine exposure in rats (56). Importantly, this finding could also partly account for the decrease in D2R binding readily observed with PET imaging of the striatum of drug abusers (44–48). Despite such downregulation of D2R protein, the proportion of D2R forming heteromers with GluN2B was three-fold higher in psychostimulant abusers compared to healthy subjects. Strikingly, addict individuals bearing the lowest D2R expression displayed the highest density of D2R-GluN2B. This raises questions regarding the underlying molecular mechanism of D2R-GluN2B formation in response to psychostimulant exposure in human. Based on our findings in mice that cocaine-induced D2R-GluN2B heteromerization depends on D2R stimulation, it is tempting to speculate that repeated increases of phasic dopamine levels resulting from recurrent psychostimulant consumption by addict individuals could be responsible for the higher D2R-GluN2B receptor proximity. The increased formation of D2R-GluN2B heteromerization we observed in human samples from psychostimulant abusers, together with interventional approaches in mice, emphasize their roles in the persistence of cocaine’s behavioral effects. These important findings constitute a significant breakthrough in understanding of the molecular bases of cocaine-induced adaptations and highlight the potential benefit of targeting D2R-Glu2NB heteromerization, not only in the field of addiction, but also potentially for multiple neuropsychiatric disorders associated with an imbalance of DA and glutamate transmission.
Materials & Methods

Animals
6-week-old C57BL/6J male mice were purchased from Janvier labs (Le Genest, St Isle, France). The animals were housed four per cage, in a 12-hour light-dark cycle, in stable temperature (22°C) and humidity (60%) conditions with ad libitum access to food and water. They were acclimatized to the animal facility for at least 1 week. All experiments were carried out in accordance with the standard ethical guidelines (European Community Council Directive on the Care and Use of Laboratory Animals (86/609/EEC) and the French National Committee (2010/63)).

Drugs
Drugs were administrated intraperitoneally in a volume of 10 ml/kg. Cocaine hydrochloride (Sigma Aldrich, St. Louis, MO) was dissolved in a saline solution (0.9% NaCl w/v). 9-tert-butyl doxycycline hydrochloride (9-TB-dox; Tebu-bio, Le Perray-en-Yvelines, France) was dissolved in a saline solution containing DMSO (5%) and Tween20 (5%). SCH23390 (0.25 mg/kg) or Eticlopride (0.5 mg/kg) dissolved in a saline solution (0.9% NaCl w/v) were administered 30 min prior to the challenge cocaine injection.

Viral constructions
All AAV recombinant genomes were packaged in serotype 9 capsids. AAV-Tet-On-GluN1C1 expresses bicistronically the fluorescent reporter protein RFP and the C1 cassette of the GluN1 subunit (864DRKSGRAEPDPKKKATFRAITSTLASDT900) upon doxycycline (dox) treatment. The related control virus AAV-Tet-On-GluN1C1Δ expresses a truncated version of C1 that is deleted from a stretch of 9 positively charged amino acids (890S890FKRRRSSK898), which are required for D1R-GluN1 interaction55. The AAV-Tet-On-D2R-IL3 encodes a sequence of the third intracellular loop of the D2R (225TKRSSRAFRA234) interacting with GluN2B. The control 9AAV-Tet-On-D2R-scr expresses a scrambled sequence (KFARRTSASR) of the D2R-IL3 (full AAV sequences are available upon request). All Tet-On AAV were injected bilaterally by infusing 0.7 µl of a solution at 5.1013 viral genomes/ml per hemisphere for the NAc (2 µl for the dorsal striatum). The AAV-PPTA-Cre and AAV-PPE-Cre contain an expression cassette consisting of the Cre recombinase driven by the promoter of the PPTA gene (prepro-tachykinin) or the PPE gene (preproenkephalin), which are specifically expressed in D1R-MSN and D2R-MSN, respectively (7, 39, 40) (see supplementary Fig. 3). AAV PPTA-cre or AAV-PPE-cre were co-injected with the AAV-pCAG-DIO-eGFP-WRPE (Upenn) expressing flexed eGFP under the CMV/actin hybrid promoter (CAG). All viruses were diluted in PBS pluronic 0.001%.

Stereotaxic injections
Mice were anesthetized with ketamine (150 mg/kg) and xylazine (10 mg/kg) and placed on a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA). Craniotomies were realized using the following coordinates: 1.7 mm rostral to the bregma, 1.2 mm lateral to midline and 4.6 mm ventral to the skull surface to target the NAc and 1mm rostral to the bregma, 1.8 mm lateral to midline and 3.25 mm ventral to the skull surface for the dorsal striatum. Viral injections were performed bilaterally at a rate of 0.15 µl/min using a 10 µl-syringe (Hamilton 1700 series, Phymep, Paris, France) with a 200 µm gauge needle (Phymep, Paris, France) mounted on a microinfusion pump (Harvard Apparatus, Holliston, MA). After the injection, the needle was left in place for an additional 8 min to avoid backflow.
**Doxycycline treatments**

Three weeks after stereotaxic injections of Tet-On AVV, the expression of the constructs was triggered by daily intraperitoneal (IP) injection of 9TB-dox (10 mg/kg) for 4 days (4d). To maintain expression mice were then supplemented with a mix containing doxycycline (dox) HCl (2 mg/ml), 9TB-dox HCl (80µg/ml) and sucrose (1%) added in drinking water.

**Behavioral testing**

All behavioral tests were conducted during the light phase (8:00–19:00). Animals were randomly assigned to the saline or cocaine groups after viral injection. Prior to behavioral testing, mice were handled daily during 7d in the experiment room. All mice were perfused with 4% (w/v) paraformaldehyde (PFA) 24h post-behavior to systematically verify the accuracy of stereotaxic injections and expression of the RFP reporter protein. Mice that did not meet quality criterion (i.e. non-bilateral expression, off-target diffusion, excessive backflow or low RFP expression) were discarded from the study.

**Locomotor activity and cocaine psychomotor sensitization**

Locomotor activity was measured in a low luminosity environment inside a circular corridor (Immetronic, Pessac, France) containing four infrared beams placed at each 90° angle. Locomotor activity was expressed as a cumulative count of crossings between quarters of the corridor for the indicated time. Mice were treated with dox 7d before and until the end of the experiment. Mice were habituated to the test apparatus for 3d; basal locomotor activity was recorded on the third day of habituation. Cocaine sensitization experiments consisted of five daily 90 min sessions during which spontaneous activity was recorded for 30 min before saline or cocaine (15 mg/kg) injections and locomotor activity was then measured for 60 min post-injections. To study the consequences of uncoupling DAR from NMDAR on the maintenance of cocaine-induced locomotor sensitization, mice were treated for 5 consecutive days with saline or cocaine in the absence of dox. After the last injections, mice were supplemented with dox during a withdrawal period followed by a challenge injection of saline or cocaine.

**Cocaine Conditioned Place Preference (CPP)**

To study the impact of DAR-NMDAR heteromerization on the development of CPP, mice were treated with dox for 7d before and until the end of the experiment. The CPP was performed in a two-compartment Plexiglas Y-maze apparatus (Imetronic). Each compartment contains different visual cues and floor textures for which mice did not show any preference on average before conditioning. All sessions lasted 20min. On day 1, mice were placed in the center of the apparatus and allowed to explore freely both compartments. Time spent in each compartment was automatically recorded. Mice spending more than 70% of the time in one compartment were excluded. On day 2, to avoid any initial preference bias, mice were randomly assigned to one or the other compartment for each group. Mice were injected with saline and placed immediately in the assigned closed compartment for 20 min. After 1h, mice were injected with saline or cocaine and placed in the other closed compartment. This was repeated on day 3. The test was performed on day 4, during which mice had a free access to both chambers. The CPP score was calculated as the difference between the time spent in the cocaine-paired chamber during day 4 minus the time spent in this compartment on day 1. CPP extinction and maintenance experiments were performed on the cocaine groups of mice injected with Tet-On-AVV that developed a preference for the cocaine-paired chamber in the absence of dox. Mice were then treated with dox until of the behavioral assessment. For the extinction phase, mice were injected with saline and put back
in the apparatus with free access to both compartments for 20 min daily for 8 days. On the ninth day, mice were injected with cocaine and allowed to explore both compartments. For palatable food-induced CPP, mice were food-deprived to 90% of initial ad libitum weight and treated with dox 7d prior and during behavioral assessment. Experiments were performed in the same apparatus and conditions as for cocaine-induced CPP with the following modifications: On day 2, after random group assignment. Mice were placed immediately in the assigned closed compartment containing chocolate crisps (Chocapic, Nestlé, Vevey, Switzerland) or nothing for 20 min. After 1 h, mice were placed in the other closed compartment. This was repeated on day 3 and 4. The test was performed on day 5.

**Mouse tissue preparation**
Mice were anesthetized with an I.P injection of Euthasol (100 mg/kg; Le Vet, Oudewater, Netherlands) and perfused transcardially with 0.1 M Na2HPO4/ Na2HPO4, pH 7.5 containing 4% PFA at 4°C. delivered with a peristaltic pump at 20 ml/min for 5 min. Brains were then extracted, post-fixed overnight in 4% PFA, and stored at 4°C. 30 µm-thick coronal sections were performed with a vibratome (Leica, Nussloch, Germany) and kept at -20°C in a cryoprotective solution containing 30% ethylene glycol (v/v), 30% glycerol (v/v) and 0.1 M PBS.

**Immunohistochemistry**
On day 1, free-floating sections were rinsed three times for 5 min in Tris-buffered saline (TBS, 0.9% NaCl, 0.1 M Tris base, pH 7.5). Sections were then incubated in blocking solution containing 3% normal goat serum, 0.2% triton X-100 (Sigma, Aldrich) and 50 mM NaF for 2 h at room temperature (RT) before overnight 4°C incubation with primary antibodies (see antibody table) diluted in the blocking buffer. On day 2, after three 10-min rinses in TBS, sections were incubated for 90 min with secondary antibodies (see antibody table). The anti-FosB antibody recognizes full-length FosB as well as ΔFosB but at the time point studied (24h post cocaine) all FosB-immunoreactive protein represents ΔFosB (57). After three 5-min rinses in TBS, sections were incubated for 5 min with Hoechst (Invitrogen) for nuclei counterstaining. Three 5-min TBS and two Tris buffer (0.1 M Tris base, pH 7.5) washes were performed before sections were mounted in Prolong Gold (Invitrogen).

**Immunoblotting**
Mice were killed by decapitation and their heads were immediately snap frozen in liquid nitrogen. Microdiscs of NAc were punched out using disposable biopsy punches (1 mm diameter) (Kai medical) and stored in individual tubes at -80°C. Microdiscs were then homogenized by sonication in a lysis solution containing 50 mM Tris Hcl, 2% SDS, 0.5 M urea diluted in water. Protein concentrations were determined using the BCA assay kit (Pierce, Rockford, IL). A fixed amount of protein (30 µg per lane) was separated by SDS-polyacrylamide gel electrophoresis (12%) before electrophoretic transfer onto nitrocellulose membranes. Membranes were incubated 1 h at RT in a blocking solution containing TBS with 0.1% tween and 5% non-fat dry milk. Membranes were incubated at 4°C overnight with primary antibodies (see antibody table). Membranes were then washed three times in TBS-Tween and then incubated for 1h at RT with secondary antibodies (see antibody table) coupled to horseradish peroxidase (HRP). Immunoreactive bands were detected by chemoluminescent detection (ECL kit, GE Healthcare) and images were acquired using the ImageQuant LAS 4000 (GE Healthcare Life Science). The densitometry of immunoreactive bands was quantified using ImageJ and normalized to the loading control.
Human brain samples
Brain samples from individuals with a history of substance dependence, with toxicological evidence of current psychostimulant use, and from matched healthy controls (n = 13/group; Table S1) were provided by the Suicide section of the Douglas-Bell Canada Brain Bank (DBCBB). Brains were donated to the DBCBB by familial consent through the Quebec Coroner’s Office, which ascertained the cause of death. Two months after death, psychological autopsies with next-of-kin were conducted, as previously described (58).
Dissections of ventral striatum were performed with the guidance of a human brain atlas (59) on 0.5 cm-thick formalin-fixed coronal brain sections at an anatomical level equivalent to plate 15 of this atlas (-7.5 mm from the center of the anterior commissure). Tissue was extracted rostral to the anterior commissure and ventral to the tip of the anterior limb of internal capsule and fixed by immersion in formalin until paraffin embedding. The latter was completed using a Leica ASP200S automated processor. Tissue blocks were dehydrated in increasing gradients of alcohol (70%, 95%, 3x100%) for 1.5 hr each, followed by clearing in 3 changes of 100% xylene (2 hr, 2x1.5 hr). The samples were then infiltrated in 3 changes molten paraffin, 3hrs each before embedding. Slices of 6 µm thickness when then prepared with microtome at the histology facility of the ICM institute (Paris).

Proximity Ligation Assay
Proximity ligation assay (PLA) on mouse brain sections (30 µm-thick) was performed in 48-well plates according to the manufacturer’s instructions for free-floating sections. PLA on human post-mortem paraffin-embedded tissue was performed on sections mounted on superfrost plus slides (Thermo Scientific). 6 µm-thick human caudate-putamen sections were deparaffinized in xylene, rehydrated in graded ethanol series and washed briefly in TBS. For antigen retrieval, sections were boiled for 6 min in sodium citrate buffer (10 mM, pH 6). For brightfield PLA, sections were incubated for 30 min at RT in TBS containing 1% H2O2 to block endogenous peroxidase. After three 5 min rinses with TBS containing 0.1% triton X-100 (TBS-T), sections were incubated 1h at RT with blocking buffer (Duolink blocking buffer for PLA) then with primary antibodies (see antibody table) diluted in the Duolink antibody dilution buffer overnight at 4°C. Anti-rat PLA plus and minus probes were made using the PLA probemaker kit (Sigma Aldrich) with a Goat anti-rat IgG antibody (Jackson Immunoresearch) according to manufacturer’s instructions. Immunofluorescent PLA and the remaining procedures for brightfield PLA were performed as previously described (31). For brightfield PLA nuclei were counterstained using the duolink nuclear stain. In this study, we used both single-recognition PLA, using only one primary antibody to detect single antigen and dual-recognition to detect DAR-NMDAR complexes using two primary antibodies.
### Antibodies

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<th>Secondary antibody</th>
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### Image acquisition and analysis

For immunochemistry and fluorescent PLA staining, images were taken with a confocal laser scanning microscope (SP5, Leica) using a 20X and a 63X objective (oil immersion, Leica) respectively. The pinhole was set to 1 Airy unit, excitation wavelength and emission range were 495 and 500-550 nm for green PLA signal, 488 and 500-550 nm for Alexa fluo 488 and 590-650 nm for RFP. Laser intensity and detector gain were constant for all image acquisitions. Images were acquired in a range of 5 µm with a z-step of 0.2 µm. Conditions were run in duplicate and quantifications were made from at least 4 images per condition. Four mice were used for each condition. Maximum projection images were analyzed using ImageJ (National Institutes of Health, Bethesda, MD).

For PLA images quantification, ImageJ was used to construct a mask from RFP positive cells. The mask was then fused with the PLA signal-containing channel using the image multiply
function. The PLA punctate signal was quantified on the resulting image in ICY using the spot
detector function (detector = scale 3: 65; filtering = Min size: 6; Max size: 30); these parameters
were chosen manually from random images to obtain optimal signal-to-noise ratio and minimal
false positive in images from negative control conditions. RFP-positive cells showing at least one
PLA puncta for one or the other heteromer subtypes were included as this allowed us to identify
the MSN subtype analyzed (D1R-MSN v.s D2R-MSN). RFP positive cells which did not present
any D1R-GluN1 or D2R-GluN2B PLA signal were discarded.

Mouse brightfield PLA images were taken using a microscope (Leica, DM4000) with a 63X
objective. A minimum of 6 random fields per structure per mice were taken. For human brightfield
PLA, whole-slide images were taken using a Zeiss, Axioscan with a 40X magnification and z-
stack with a 1 µm step interval. Whole-slide scanned images were visualized with ZEN Blue
edition lite software (Zeiss, version 3.1). 25 region of interest (ROI) of 500 µm x 500 µm were
generated randomly in the NAc and were exported as TIFF files using the image export plugin.
The z-stack with the maximum number of PLA signal in the focus was chosen for each image.

Human and mouse brightfield PLA Images were analyzed using an ImageJ homemade macro
that use the Find Maxima tool to detect the PLA punctate with prominence set at >40. Results are
represented as the mean PLA signal density per field of views.
For quantification of immunohistochemistry experiments, immunoreactive cells, were analyzed
using ImageJ considering the cells with immunofluorescence above a fixed threshold.

**Neuronal survival**
Neuronal survival was quantified manually based on Hoescht-counterstained nuclei. Survival was
defined as the percentage of viable neurons exhibiting large, uniform nuclei and even distribution
of Hoescht among RFP positive cells over RFP positive cells classified as apoptotic based on, at
least, two of the following criteria: condensed nuclei, single chromatin clump, small nuclei size,
non-circular nuclei shape and increased Hoescht intensity.

**Spine density analysis**
Twenty-four hours after the CPP paradigm, mice were perfused and brains were sliced as
described above. Dendrite spine analysis was performed on RFP-positive dendrites from D1R-
MSN and D2R-MSN identified based on the presence of GFP driven by the co-injection of AAV-
PPTA-cre/AAV-pCAG-DIO-eGFP or AAV-PPE-cre/pCAG-DIO-eGFP, respectively. Image stacks
were taken using a confocal laser scanning microscope (SP5, Leica). For analysis of D1R-MSN,
images were collected through 63x objective with pixel size of 65 nm and z-step of 200 nm. For
D2R-MSN, images were collected through 40x objective with pixel size of 95 nm and z-step of
300 nm. The excitation wavelength was 488 for GFP and 561 for RFP, with emission range 500-
550 nm and 570–650 nm, respectively. Images were acquired in sequential mode with a Hybrid
detector (HyD, Leica). Deconvolution with experimental point spread function from fluorescent
beads using a maximum likelihood estimation algorithm was performed with Huygens software
(Scientific Volume Imaging). Neuronstudio software was used to reconstruct the dendrite and
detect dendritic spines with manual correction.
Patch clamp recordings
Mice were anesthetized (Ketamine 150 mg/kg / Xylazine 10 mg/kg) and transcardially perfused with aCSF for slice preparation. Coronal 250 μm slices containing the nucleus accumbens were obtained in bubbled ice-cold 95% O2/5% CO2 aCSF containing (in mM): KCl 2.5, NaH2PO4 1.25, MgSO4 10, CaCl2 0.5, glucose 11, sucrose 234, NaHCO3 26, using a HM650V vibratome (Microm, France). Slices were then incubated in aCSF containing (in mM): NaCl 119, KCl 2.5, NaH2PO4 1.25, MgSO4 1.3, CaCl2 2.5, NaHCO3 26, glucose 11, at 37°C for 1 h, and then kept at room temperature. Slices were transferred and kept at 31°C in a recording chamber superfused with 2 ml/min aCSF in the continuous presence of 50 μM picrotoxin (Sigma-Aldrich, France, dissolved in DMSO) to block GABAergic transmission. Neurons were visualized by combined epifluorescent and infrared/differential interference contrast visualization using an Olympus upright microscope holding 5x and 40x objectives. Whole-cell voltage-clamp recording techniques were used to measure synaptic responses using a Multiclamp 700B (Molecular Devices, Sunnyvale, CA). Signals were collected and stored using a Digidata 1440A converter and pCLAMP 10.2 software (Molecular Devices, CA). AMPA-R/NMDA-R ratio was assessed using an internal solution containing (in mM) 130 CsCl, 4 NaCl, 2 MgCl2, 1.1 EGTA, 5 HEPES, 2 Na2ATP, 5 sodium creatine phosphate, 0.6 Na3GTP and 0.1 spermine. Synaptic currents were evoked by stimuli (60 μs) at 0.1 Hz through a glass pipette placed 200 μm from the patched neurons. Evoked-EPSCs were obtained at V = +40mV in the absence and presence of the AMPA-R antagonist DNQX. In all cases, 30 consecutive EPSCs were averaged and offline analyses was performed using Clampfit 10.2 (Axon Instruments, USA) and Prism (Graphpad, USA). Pharmacologically isolated EPSC NMDAR decay time, recorded from cells voltage clamped at +40 mV, was fitted with a double exponential function, using Clampfit software, to calculate both slow and fast decay time constants, τfast and τslow, respectively. The weighted time constant (τweighted) was calculated using the relative contribution from each of these components, applying the formula: τw = [(af.τf) + (as.τs)] / (af + as), where af and as are the relative amplitudes of the two exponential components, and τf and τs are the corresponding time constants.

Cyclic AMP accumulation assay
HEK 293 cells stably expressing the D2R (60) were grown on polylysine (Poly-D-lysine hydrobromide, Sigma) and transfected with Tet-On plasmids encoding either the D2R-IL3 or D2R-IL3-scr peptides and the Tag RFP (4 µg per well), complexed with Lipofectamine® 2000 transfection reagent (Invitrogen™), in DMEM (DMEM high glucose GlutaMAX™ Supplement pyruvate, Gibco), and incubated for 5 h. Post-transfection, cells were rinsed and wells filled with DMEM supplemented with 10% FBS and antibiotics to induce D2R expression (Hygromycine 2 µl/ml (Sigma Aldrich); Blasticidin 1.5 µl/ml (Cayla Invivogen ant-bl-1); Tetracycline (Sigma T7660-5g) 1 µl/ml) and incubated overnight. Peptide expression was induced by addition of 1 µg/ml dox solution, incubating for 48 hours. Following the dox treatment, cells were rinsed in DMEM before pretreatment with 1mM IsoButylMethylXanthine (IBMX, Sigma Aldrich) for 15 min. Cells were then stimulated for 30 min with the indicated concentrations of agonist Quinpirole (Tocris), in the presence of 1 mM IBMX and 10 µM Forskolin (Tocris). Endogenous phosphodiesterase activity was stopped by aspiration of the medium and the addition of 0.1 M HCL (300 µl/ well). After centrifugation at 600 g during 10 min, protein concentration of supernatants was quantified by BCA (Uptima, Interchim). Cyclic AMP levels were determined in samples containing 10 µg of protein. The accumulation of cAMP was measured by using a CAMP Enzyme Immunoassay kit (Sigma Aldrich) as described by the manufacturer using Victor3 (Perkin Elmer) plate reader. The curve fit was obtained by GraphPad Prism 8 (GraphPad Software, Inc.).
Statistical analysis
Results were analyzed with Graphpad Prism (version 8.0.1). Sample size was predetermined on the basis of published studies, pilot experiments and in-house expertise. All data are displayed as mean +/- SEM. Two-tailed Student’s test was used for the comparison of two independent groups. For more than two groups comparison, one-way, two-way or three-way repeated-measures ANOVA were performed followed by Bonferoni Post-hoc test. Data distribution was assumed to be normal and variances were assumed to be homogenous. The main effect and post-hoc statistical significances are given in the appropriate figure legend for each experiment.
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Authors contributions
A.A performed most PLA, viral injections, behavioral studies, immunohistochemistry, confocal imaging, biochemistry and statistical analysis with the help of E.S.J; M.C.A; V.K; S.B, V.O and R.W. Dendritic spines analysis was performed by N.H and A.A. Electrophysiological recordings were performed by P.P and S.P.F. Viruses were designed by P.V and A.P.B and produced by C.J and A.P.B and V.S.P and A.P performed cAMP assays. G.T and N.M obtained, characterized and provided human brain samples. Y.Z and J.A.J. helped A.A for PLA experiments from human tissues and related quantifications. A.A; J.C; P.T; J.B and P.V designed experiments and wrote the manuscript, which was edited by the other authors.

Competing interests
The authors declare no competing interests
Figures & Legends

Andrianarivelo et al. Figure 1

A

Days

Saline Cocaine

Pla signal (Fold)

Locomotor activity (14 turn/min)

(Days)

B

Neg. Cont D1R-GluN1

D2R-GluN2B

Neg. Cont D2R-GluN2B

C

D1R-GluN1

Saline Cocaine

D

D2R-GluN2B

Saline Cocaine

E

Days

Saline Cocaine

F

Days

Saline Cocaine

D1-R or D2-R antagonist

Loci-motor activity (14 turn/min)

(Days)

PLA signal (Fold)

P.L.A signal (Fold)

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Figure 1. Behavioral sensitization to cocaine is associated with transient D1R-NMDAR heteromerization and prolonged D2R-GluN2B heteromerization in the NAc. (A) Experimental time frame (DL Str: dorso-lateral striatum; DM Str dorso-medial striatum; nucleus accumbens core (NAc core) and shell (NAc shell) and measurements of locomotor activity prior and during 5 days of saline or cocaine (15 mg/kg) injections. Two-way ANOVA, treatment effect, F (1, 24) = 67.79, *** P < 0.0001 saline vs cocaine on day 5, n=13 mice/group. (B) Example images of D1R-GluN1 and D2R-GluN2B heteromer PLA detection and related negative (Neg. Cont) showing the absence of signal when one primary antibody is omitted. (C) Detection and quantifications of D1R-GluN1 heteromerization in saline- and cocaine-treated groups. PLA signal is represented as fold increase normalized to the saline group. Two-sided Student’s t-test. * P < 0.05; ** P < 0.01; *** P < 0.001 saline vs cocaine, n=28-84 fields of view/structure (NAc core: 4, NAc shell: 12; DM: 6, DL: 6, fields of view/mice) from 7 mice/group. (D) Same as for (C) for D2R-GluN2B heteromerization. (E) Experimental time frame, measurements of locomotor activity and quantifications of D1R-GluN1 and D2R-GluN2B heteromerization. PLA signal is represented as fold increase normalized to the saline group. One-way ANOVA. * P < 0.05; ** P < 0.01; *** P < 0.001 saline-saline vs cocaine-saline/cocaine-cocaine, ## p<0.01 cocaine-saline vs cocaine-cocaine, n=28-84 fields of view/structure from 7 mice/group. (F) Same as for (E) except that mice received either a vehicle solution (veh) or the D1R antagonist SCH23390 (SCH) or the D2R antagonist Eticlopride (Etic) prior to a cocaine challenge. One-way ANOVA. * P < 0.05; ** P < 0.01; *** P < 0.001 vehicle-saline vs vehicle-cocaine, # p < 0.05, ## p<0.01 vehicle-cocaine vs SCH-cocaine or cocaine vs Etic-cocaine, n.s: not significant, n=28-84 fields of view/structure from 7 mice/group. (B-D) scale bar: 10 μm. Error bars denote s.e.m.
Andrianarivelo et al. Figure 2

A. Tet-On-GluN1C1

B. RFP

C. PLA (p1R/GluN1)

D. Merge

E. Tet-On-GluN1C1

F. AMPA+NMDA

G. NMDA

H. % survival (RFP+ cells)

I. Tet-On-GluN1C1

J. Basal locomotor activity

K. Locomotor activity

Andrianarivelo et al. Figure 2
Figure 2. D1R-GluN1 heteromerization controls cocaine-evoked potentiation of glutamate transmission onto D1R-MSN and the development of behavioral sensitization. (A) Top: Viral-based strategy for expression of interfering peptide to disrupt D1R-GluN1 interaction (Tet-On-GluNC1; C1) and control (Tet-On-GluN1C1Δ; C1Δ) in the NAc. Bottom: Example image of doxycycline (+dox)-mediated RFP expression (ac: anterior commissure). (B) Example image of D1R-GluN1 heteromer detection by PLA in C1Δ- and C1-transduced neurons. Scale bar: 10 μm. (C) Quantifications of the PLA signal in C1Δ- and C1-transduced neurons. Two-sided Student's t-test, t = 4.078 df. = 13, ** P = 0.0013, n = 7-8 cells from 4 mice/group. (D) Neuronal survival of C1Δ- and C1-transduced neurons. Two-sided Student's t-test, t = 0.354 df. = 6, P = 0.735, n = 4 mice/group. (E) Experimental design and example trace of AMPA+NMDA (black) and NMDA (grey) currents in neurons (asterisk) expressing GFP (i.e. D1R-MSN (see Fig. S3A)), and C1 or C1Δ (RFP+). (F) AMPA to NMDA (A/N) ratios. Two-way ANOVA: virus effect, F (1, 30) = 2.511, *P = 0.033; ## P = 0.0061; n = 3-4 mice/group and n = 6-13 cells/group. (G) Comparison of representative recordings of pharmacologically-isolated NMDAR EPSCs, normalized to the peak amplitude (in %). (H) Deactivation kinetics of NMDAR EPSCs. Two-way ANOVA: virus effect, F (1, 30) = 0.205, P > 0.999, n = 3-4 mice/group and n = 8-9 cells/group. (I) Experimental time frame and basal locomotor activity. Two-sided Student's t-test, t = 0.332 df. = 30, P = 0.742, n = 16 mice per group. (J) Inhibition of D1R-GluN1 heteromerization during the development of locomotor sensitization. Three-way ANOVA: virus effect, F (1, 256) = 13.72, ***P = 0.0003; n.s P > 0.9999, n = 7-11 mice/group. (K) Experimental time frame and measurement of locomotor activity in each group. Three-way ANOVA: virus effect, F (1, 243) = 0.6160, P > 0.9999, n = 7-8 mice/group. n.s not significant. Error bars denote s.e.m.
Figure 3. D2R-GluN2B heteromerization selectively controls the maintenance of cocaine sensitizing effects. (A) Viral strategy for expression of interfering peptide to prevent D2R-GluN2B heteromerization (Tet-On-D2R-IL3; IL3) and control (Tet-On-D2R-IL3-scr; IL3-scr). (B) Representative image D2R-GluN2B heteromer detection by PLA in IL3-scr- and IL3-transduced neurons. Scale bar: 10 μm. (C) Quantifications of the PLA signal in IL3-scr- and IL3 infected neurons. Two-sided Student’s t-test, t = 2.393 df. = 25, **P = 0.0246, n=11-16 cells from 4 mice/group. (D) Neuronal survival of IL3-scr- and IL3-transduced neurons. Two-sided Student’s t-test, t = 0.2767 df. = 6, P = 0.7913, n=4 mice/group. (E) Tet-On-D2R-IL3 spares quinpirole-induced inhibition of forskolin-induced accumulation of cAMP. LogIC50 is -9.78 for D2R-IL3 and -9.65 for D2R-IL3-Scr. n=3 independent experiments/condition. (F) Experimental time frame and representative traces of AMPA+NMDA (black) and NMDA (grey) currents in neurons expressing GFP (i.e. D2R-MSN (see Figure S3B)) and IL3 or IL3-scr (RFP+). (G) AMPA to NMDA (A/N) ratios; Two-sided Student's t-test, t = 0.3397 df. = 21, P = 0.7375, n=4-6 mice/group and n=11-12 cells/group. (H) Comparison of representative recordings of pharmacologically-isolated NMDAR EPSCs, normalized to the peak amplitude (in %). (I) Deactivation kinetics of NMDA EPSCs, Two-sided Student’s t-test, t = 0.5129 df. = 21, P = 0.6134, n=11-12 cells/group. (J) Top: Experimental time frame. Bottom left: basal locomotor activity; Two-sided Student’s t-test, t = 0.994 df. = 30, P = 0.3282, n=16 mice/group. Bottom right: Inhibition of D2R-GluN2B heteromerization does not impair the development of locomotor sensitization Three-way ANOVA: virus effect, F (1, 192) = 1.984, n.s P > 0.9999; n=6-8 mice/group. (K) Experimental time frame and impact of D2R-GluN2B heteromer inhibition on the maintenance of cocaine-evoked locomotor sensitization. Three-way ANOVA: virus effect, F (1, 198) = 7.278, *P = 0.0330; n=6 mice/group. (L) Representative images of ΔFosB expression in D2R-MSN (GFP+) infected with IL3-scr or IL3 (RFP+) after the cocaine challenge injection (see panel K). Arrowheads show GFP+/RFP+/ΔFosB+ D2R-MSN. Scale bar: 50 μm. (M) Quantifications of GFP+/RFP+/ΔFosB+ D2R-MSN. Two-sided Student’s t-test, t = 2.694 df. = 10, *P = 0.0225, n=6 mice/group. n.s: not significant. Error bars denote s.e.m.
Andrianarivelo et al. Figure 4
Figure 4. Differential roles of D1R-GluN1 and D2R-GluN2B heteromerization in controlling the rewarding effects of cocaine. (A) Experimental time frame and conditioned place preference (CPP) score upon inhibition of D1R-GluN1 heteromerization. Two-way ANOVA: virus effect, $F(1, 59) = 5.281$, *** $P < 0.0001$; ## $P=0.0040$, n=15-16 mice/group. (B) Top: low magnification images of D1R-MSN (GFP+; see Fig. S3A) infected (RFP) shown by the asterisks Scale bar 10 μm. Bottom: high magnification of dendritic segments. Scale bar: 5 μm. Spine density analysis. Two-way ANOVA: virus effect, $F(1,162) = 11.14$, * $P=0.0446$; ## $P =0.0043$, n=27-69 dendrites from 6 mice/group. (C) Experimental time frame to study the impact of D1R-GluN1 uncoupling on the extinction and cocaine induced relapse to CPP. Two-way ANOVA: virus effect, $F (1, 24) = 0.004$, $P > 0.999$, cocaine C1 vs cocaine C1Δ, CPP score on relapse day, n=10-16 mice/group. (D) Same as for (A) upon inhibition of D2R-GluN2B heteromerization. Two-way ANOVA: virus effect, $F (1, 57) = 2.424$, ***$P <0.0001$; #P=0.0396, n=14-16 mice per group. (E) Top: low magnification images of D2R-MSN (GFP; see Fig S3B) infected (RFP) shown by the asterisk. Scale bar 10 μm. Bottom: high magnification of dendritic segments. Scale bar: 5 μm. Spine density analysis. Two-way ANOVA: virus effect, $F (1, 166) = 0.1268$, n.s $P >0.999$ saline IL3-scr vs cocaine IL3-scr; n=30-53 dendrites from 6 mice/group. (F) CPP score upon inhibition of D2R-GluN2B during CCP during extinction and cocaine-induced relapse. Two-way ANOVA: virus effect, $F (1, 31) = 0.899$, **$P =0.0018$, cocaine IL3 vs cocaine IL3-scr CPP score on relapse day, n=16-17 mice/group. n.s: not significant. Error bars denote s.e.m.
Figure 5. Inhibiting D1R-GluN1 or D2R-GluN2B heteromerization does not alter conditioned place preference for food. (A) Experimental time frame to study the consequences of inhibition of heteromerization on the development of food-induced CPP. (B) Impact of inhibiting D1R-GluN1 heteromerization on the CPP score. Two-way ANOVA: virus effect, F (1, 27) = 2.756, ***P =0.0002, control C1∆ vs food C1∆; ###P =0.0002, control C1 vs food C1; n.s P>0.999, food C1 vs food C1∆, n=7-8 mice/group. (C) Effect of inhibiting D2R-GluN2B heteromerization on the CPP score. Two-way ANOVA: virus effect F (1, 26) = 0.007, **P <0.0098, control IL3-scr vs food IL3-scr; #P =0.0366, control IL3 vs food IL3; n.s, P>0.999 food IL3 vs food IL3-scr, n=7-8 mice/group. n.s: not significant. Error bars denote s.e.m.
Figure 6. D2R-GluN2B heteromerization is increased in post-mortem brain samples from addict subjects despite decreased D2R expression. (A) Representative images of D2R single recognition by PLA and negative control, in which the primary antibody is omitted (left panel Neg. Cont; see Fig. S5). (B) Quantifications of D2R single PLA signal represented as fold decreased compared to control subjects. Two-sided Student’s t-test, t = 3.331 df. = 24, **P = 0.0028, n=13 subjects/group. (C) Example images of GluN2B single detection and Neg. Cont. (D) Quantifications of GluN2B PLA signal. Two-sided Student’s t-test, t = 0.224 df. = 24, P = 0.8243, n=13 subjects/group. (E) Illustrative images of D2R-GluN2B heteromer detection by PLA and Neg. Cont (GluN2B antibody omitted). (F) Quantifications of D2R-GluN2B PLA signal. Two-sided Student’s t-test, t = 1.868 df. = 24, P = 0.074, n=13 subjects per group. (G) Pearson correlation between D2R expression levels and D2R-GluN2B heteromerization for each sample from all addict subjects, R²=0.3219, P=0.0432. (H) Pearson correlation between GluN2B expression levels and D2R-GluN2B heteromerization for each sample from all addict subjects R²=0.0087, P=0.761. (I) Quantifications of D2R-GluN2B PLA signal normalized to D2R expression levels for each subject. Two-sided Student’s t-test, t = 2.882 df. = 24, **P = 0.0082, n=13 subjects/group. (A, C, E) Scale bar: 25 μm. Error bars denote s.e.m.
Supplementary Materials

Andrianarivelo et al. Figure S1

**Fig. S1.** Repeated cocaine exposure does not alter expression levels of D1R, D2R and GluN1/2B subunits of NMDAR in mice. (A) Top: Experimental time frame of saline or cocaine (15mg/kg) treatments. Bottom: representative D1R, GluN1, D2R, GluN2B and actin immunoblots performed 24h after the last saline (sal.) or cocaine (coc.) injection. (B) Quantifications of D1R expression levels: Two-sided Student's t-test, \( t = 0.2263 \) df. = 21, \( P = 0.8232 \) n=11-12 mice/group; GluN1 expression levels: Two-sided Student's t-test, \( t = 1.001 \) df. = 21, \( P = 0.3282 \) n=11-12 mice/group; D2R expression levels: Two-sided Student's t-test, \( t = 0.992 \) df. = 20, \( P = 0.3332 \) n=11 mice/group and GluN2B expression levels: Two-sided Student's t-test, \( t = 1.163 \) df. = 20, \( P = 0.2587 \) n=11 mice/group normalized to actin levels and represented as fold relative to the saline-treated group. (C) Representative PSD-95 and actin immunoblots and quantifications of PSD-95 expression levels (normalized to actin) relative to the saline-treated group. Two-sided Student's t-test, \( t = 2.272 \) df. = 21, *\( P = 0.0338 \) n=11-12 mice/group. Error bars denote s.e.m.
Fig. S2. The inhibition of D1R-GluN1 heteromerization in the NAc with AAV Tet-On-GluN1C1 blocks cocaine-mediated GluN2B phosphorylation and ERK activation. (A) Top: Time frame of AAV-Tet-On injection, doxycycline (dox) treatment and saline or cocaine (15 mg/kg) administration. Mice were perfused 10 min after a single injection of saline or cocaine. Bottom: Representative images of RFP expression and GluN2B phosphorylation (pGluN2B) in saline or cocaine-treated mice infected with Tet-On-GluN1C1Δ or Tet-On GluN1C1. Scale bar: 30 μm. (B) Quantifications of pGluN2B in RFP positive neurons infected with the Tet-On-GluN1C1Δ (C1Δ) or Tet-On-GluN1C1 (C1) AAVs represented as fold relative to the saline-treated group infected with C1Δ. Two-way ANOVA: virus effect, F (1, 12) = 10.36, **P =0.0083, saline C1Δ vs cocaine C1Δ; ##P=0.0035, cocaine C1 vs cocaine C1Δ, n=4 mice/group. (C) Quantifications of ERK phosphorylation (pERK) in RFP positive neurons infected with the Tet-On-GluN1C1Δ (C1Δ) or Tet-On-GluN1C1 (C1) AAVs represented as fold relative to the saline-treated group infected with C1Δ. Two-way ANOVA: virus effect, F (1, 12) = 7.031, *P =0.0164, saline C1Δ vs cocaine C1Δ; ##P=0.010, cocaine C1 vs cocaine C1Δ, n=4 mice/group. Error bars denote s.e.m.
Fig. S3. Virally-mediated tagging of D1R- and D2R-MSN. (A) Illustrative coronal sections obtained from mice injected with the mixture of AAV-PPTA-Cre and AAV-DIO-GFP in the NAc and the dorso-medial striatum to tag D1R-MSN. Note that, this combination of AAV yielded, as expected, a GFP signal in projections structures of D1R-MSN, e.g. the GPi, the SNR and the VTA, as expected for D1R-MSN. (B) Mice injected with the mixture of AAV-PPE-Cre and AAV-DIO-GFP in the NAc and the dorsal striatum to tag D2R-MSN. This combination of AAV leads to GFP staining in the GPe and VP, but in the SNR and the VTA. A D1R immunolabeling (red) was performed to facilitate the visualization of distinct brain regions. Abbreviations: 3V: 3rd ventricle; AcbC: accumbens nucleus core; Cpu: Caudate putamen; Cx: cortex; GPe: external globus pallidus; GPi: internal globus pallidus; HPC: hippocampus; ic: internal capsule; LAcbSh: lateral accumbens shell; LV: lateral ventricle; mAcbSh: medial accumbens shell; Tu: olfactory tubercle; VP: ventral pallidum; Rt: reticular nucleus; SNR: substantia nigra, pars reticulata; SNC: substantia nigra, pars compacta; VTA: ventral tegmental area
Andrianarivelo et al. Figure S4

A

B

C

D

E

Weight (g)
(Days)

Basal locomotor activity (1/4 turn/60min)
(Days)

Locomotor activity (1/4 turn/60min)
(Days)

Tet-On-GluN1C1

Tet-On-GluN1C1

saline

cocaine

dox

sal. C1
coc. C1

Basal locomotor activity (1/4 turn/60min)

Locomotor activity (1/4 turn/60min)

sal. C1
coc. C1

sal. C1
coc. C1

n.s

Tet-On-GluN1C1

Tet-On-GluN1C1

saline

cocaine

dox

sal. C1
coc. C1

sal. C1
coc. C1

n.s

Andrianarivelo et al. Figure S4
Fig.S4. Innocuity of doxycycline treatment in mice that are not injected with an AAV-Tet-On and impairment of the development, but not maintenance, of cocaine-induced locomotor sensitization upon inhibition of D1R-GluN1 heteromerization in the dorsal striatum. (A) Mice were treated for 7d with doxycycline (dox) prior to and during body weight measurement. Two-way ANOVA: doxycycline effect, F (1, 30) = 0.6105, P >0.999, dox vs vehicle, n=16 mice/group. (B) Lack of effect of dox administration on basal locomotion. Two-sided Student's t-test, t=0.356 df. = 30, P =0.723 n=16 mice/group. (C) Cocaine-induced locomotor sensitization of mice treated or not with dox prior and during cocaine injections. Two-way ANOVA: doxycycline effect, F (1, 13) = 0.152, n. P >0.999, cocaine dox vs cocaine vehicle locomotion on d5, n=7-8 mice/group. (D) Experimental time frame to study the consequences of inhibiting D1R-GluN1 heteromerization in the dorsal striatum on basal locomotion and development of cocaine-induced locomotor sensitization. Basal locomotor activity: Two-sided Student's t-test, t = 1.653 df. = 29, P =0.1092, n=15-16 mice/group. Cocaine-induced locomotor sensitization: Three-way ANOVA: virus effect, F (1, 224) = 9.643, **P<0.001, n.s P>0.999, n=6-10 mice/group. (E) Experimental design to study the role of D1R-GluN1 heteromerization in the dorsal striatum on the maintenance of cocaine-induced locomotor sensitization. Locomotor activity in each group: Three-way ANOVA: virus effect, F (1, 243) = 0.2522, n.s P >0.999, n=7-8 mice/group. n.s not significant. Error bars denote s.e.m.
Fig.S5. Analysis of the PLA signal from human post-mortem caudate putamen samples. (A) From a whole-slide image of human caudate putamen slice (left), the ZEN software allows the extraction of 25 high magnification images randomly selected in a region of interest corresponding to the nucleus accumbens (middle). Extracted images are used to automatically detect the PLA signal (right). (B) Example image of the D2R-GluN2B PLA signal (left) and its detection by a custom-made macro for local maxima detection (middle panel). Right panel: example image showing the absence of PLA signal in the negative control where the GluN2B antibody is omitted during the PLA. (C) Same as B in the case of D2R single recognition. Abbreviations: Cdt: caudate; NAc: nucleus accumbens; Ptm: putamen;
### Table S1. Detailed human subject information. PMI: post-mortem interval

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**Control samples**

- **Gender**: Male
- **Age**: 28
- **Cause of death**: Suicide
- **PMI**: 16
- **pH value**: 6.93
- **Substances at death**: Nil
- **Axis 1 dependence**: Nil

**Samples from addict individuals**

- **Gender**: Male
- **Age**: 39
- **Cause of death**: Suicide
- **PMI**: 36.75
- **pH value**: 6.74
- **Substances at death**: Cocaine and metabolites
- **Axis 1 dependence**: Substance dependence (alcohol)

- **Gender**: Female
- **Age**: 51
- **Cause of death**: Suicide
- **PMI**: 33.75
- **pH value**: N/A
- **Substances at death**: Ethanol, cocaine and metabolites
- **Axis 1 dependence**: Substance dependence
Introduction Article II

Multiple evidences indicate that nuclear calcium-dependent signaling is a main route for synapse-to-nucleus communication, which plays a key role in numerous models of transcription-dependent long-term neuronal adaptations. In hippocampal neurons, nuclear calcium transients, generated as a consequence of increases in neuronal activity, have been shown to shape a transcriptional program favoring neuroprotection, long-term synaptic plasticity and memory consolidation (Bading, 2013). In spinal excitatory neurons, nuclear calcium signaling also participates to pathological forms of long-lasting neuroadaptations such as the transition from acute to chronic inflammatory pain (Simonetti et al., 2013). Because striatal signaling is a key driver of pathological drug-adaptive behavior, the main objective of the present work was to investigate the dynamics and roles of nuclear calcium signals in striatal neurons in cocaine-evoked responses.

We first studied nuclear calcium dynamics owing to a genetically-encoded nuclear calcium sensor, GCaMP3-NLS, in cultured striatal MSN. Neurons were treated with a D1R agonist in the presence of a low dose of glutamate, a co-stimulation paradigm that recapitulates the main features of signaling events induced by acute cocaine in vivo in the striatum (Cahill, Pascoli, et al., 2014; Pascoli et al., 2011). We found that this co-stimulation triggered a fast NMDAR-dependent increase in nuclear calcium concentration. By contrast, incubating striatal neurons with a D1R agonist or a low glutamate concentration separately, did not yield nuclear calcium changes, therefore demonstrating a synergistic effect of D1R and NMDAR stimulation on nuclear calcium signals. Furthermore, we demonstrated that this synergy requires the D1R/GluN1 heteromerization. Thereafter, we performed ex vivo studies which revealed that this synergistic effect of D1R and NMDAR stimulation on nuclear calcium was also detectable in acute striatal slices expressing GCaMP3-NLS, specifically in NAc D1R-MSN. Next, nuclear calcium imaging was performed in freely-moving mice, which revealed that acute cocaine administration triggered a sustained increase of nuclear calcium in NAc D1R-MSN in vivo.

We then studied the role of nuclear calcium-dependent signaling in D1R-MSN and D2R-MSN on cocaine-evoked molecular, cellular and behavioral responses. To do so, we selectively expressed in NAc D1R-MSN or D2R-MSN, the protein CaMBP4, a nuclear Ca^{2+}/CaM-dependent signaling blocker. This allowed us to show that nuclear calcium-dependent signaling in D1R-MSN, but not in D2R-MSN, alters the rewarding effects of cocaine. In addition, we demonstrated that the increase in dendritic spine induced by cocaine in D1R-MSN requires the activation of nuclear calcium signaling in this MSN subtype. Finally, we established that nuclear calcium-signaling in D1R-MSN is necessary for cocaine-induced expression of c-Fos and Arc IEGs and ERK activation.

Our study reveals a central role of nuclear calcium-dependent signaling in D1R-MSN in response to cocaine, inducing molecular, cellular and behavioral adaptations. Altogether, these results represent a significant progress as it contributes to a better understanding of the molecular basis addiction.
Article II

Manuscript to be submitted in September 2021 to Biological Psychiatry

Full title:
Nuclear calcium signaling in D1 receptor-expressing neurons of the nucleus accumbens regulates molecular, cellular and behavioral adaptations to cocaine

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Key words: striatum, medium-sized spiny neurons (MSN), signaling, nuclear calcium, extracellular signal-regulated kinase (ERK), addiction

Running title: Cell-type-specific role of nuclear calcium in addiction
Article II

Abstract

Background: Cocaine-induced long-lasting behavioral adaptations depend on gene regulations within the reward circuit. In particular, activation of the extracellular signal-regulated kinase (ERK) in the striatum is known to launch a transcriptional program shaping long-term adaptations to cocaine. Nuclear calcium signaling has also been shown to control multiple forms of transcription-dependent long-lasting neuroadaptations in physiological and pathological conditions. However, the dynamics and roles of nuclear calcium signaling in the context of addiction remains unknown.

Methods: Nuclear calcium imaging was performed in striatal neurons in vitro and in vivo in cocaine-treated mice, using a genetically-encoded cell-type-specific nuclear calcium probe. Stereotaxic injections of a cell-type-specific inhibitor of nuclear calcium signaling, combined with immunohistochemistry, cellular imaging and behavioral studies were used to unravel the role of nuclear calcium in medium-sized spiny neurons (MSN) expressing the dopamine D1 (D1R) or D2 (D2R) on cocaine-evoked adaptations.

Results: The D1R-mediated potentiation of calcium influx through glutamate N-methyl-D-aspartate receptor (NMDAR), which drives cocaine-induced ERK activation and behavioral adaptations, also generates nuclear calcium transients in vitro. Accordingly, in cocaine-treated mice a sustained nuclear calcium increase was observed in NAc D1R-MSN using fiber photometry. Blocking nuclear calcium in D1R-MSN, but not in D2R-MSN, impaired cocaine-evoked morphological changes of MSN, gene expression, and ERK activation in D1R-MSN.

Conclusions: Our study unravels a key role of cocaine-induced nuclear calcium signaling increase in D1R-MSN on molecular, cellular and behavioral adaptations to cocaine, and brings a significant breakthrough as it could contribute to the development of innovative strategies with therapeutic potentials to alleviate addiction symptoms.
Introduction

Drug addiction is defined a compulsive pattern of drug-seeking- and taking- behavior despite deleterious consequences and recurrent episodes of abstinence and relapse. Addictive drugs hijack reward processing by increasing dopamine (DA) in the mesocorticlimbic system (1), which persistently modulates excitatory glutamate transmission within the reward circuitry, especially in the nucleus accumbens (NAc) (2)(3). The NAc is a key target structure of drugs of abuse, notably because it receives converging glutamate inputs from limbic, thalamic and cortical regions, encoding components of drug-associated stimuli and environment, together with DA signals that mediate reward prediction error and incentive values. Signal integration is achieved by GABAergic striatal medium-sized spiny neurons (MSN) receiving glutamate and DA axons congregating onto their dendritic spines (4)(5).

Based on the preferential expression of either DA-D1 (D1R) or DA-D2 (D2R) receptors, MSN primarily form two segregated populations (6)(7) with distinct roles in reward processing and drug-adaptive behavior (8)(9). It is well-acknowledged that drug-evoked changes in synaptic transmission in the NAc trigger cell-type-specific activation of intracellular signaling pathways driving genetic and epigenetic responses likely participating to the transition from casual to compulsive drug intake and addiction (9)(10). Accordingly, virtually all drugs of abuse have been shown to activate the extracellular-signal regulated kinase (ERK) pathway in the striatum (11). This activation specifically occurs in D1R-MSN (12) and requires the stimulation of both D1R and glutamate receptors of the NMDA subtype (NMDAR; (13)(14), which makes ERK activation a coincidence detector of glutamate and DA signaling (15). We previously showed that a D1R-mediated facilitation of NMDAR functions was the trigger for a calcium-dependent activation of ERK by cocaine in the striatum in vivo (16). Once activated locally in dendrites, ERK translocates to nucleus (17)(18), where it controls cocaine-induced immediate early gene (IEG) expression and morphological changes of MSN, notably through the direct and indirect targeting of the transcription factors Elk-1(19). This cytoplasm-to-nucleus shuttling has important functional consequences since inhibiting the potentiation of NMDAR by D1R driving ERK activation, or pharmacological blockade of ERK activity, blunts the sensitizing and rewarding effects of cocaine (13)(16) and the reconsolidation of drug-associated memories (20)(21)(22).

Signal propagation along the synapse-to-nucleus axis is not solely mediated by calcium-mediated activation of the ERK pathway. Multiple evidence indicates that calcium ions are themselves key actors of the synapse-to-nucleus communication. Calcium signals generated by synaptic activity, as well as the opening of NMDAR and voltage-gated calcium channels (VGCC), evoke calcium signals within the nucleus (23). The generation of such nuclear calcium transients is a major signaling endpoint in synapse-to-nucleus communication and functions as a master switch for transcription-dependent neuronal plasticity in multiple models of long-term adaptations in the brain (24). For instance, nuclear calcium signaling has been shown to sustain ‘acquired neuroprotection’ in hippocampal neurons (25), an activity-driven form of adaptation in which neurons that have been electrically activated are more resistant to cell death-inducing conditions. Similarly, the consolidation of memories, both in the fruit fly Drosophila melanogaster and in rodents, as well as the development of chronic pain in mice are critically dependent on nuclear calcium signaling (26)(27)(28). Based on multiple similarities, both in concepts and mechanisms, between cellular processes underlying addiction and those responsible for other forms of long-
lasting adaptations in the brain, we investigated the dynamics of roles of nuclear calcium signaling in the NAc in cocaine-mediated adaptations in vivo.

Cell-type-specific imaging of nuclear calcium signals revealed that the facilitation of NMDAR functions by D1R stimulation, which is critical for cocaine-evoked adaptive behavior (14)(16)(22), triggers nuclear calcium transients in NAc D1R-MSN. Furthermore, freely-moving mice exposed to cocaine display a sustained increase of nuclear calcium in D1R-MSN. In drug-naïve mice, inhibition of nuclear calcium signaling in either MSN subtype, does not compromise the survival rate integrity of MSN and spared basal locomotor activity. By contrast, the rewarding properties of cocaine were impaired upon blockade of nuclear calcium signaling in D1R-MSN, whereas the same manipulation in D2R-MSN had no effect. We also established that nuclear calcium blockade in D1R-MSN fully inhibits cocaine-evoked morphological changes of this MSN subtype and IEGs expression. Finally, we highlight that nuclear calcium signaling exerts a permissive role on the activation of the ERK pathway by cocaine. These findings unravel the key role of nuclear calcium signals in D1R-MSN in molecular and cellular adaptations induced by cocaine and constitute a significant breakthrough in our understanding of the molecular basis of addiction.
Materials & Methods

Animals & treatment
6-week-old C57BL/6J male mice were housed four per cage, in a 12-hour light-dark cycle, in stable temperature (22°C) and humidity (60%) conditions with ad libitum access to food and water. They were acclimatized to the animal facility for at least 1 week. All experiments were carried out in accordance with the standard ethical guidelines (European Community Council Directive on the Care and Use of Laboratory Animals (86/609/EEC) and the French National Committee (2010/63)). Intraperitoneal injections (i.p) of cocaine (15mg/kg) dissolved in a saline solution (0.9% NaCl w/v) were performed in a volume of 10 ml/kg.

Nuclear calcium imaging in vivo
Stereotaxic injections were performed in the NAc with a serotype ½ recombinant AAV driving expression of the calcium probe GCaMP3 targeted to nuclei owing to the fusion of three nuclear localization signals (NLS) (27)(29) in a Cre-recombinase-dependent manner (AAV-DIO-GCaMP3-NLS). D1R-MSN-specific expression of GCaMP3-NLS was achieved by co-injecting AAV-DIO-GCaMP3-NLS with AAV-PPTA-cre, which driving the expression of the Cre recombinase under the D1R-MSN-specific preprotachykinin promoter (30). Three weeks after stereotaxic injections of AAV, a chronically implantable cannula composed of a bare optical fiber and a fiber ferrule was implanted at the location of the viral injection site (1.7 mm rostral to the bregma, 1.2 mm lateral to midline and 4.6 mm ventral to the skull). Mice were then habituated to the open field arena. On test day, the fiber was connected but not switched on for habituation to the open field for at least for 30 min. Recordings started 10 min before the i.p. injection of cocaine or saline and lasted for 30-35 min post-injection. For each trial, ΔF/F signal analysis was performed from -5 to +20 min around the time of injection.

Cell-type-specific inhibition of nuclear calcium signaling
The AAV-DIO-mScarlet-T2A-CaMBP4-HA (AAV-mScarlet-CaMBP4) has been injected into the NAc to drive a Cre-recombinase-dependent bicistronic expression of the fluorescent reporter protein mScarlet and CaMBP4 fused to a hemagglutinin tag (HA). CaMBP4 is a nuclear peptide bearing several nuclear localization sequences (NLS) that binds to the Ca2+/CaM complexes and inhibits nuclear Ca2+/calmodulin-dependent transcription (25)(26)(31)(32). To alter nuclear calcium signaling in D1R-MSN or D2R-MSN, mice were injected in the NAc with a mixture of AAV-mScarlet-CaMBP4 and AAV-PPTA-Cre (D1R-MSN-specific expression of Cre recombinase) or the AAV-PPE-cre, which allows a D2R-MSN-specific expression of the Cre recombinase (33). As a control, we co-injected the AVV-mScarlet-T2A-3XNLS, driving the a bicistronic expression of 3 NLS repeats, together with AAV-PPTA-Cre or AAV-PPE-cre. All experiments were carried out 3 weeks after stereotaxic injections.

Conditioned place preference
Conditioned place preference (CPP) was performed in a two-compartment Plexiglas Y-maze apparatus. On day 1 (pre-test session), mice were placed in the center of the apparatus and allowed to explore freely both compartments. Time spent in each compartment was automatically recorded. On day 2, mice were injected with saline and placed immediately in the assigned closed compartment for 20 min. After 1h, mice were injected with saline or cocaine and placed in the other closed compartment. This was repeated on day 3. On day 4 (test session) mice had a free
access to both chambers. The CPP score was calculated as the difference between the time spent in each compartment during day 4 minus the same time difference but on day 1.

**Tissue preparation**
Mice were anesthetized with an i.p injection of Euthasol (100 mg/kg) and perfused transcardially with 0.1 M Na2HPO4/Na2HPO4, pH 7.5 containing 4% PFA at 4°C delivered with a peristaltic pump at 20 ml/min for 5 min. Brains were then extracted, post-fixed overnight in 4% PFA, and stored at 4°C. 40 μm-thick coronal sections were performed with a vibratome and kept at -20°C in a cryoprotective solution containing 30% ethylene glycol (v/v), 30% glycerol (v/v) and 0.1 M PBS until slices were processed for immunohistochemistry. 3D-analysis of MSN dendritic spine density was performed through gene gun-mediated delivery of the green lipophilic plasma membrane dye DIO to stochastically label dendritic fragments (34).

See supplementary information for detailed experimental procedures and statistical analysis.
Results

Synergistic action of D1R and NMDAR stimulation on nuclear calcium transients in cultured MSN in vitro

A prerequisite for the study of the role of striatal nuclear calcium signaling in cocaine-evoked adaptations in vivo was to characterize the yet unknown dynamics of nuclear calcium in the striatum. We first used an easy-to-handle model of cultured striatal neurons stimulated with a low dose of glutamate (0.3 mM) or the D1R agonist SKF38393 (3 mM) used separately or in combination, a treatment hereafter referred to as co-stimulation. We previously validated this co-stimulation paradigm as instrumental for the identification of striatal signaling events controlling long-lasting behavioral responses to cocaine in vivo (16)(35). Notably, this approach allowed us to show that D1R stimulation triggers the facilitation of calcium influx through GluN2B-containing NMDAR, which drives downstream ERK activation and cocaine-evoked adaptations in vivo (16)(35). Herein, we asked whether the D1R-mediated facilitation of NMDAR could yield calcium transients in nuclei. Cultured MSN were infected with AAV-GCaMP3-NLS encoding the calcium probe GCaMP3 targeted specifically to nuclei owing to the fusion of three nuclear localization signals at its C-terminus end, as already described (29)(27) (see Figure 1A). We found that glutamate or SKF38393 applied alone to cultured neurons did not trigger any fluorescence intensity changes whereas the co-stimulation produced a transient, but significant, increase in nuclear calcium signals that was blocked by the NMDAR antagonist APV (Figure 1A-C).

The heteromerization (i.e. direct interaction) of D1R with NMDAR GluN1 subunits has been shown to control the D1R-mediated potentiation of NMDA currents, ERK activation and the sensitizing and rewarding properties of cocaine (35)(36). We thus asked whether D1R-GluN1 heteromerization could participate to this synergistic action of D1R and NMDAR on nuclear calcium signals. This appeared to be the case since a cell-penetrating interfering peptide (TAT-GluN1C1), which disrupts D1R-GluN1 interaction (35), blocked nuclear calcium transients induced by the co-stimulation of D1R and NMDAR. By contrast, the control peptide (TAT-GluN1C1C), which spares D1R-GluN1 interaction (35), preserves nuclear calcium increases induced by the co-stimulation paradigm (Figure 1D-F). Altogether, these results show that the D1R-mediated facilitation of NMDAR, which we identified as a key event for cocaine response in vivo (16), triggers calcium transients within nuclei of cultured MSN.

Cocaine induces a sustained increase of nuclear calcium signals in D1R-MSN in vivo

In anesthetized mice, cell-type-specific imaging of whole-cell calcium signals revealed that calcium concentrations increase in D1R-MSN, but not D2R-MSN, in response to a single cocaine injection (37). This calcium rise was progressive and sustained, which drastically differs from the transient calcium changes observed in cultured MSN (16)(35) (see Figure 1), where the segregation of MSN subtypes is not observed. This led us to study, in a cell-type-specific manner, changes in nuclear calcium signals in a more physiological ex vivo model of cocaine exposure that we recently validated (30). Nuclear calcium imaging was performed from acute striatal slices prepared from adult mice incubated with either glutamate (3 mM) or SKF38393 (10 mM) or both. In this model, where D1R- and D2R-MSN are largely segregated, this co-stimulation paradigm triggers main adaptations induced by cocaine in D1R-MSN in vivo, including increase in dendritic spine density, ERK activation and downstream IEGs induction (30). To monitor nuclear calcium
in D1R-MSN, mice were injected in the NAc with a mixture of AAV-DIO-GCaMP3-NLS and the AAV-PPTA-cre, driving the expression of the Cre recombinase under the D1R-MSN-specific preprotachykinin promoter (30)(33)(38). Using two-photon imaging, we observed that glutamate or SKF38393 used alone did not impact nuclear calcium contents in D1R-MSN. By contrast, the co-stimulation induced a progressive and sustained nuclear calcium increase in D1R-MSN (Figure2A-C), as observed for global calcium signal in D1R-MSN in anesthetized mice exposed to cocaine (37).

More recently, calcium imaging in freely-moving mice established that a single cocaine injection triggered an increased frequency of calcium transients in D1R-MSN (39). We thus probed whether this increased occurrence of calcium events could affect the nuclear calcium pool in D1R-MSN. Mice were injected with AAV-DIO-GCaMP3-NLS and AAV-PPTA-cre and nuclear calcium dynamics was monitored in the NAc by fiber photometry before and after a saline or a cocaine injection (Figure 2D). A single cocaine injection triggered a progressive increase of nuclear calcium signals in D1R-MSN during the first 10 min post-injection, which reached a plateau between 10 to 20 min post-injection (Figure 2D). This increase was significant when compared to mice receiving saline that do not displayed any persistent changes in nuclear calcium signals (Figure 2E), thus demonstrating that cocaine activates nuclear calcium signaling in D1R-MSN in vivo.

Nuclear calcium signaling in D1R-MSN, but not D2R-MSN, is involved in the rewarding effects of cocaine

In light of the effects of cocaine on nuclear calcium in NAc D1R-MSN in vivo, we investigated the role of the calcium pool in cocaine-evoked molecular, morphological and behavioral adaptations. We used the CaMBP4 protein, which contains four repeats of the M13 calmodulin (CaM) binding derived from the rabbit skeletal muscle myosin light chain kinase (31). As CaMBP4 bears several nuclear localization signals (NLS)(40), this protein binds with a high affinity to the Ca2+/CaM complexes in the nucleus and inhibits nuclear Ca2+/calmodulin-dependent transcription, as previously described(25)(26)(31)(32). We designed AAV-DIO-mScarlet-P2A-CaMBP4-HA (AAV-mScarlet-CaMBP4) to drive a Cre-recombinase-dependent bicistronic expression of the fluorescent reporter protein mScarlet and CaMBP4 fused to a hemagglutinin tag (HA). To alter nuclear calcium signaling in D1R-MSN, mice were injected in the NAc with AAV-mScarlet-CaMBP4 and AAV-PPTA-Cre (Figure 3A). As a control, we co-injected the AVV-mScarlet-NLS, driving the bicistronic expression of 3 NLS repeats, together with AAV-PPTA-Cre. Three weeks after stereotaxic injections, D1R-MSN infected with AVV-mScarlet-CaMBP4 displayed a strong somatic expression of mScarlet and lesser fluorescence levels in dendrites, whereas the HA immunostaining confirmed the nuclear localization of CaMBP4, as expected (Figure 3B).

Nuclear calcium signaling has been shown to control a genetic program involved in neuroprotection in cultured hippocampal neurons (25). We thus assessed the integrity of NAc D1R-MSN expressing CaMBP4 compared to the ones expressing NLS. Analysis of the morphology of nuclei counterstained with Hoechst (Figure 3D) and expression of the nuclear neuronal marker (NeuN; Figure 3E) revealed that nuclear calcium blockade preserved the viability of D1R-MSN in vivo. At the behavioral level, inhibition of nuclear calcium signaling in NAc D1R-MSN did not alter basal locomotor activity when compared to the control group (Figure 3F). We then tested whether cocaine-evoked increase of nuclear calcium in D1R-MSN could contribute to
the rewarding effects of cocaine using the conditioned place preference (CCP) paradigm. As expected, mice infected with the control AAV developed a significant cocaine induced CPP (Figure 3G). This was not the case for mice expressing CaMBP4 in D1R-MSN (Figure 3H), thus indicating that activation of nuclear calcium signaling in D1R-MSN participates to the development of the rewarding effects of cocaine.

Because calcium signals are differentially modulated by cocaine in both MSN populations and play distinct roles in the formation of associations between cocaine and contextual cues (37)(39), we also studied the consequences of nuclear calcium blockade in D2R-MSN. We verified that inhibition of nuclear calcium-dependent signaling in NAc D2R-MSN preserved their viability (Figure 4A-C) and spared basal locomotor activity (Figure 4D). In direct contrast to what we observed for D1R-MSN, nuclear calcium blockade in D2R-MSN did not alter the rewarding properties of cocaine since mice expressing CaMBP4 in NAc D2-R-MSN developed a significant CPP (Figure 4E-F). Altogether, this demonstrates that inhibition of nuclear calcium signaling does not impact MSN viability or basal locomotor activity. By contrast, nuclear calcium blockade in D1R-MSN, but not in D2R-MSN, impairs the rewarding effects of cocaine.

**Cocaine-evoked morphological changes of D1R-MSN require nuclear calcium signaling**

Acute or repeated cocaine administrations trigger an increase of dendritic spine density that is confined to D1R-MSN (30)(41), leading to an enhancement of glutamatergic connectivity of NAc D1R-MSN (30)(42). This increased dendritic spine density has been shown to strictly depend on the activation of the ERK pathway downstream D1R and NMDAR stimulation (43). Because, nuclear calcium-CaMKIV signaling controls neuronal differentiation and spine density in the hippocampus (44)(26), we asked whether nuclear calcium could contribute to the cocaine-evoked morphological changes in D1R-MSN. Three weeks before being subjected to five daily saline or cocaine injections, mice were injected with AAV-mScarlet-CaMBP4, or related control AAV, together with AAV-PPTA-Cre or PPE-Cre to alter nuclear calcium signals in D1R-MSN or D2R-MSN, respectively (Figure 5A; left). Based on the mScarlet signal, we were not able to analyze spine density in identified MSN subpopulation. The density of mScarlet-positive cells was indeed too high to detect isolated dendritic fragments and the fluorescence intensity in dendrites was too dim to accurately detect spines in identified D1R- or D2R-MSN. As an alternative, we chose a gene gun-mediated delivery of the green lipophilic plasma membrane dye DIO to stochastically label dendritic fragments of both MSN subtypes upon inhibition of nuclear calcium in either D1R or D2R-MSN (Figure 5A; right). As expected, mice infected with the control AAV in D1R-MSN displayed a cocaine-mediated increase of dendritic spine density. This increase was fully blocked upon inhibition of nuclear calcium signaling in D1R-MSN (Figure 5B). By contrast, the increase of MSN spine density induced by cocaine was spared when CaMBP4 was expressed in D2R-MSN (Figure 5C). These results establish that the increase in dendritic spine induced by cocaine in D1R-MSN requires the activation of nuclear calcium signaling in this MSN subtype.

**Nuclear calcium-dependent signaling controls cocaine-mediated immediate early induction and ERK activation.**

Multiple evidence supports that nuclear calcium signaling is a main route linking changes in synaptic activity to transcription in multiple models of long-lasting neuronal adaptation (24). We thus interrogate its functional role on cocaine-mediated gene expression. We focus on two
immediate early genes (IEGs), c-fos and arc, which induction is involved in the development of enduring behavioral adaptations to cocaine (9)(45)(46). Mice were injected in the NAc with the mixture of AAV used to alter nuclear calcium signaling in D1R-MSN as described above. Three weeks later, they received a single injection of saline or cocaine and were perfused 1h later to study IEGs expression levels in mScarlet-D1R-MSN (Figure 6A). In mScarlet-positive D1R-MSN infected with the control AAV, cocaine evoked an induction of c-Fos and Arc proteins, which was fully blocked upon nuclear calcium inhibition (Figure 6A-D). Because the induction of these IEGs in the striatum in response to cocaine has been shown to strictly depend on ERK activation (13)(46), we examined whether a crosstalk between nuclear calcium signaling and the ERK pathway could exist. Another cohort of mice has been injected in the NAc with the combination of AAV driving nuclear calcium inhibition in D1R-MSN and mice were sacrificed 10 minutes after a single injection saline or cocaine, which corresponds to the pic of ERK activation(13). We surprisingly found that cocaine-mediated ERK activation (i.e phosphorylation) was blocked upon inhibition of nuclear calcium in D1R-MSN (Figure 6E-F). This inhibitory effect of CaMBP4 is unlikely due to an inhibition of ERK transcription since the intensity of ERK immunofluorescence in the soma of infected D1R-MSN was not decreased in upon nuclear calcium blockade (Figure 6G-H), therefore suggesting that nuclear calcium signaling controls molecular events upstream from ERK to gate its activation by cocaine. Altogether, these results demonstrate that cocaine-induced increase in nuclear calcium controls both IEGs expression and ERK activation in NAc D1R-MSN.
Discussion

Growing evidence indicate that nuclear calcium signaling is a main route for synapse-to-nucleus communication playing a key role in multiple forms of long-term neuronal adaptations. Nuclear calcium transients in hippocampal neurons indeed drive a transcriptional program supporting neuroprotection, long-term synaptic plasticity and memory consolidation (24). In spinal excitatory neurons, nuclear calcium signaling also participates to pathological neuroadaptations since it controls the transition from acute to chronic inflammatory pain (27). Because striatal signaling is a main driver of pathological drug-adaptive behavior (9)(10), we investigated the dynamics and roles of nuclear calcium signals in striatal neurons on cocaine-evoked responses. We established that acute cocaine administration indeed triggers sustained increases of nuclear calcium in NAc D1R-MSN in vivo. We also provided multiple evidence that nuclear calcium-dependent signaling in D1R-MSN, but not in D2R-MSN, controls cocaine-evoked molecular, cellular and behavioral responses.

Nuclear calcium dynamics in the striatum was first monitored in vitro in cultured striatal neurons incubated with a D1R agonist and a low dose of glutamate, which recapitulates most of the signaling events triggered by cocaine in D1R-MSN in vivo (16)(35), including a D1R-mediated potentiation of NMDAR-dependent calcium influx and ERK activation in D1R-MSN. Of interest, these signaling events are critical for the sensitizing and rewarding effects in vivo (16). Herein, we show that the crosstalk between D1R- and NMDAR-dependent signaling, which is functionally relevant for cocaine responses in vivo (22), not only triggers ERK activation but also generates NMDAR-dependent nuclear calcium transients. Furthermore, disrupting D1R-NMDAR heteromerization, which alters the sensitizing effects of cocaine in vivo (35), also blocked nuclear calcium increases induced downstream of D1R and NMDAR, therefore opening the possibility that nuclear calcium signaling in D1R-MSN may be functionally relevant for drug-evoked responses in vivo. Of interest, cell-type-specific nuclear calcium imaging in the NAc of freely-moving mice showed that a single injection of cocaine induced a progressive and long-lasting increase of nuclear calcium in D1R-MSN in vivo, with a kinetic paralleling the one reported for whole-cell calcium imaging performed in D1R-MSN after acute cocaine administration (37). By contrast, in D2R-MSN, cocaine and cocaine-associated cues have been shown to decrease calcium levels (37)(39). Since autonomous nuclear calcium transients, independently on cytosolic calcium rise, have not been reported yet (24), it is unlikely that cocaine exposure could increase nuclear calcium in D2R-MSN. In agreement with this hypothesis, inhibiting nuclear calcium signaling in D2R-MSN did not impact on any of the cocaine-evoked adaptations tested in our study (see below), thus supporting that nuclear calcium rise does not occur in D2R-MSN in response to cocaine or, alternatively, that they are not functionally relevant for the cocaine-induced responses studied. Altogether these data suggest that nuclear calcium increases may originate from local NMDAR-mediated calcium rises at glutamatergic synapses onto D1R-MSN, and spreads rapidly towards the nucleus as a consequence of VGCC activation by propagating excitatory postsynaptic potentials, as already proposed (24).

To characterize the roles of nuclear calcium signaling in NAc MSN, we used a cell-type-specific AAV-mediated expression of the CaMBP4 peptide, which has been shown to efficiently inhibit nuclear calcium/calmodulin complexes both in vitro and in vivo (25)(26)(27)(28). A limitation of this strategy is that it preserves calmodulin-independent functions of the nuclear calcium pool. Nuclear calcium ions are indeed able to directly bind to the EF motifs of the transcription repressor
DREAM. Upon nuclear calcium increase, the direct binding of nuclear calcium ions to DREAM relieves its binding to DNA and promotes the activation of the DRE-driven transcription in calmodulin-independent manner (47)(48). Although overexpressing CaMBP4 allowed us to highlight that nuclear calcium is key for the nuclear calcium signaling in cocaine-evoked adaptations (see below), we may have underestimated the full impact of nuclear calcium signaling by using this interfering approach.

Because nuclear calcium has been shown to promote acquired neuroprotection in cultured hippocampal neurons (25), we first assessed whether nuclear calcium blockade in vivo could alter MSN survival rate in naïve mice expressing CaMBP4 in either NAc D1R-MSN or D2R-MSN. We found that the AAV-mediated long-term expression of CaMBP4 was not harmful for either type of MSN. This is likely due to the low basal activity of MSN that did not yield spontaneous nuclear calcium transients in freely-moving naïve mice (see Fig2), as opposed to cultured hippocampal neurons displaying spontaneous bursts of activity (49), which may trigger spontaneous nuclear calcium transients. Accordingly, inhibiting nuclear calcium signaling in both MSN subtypes spared basal locomotor activity. By contrast, blocking cocaine-induced increase in nuclear calcium signaling in D1R-MSN impaired the development of cocaine-induced CPP, whereas the same manipulation in D2R-MSN did not impact on the rewarding effects of cocaine. This shows that disrupting nuclear calcium signaling in NAc D1R-MSN, alters the association between the rewarding effects of the drug and the drug-paired environment, further supporting a privileged role nuclear calcium in associative learning and memory (26)(28), but here in the context of drug reward processing.

Acute or repeated cocaine exposures enhance dendritic spine density in D1R-MSN selectively (30)(41). This is associated with an increased formation of glutamate synapses onto D1R-MSN (30), which is likely involved in the long-term effects of cocaine on glutamate transmission onto D1R-MSN and drug-adaptive behavior (22)(50). The inhibition of nuclear calcium signaling in either D1R-MSN or D2R-MSN did not impact on the dendritic spine density in saline-treated mice. By contrast, the increase in dendritic spine induced by cocaine in D1R-MSN required the activation of nuclear calcium signaling in this MSN subtype, thus agreeing with the observation that a constitutive activation of nuclear calcium-CaMKIV signaling increases spine density in hippocampal neurons (44).

At the molecular levels, the inhibition of nuclear calcium-CaMKIV signaling fully blocked the induction of the IEGs c-Fos and Arc, two neuronal-plasticity regulated genes involved in the development of cocaine-adaptive behavior (9). We previously showed that an inhibitor of the ERK pathway was also able to fully blocked the induction of these IEGs (13)(46). This led us to test a possible link between nuclear calcium signaling and the ERK pathway in D1R-MSN. This appeared to be the case since overexpression of CaMBP4 in D1R-MSN blocked the activation of ERK induced by acute cocaine in the NAc. This is unlikely due to a direct transcriptional control of nuclear calcium signaling on the gene encoding the ERK protein since we did not find any decrease of ERK protein expression in D1R-MSN infected with CaMB4. Since ERK is locally activated in dendrites in a calcium-dependent manner before its nuclear translocation (13)(18)(16), once could envision that the inhibitory effect of CaMBP4 on ERK activation could be due to an artefactual inhibition of dendritic and cytosolic calcium resulting from a leakage of CaMBP4 outside nuclei. This is unlikely since immunofluorescence detection of the HA tag fused to the CaMBP4 construct showed its absence from dendrites and confirmed its nuclear
expression. As an alternative, nuclear calcium signaling could control expression levels of one, or more, protein(s) involved in the activation of ERK induced by a single cocaine administration. However, ERK activation by cocaine is controlled by a complex signaling network, which makes the identification of molecular mechanisms linking nuclear calcium to the modulation of ERK activation difficult. For instance, we showed that cocaine triggers a calcium-dependent activation of ERK activation relying on a D1R-mediated potentiation of calcium influx through GluN2B-containing NMDAR, via the activation the tyrosine kinase Fyn (16). A control by nuclear calcium signaling of expression levels of D1R, NMDAR subunits or Fyn could therefore explain the inhibition of ERK activation by cocaine upon CaMBP4 overexpression. Other actors involved in the cocaine-induced activation of ERK are the protein Ras-GRF1 (51), which is activated downstream GluN2B-containing NMDAR (52), the cAMP-regulated phosphoprotein of 32 kDa (DARPP32), protein phosphatase 1 (PP1) and the phosphatase STEP (53). Elucidating a possible transcriptional control of these signaling proteins by nuclear calcium could unravel new mechanisms involved in cocaine-induced activation of ERK.

To conclude, our study unravels an important and key role of cocaine-induced nuclear calcium signaling increase in D1R-MSN on molecular, cellular and behavioral adaptations to cocaine, and brings a significant breakthrough as it could contribute to the development of innovative strategies with therapeutic potentials to alleviate addiction symptoms.
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References


Figure 1: Synergistic action of D1R and NMDAR stimulation on nuclear calcium transients in cultured MSN in vitro. (A) To image nuclear calcium signals, cultured striatal neurons were infected with AAV-GCaMP3-NLS, driving the expression of the calcium probe GCaMP3 in nuclei (top panels). Changes of nuclear calcium signal intensity (ΔF/F) in basal conditions and during incubations with glutamate (0.3 μM) or the D1R agonist SKF38393 (3 μM), used separately or in combination, in the absence (co-stim) or presence of the NMDAR antagonist APV (co-stim+APV; bottom panels). (B) Traces of nuclear calcium intensity changes (ΔF/F) before and during the stimulation in each group (n=15-23 wells per treatment from at least n = 5 independent neuronal cultures, SKF38393: n = 15 wells, glutamate: n = 16 wells, co-stim: n = 23 wells, co-stim + APV: n = 16 wells). (C) Quantifications of the area under the curves (A.U.C; arbitrary unit) in each group. One-way ANOVA followed by a Bonferroni post-hoc test: treatment effect, F (3,65) = 28.36, *** P < 0.001. (D) Representative pictures of GCaMP3-NLS expression (top panels) and (ΔF/F) of nuclear calcium signal in cultured MSN incubated with either a vehicle solution, a peptide disrupting D1R-GluN1 NMDAR subunit heteromerization (Tat-GluN1C1), or the related control peptide Tat-GluN1C1 prior and during the co-stim paradigm (bottom panels). (E) Nuclear calcium traces obtained in each group (n=15-23 wells per treatment from at least n = 3 independent neuronal cultures, Tat-GluN1C1=15 wells, Tat-GluN1C1=15 wells, co-stim=23 wells). (F) Quantifications of A.U.C. Nuclear calcium transients induced by the co-stim paradigm require D1R-GluN1 NMDAR subunit heteromerization. One-way ANOVA followed by a Bonferroni post-hoc test: peptide effect F(2,50) = 13.10, * P < 0.0127; *** P < 0.001. Error bars denote s.e.m; n.s: not significant.
Saint-Jour et al. Figure 2

**A**

![Image of GCaMP3-NLS and glutamate](image)

**B**

![Graph showing time course of Ca²⁺ fluorescence](image)

**C**

![Bar graph comparing AUC of glutamate, SKF38393, and co-stim](image)

**D**

![Diagram showing saline and cocaine](image)

**E**

![Graph showing time course of Ca²⁺ fluorescence](image)

**F**

![Bar graph comparing AUC of saline and cocaine](image)
Figure 2: Cocaine induces a sustained increase of nuclear calcium signals in D1R-MSN in vivo. (A) Mice were injected in the NAc with a mixture of AAV-DIO-GCaMP3-NLS and AAV-PPTA to study nuclear calcium changes in D1R-MSN by two-photon imaging in acute striatum slices incubated with either glutamate (3 μM) or SKF38393 (10 μM) or both in combination. (B) Changes of nuclear calcium signals (ΔF/F) before and during pharmacological treatments in each group (n = 4-9 slices from 4-5 mice, SKF38393: n = 6, glutamate n = 4, co-stim: n = 9). (C) Quantifications of the related area under the curves (A.U.C; arbitrary unit). One-way ANOVA followed by a Bonferroni post-hoc test: treatment effect, F (2,16) = 15.76, ** P < 0.01; *** P < 0.001. (D) Mice were injected in the NAc with AAV-DIO-GCaMP3-NLS and AAV-PPTA-Cre. Variations of nuclear calcium in D1R-MSN were monitored by fiber photometry in mice freely moving receiving a first administration of saline solution and then a single injection of cocaine (n = 5 mice per group). (F) Quantifications of related A.U.C. Two-sided Student's t-test, t=19.13 df=4, *** P < 0.001. Error bars denote s.e.m.
Saint-Jour et al. Figure 3

A

B

C

D

E

F

G

H

Survival of mScarlet positive D1R-MSN

3 weeks

mScarlet

HA (CaMBP4)

mScarlet/HA

mScarlet

Hoescht

mScarlet/NeuN

NeuN

Healthy mScarlet-positive D1R-MSN

Basal locomotion

CPP score (sec)

NeuN/mScarlet-positive D1R-MSN

NLS

CaMBP4

NLS

CaMBP4

NLS+cocaine

NLS+saline

CPP score (sec)

CaMBP4+cocaine

CaMBP4+saline

pre-test
test

pre-test
test

n.s

n.s

n.s

n.s

n.s

n.s
Figure 3: Inhibition of nuclear calcium signaling in NAc D1R-MSN alters the rewarding effects of cocaine. (A) To study the impact of nuclear calcium signaling inhibition on the viability of in D1R-MSN, mice were injected in the NAc with a mixture of AAV-DIO-mScarlet-T2A-CaMBP4-HA (CaMBP4) and AAV-PPTA-Cre. As a control, mice were infected with AAV-DIO-mScarlet-T2A-NLS-Myc (NLS) and AAV-PPTA-Cre. Neuronal survival of mScarlet-positive D1R-MSN has been studied 3 weeks after AVV injections. (B) Illustrative images of the bicistronic expression of mScarlet, which is localized in all sub-cellular compartments, whereas the immunostaining of HA tag, fused to CaMBP4, revealed it’s strictly nuclear targeting. (C) Representative images of mScarlet expression, Hoechst counterstaining, and NeuN immunolabeling obtained from mice infected with DIO-mScarlet-T2A-NLS-Myc and PPTA-Cre (top panels) or DIO-mScarlet-T2A-CaMBP4-HA and PPTA-Cre (bottom panels). Quantifications of mScarlet-positive D1R-MSN viability based on a nuclear counterstaining with Hoechst (D) and (E) the expression of NeuN. Results are expressed as ratios compared to the control group in which D1R-MSN are infected with the control AAV (NLS) (n = 3 mice per group). Hoechst: Two-sided Student’s t-test, t=0.3619 df=2, P =0.7521. NeuN: Two-sided Student’s t-test, t=3.384 df=2, P =0.0773. (F) Basal locomotor activity of mice expressing NLS or CaMBP4 in D1R-MSN n = 16 mice per group. Two-sided Student’s t-test, t=1.662 df=15, P = 0.1173. (G) CPP scores of mice infected in D1R-MSN with the NLS and treated with saline (grey) or cocaine (orange) (n = 8 mice per group). Two-way ANOVA followed by a Bonferroni post-hoc test: treatment effect, F(1,28) = 8.966, **P<0.01 for pre-test vs test cocaine groups. (H) Same as in (G) for mice expressing CaMBP4 in D1R-MSN (n = 8 mice per group). Two-way ANOVA followed by a Bonferroni post-hoc comparisons: treatment effect, F (1,28) = 8.089, P=0.3657 for pre-test vs test cocaine groups. Error bars denote s.e.m.; n.s: not significant.
Figure 4: Inhibition of nuclear calcium signaling in NAc D2R-MSN preserves cocaine-mediated conditioned place preference. (A) Top: Experimental time frame to study the impact of nuclear calcium signaling inhibition on the viability of D2R-MSN. Mice were injected in the NAc with a mixture of AAV-DIO-mScarlet-T2A-CaMBP4-HA (CaMBP4) with AAV-PPE-Cre. As a control, mice were infected with AAV-DIO-mScarlet-T2A-NLS-Myc (NLS) and AAV-PPE-Cre. Bottom: representative images of mScarlet expression, Hoechst counterstaining, and NeuN immunolabeling obtained from mice infected with DIO-mScarlet-T2A-NLS-Myc and PPE-Cre (top panels) or DIO-mScarlet-T2A-CaMBP4-HA and PPE-Cre (bottom panels). Quantifications of mScarlet-positive D2R-MSN viability based on a nuclear counterstaining with Hoechst (B) and (E) the expression of NeuN. Results are expressed as ratios compared to the control group in which D2R-MSN are infected with the control AAV (NLS) (n = 4 mice per group). Hoechst: Two-sided Student's t-test, t=0.8847 df=3, P =0.4415. NeuN: Two-sided Student's t-test, t=0.5600 df=3, P =0.6145. (D) Basal locomotor activity of mice expressing the control AVV (NLS) or CaMBP4 in D2R-MSN (n = 16 mice per group). Two-sided Student's t-test, t=1.006 df=15, P = 0.3305. (E) CPP scores of mice infected in D2R-MSN with NLS and treated with saline (grey) or cocaine (orange) (n=8 per group). Two-way ANOVA followed by a Bonferroni post-hoc test: treatment effect, F (1,28) = 6.725, **P<0.01 for pre-test vs test cocaine group. (F) Same as in (E) for mice expressing CAMBP4 in D2R-MSN (n = 7 mice per group). Two-way ANOVA followed by a Bonferroni post-hoc test: treatment effect, F (1,24) = 4.329, P =0.1112 for pre-test vs test cocaine groups. Error bars denote s.e.m; n.s: not significant.
Saint-Jour et al. Figure 5

A

B

C

DiO-mScarlet-P2A-CaMBP4-HA or DiO-mScarlet-P2A-NLS-Myc
+PPTA-cre or PPE-cre

Stochastic MSN dendrite labeling

Day -21 -2 -1 0 1 2 3 4 5

mScarlet

DiO labeling

merge

DIO-mScarlet-NLS+ PPTA-cre

DIO-mScarlet-CaMBP4+ PPTA-cre

saline

cocaine

Saline

Cocaine

Spine density

0 5 10 15 20 25

sal coc sal coc

NLS

CaMBP4

***

***

***

127
Figure 5: Nuclear calcium signaling in NAc D1R-MSN, but not D2R-MSN, controls cocaine-evoked increase in dendritic spine density of MSN. (A) To analyze the role of nuclear calcium signaling in D1R-MSN or D2R-MSN on cocaine-mediated changes of dendritic spine density in MSN, mice were injected in the NAc with a mixture of AAV-DIO-mScarlet-T2A-CaMBP4-HA (CaMBP4), or the related control virus (AAV-DIO-mScarlet-T2A-NLS-Myc; NLS), with AAV-PPTA-Cre or AAV-PPE-Cre, respectively. Three weeks after viral injections, mice were habituated (hab.) for 3 days and then subjected to a daily injection of saline or cocaine for 5 days in a new cage cage. As shown in the right panels, dendritic spine density has been studied owing to a stochastic labelling of both MSN subtypes with the lipophilic plasma membrane dye DIO (green). (B) Three D-rendering of MSN dendritic fragments obtained from mice expressing NLS or CaMBP4 in D1R-MSN and treated with saline or cocaine (n = 18-53 dendritic fragments from 4-6 mice per group). Note that nuclear calcium inhibition in D1R-MSN fully blocks cocaine-induced increase in dendritic spine density of MSN. Two-way ANOVA followed by a Bonferroni post-hoc test: treatment effect, F (1,113) = 22.19, *** P < 0.001 saline vs cocaine; virus effect: F (1,113) = 10.22, ### P < 0.001 cocaine NLS vs cocaine CaMBP4. (C) Same as in (B) except that NLS and CaMBP4 were expressed in D2R-MSN (n=20-34). Two-way ANOVA followed by a Bonferroni post-hoc test: treatment effect, F (1,115) = 46.59, *** P < 0.001 saline vs cocaine; virus effect: F (1,115) = 3.011, P > 0.9999 cocaine NLS vs cocaine CaMBP4. Error bars denote s.e.m.
Figure 6: Nuclear calcium-dependent signaling controls cocaine-mediated immediate early gene induction and ERK activation. (A) To characterize the impact of nuclear calcium signaling blockade on immediate early gene induction by cocaine in D1R-MSN, mice were injected in the NAc with a mixture of AAV-DIO-mScarlet-T2A-CaMBP4-HA (CaMBP4), or the related control virus (AAV-DIO-mScarlet-T2A-NLS-Myc; NLS), with AAV-PPTA-Cre. Three weeks after viral injections, mice were habituated (hab.) for 5 days and then subjected to a single injection of saline (sal) or cocaine (coc) and perfused 1h post-injection. Bottom panels show illustrative images of mScarlet fluorescence and c-Fos immunostaining performed from mice expressing NLS or CaMBP4 in D1R-MSN and treated with saline or cocaine. (B) Quantifications of mScarlet- and c-Fos-positive D1R-MSN in each group (n = 4-6 mice per group). Two-way ANOVA followed by a Bonferroni post-hoc test: treatment effect, F (1,16) = 6.693, ** P<0.001 saline NLS vs cocaine NLS; virus effect: F (1,16) = 33.33, ### P < 0.001 cocaine NLS vs cocaine CaMBP4. (C) Representative pictures of mScarlet fluorescence and Arc immunostaining performed from mice expressing NLS or CaMBP4 in D1R-MSN and treated with saline or cocaine. (D) Quantifications of mScarlet- and Arc-positive D1R-MSN in each group (n = 4-6 mice per group). Two-way ANOVA followed by a Bonferroni post-hoc test comparisons: treatment effect, F (1,15) = 42.22, ### P < 0.001 cocaine NLS vs cocaine CaMBP4. (E) Mice were injected in the NAc with a mixture of CaMBP4 or NLS, with PPTA-Cre. Three weeks after viral injections, mice were habituated (hab.) for 3 days, then subjected to a single injection of saline or cocaine and perfused 10 min post-injection. Bottom panels show illustrative images of mScarlet fluorescence and phospho-ERK (pERK) immunostaining performed from mice expressing NLS or CaMBP4 in D1R-MSN and treated with saline or cocaine (n = 4-6 mice per group). (F) Quantifications of mScarlet- and pERK-positive D1R-MSN in each group. Two-way ANOVA followed by a Bonferroni post-hoc test: treatment effect, F (1,15) = 4.038, * P<0.05 saline NLS vs cocaine NLS; virus effect: F (1,15) = 9.208, ## P<0.01 cocaine NLS vs cocaine CaMBP4. (G) Illustrations of the mScarlet fluorescence and ERK immunostaining performed from mice expressing NLS or CaMBP4 in D1R-MSN and treated with saline or cocaine. (H) Quantifications of ERK immunofluorescence intensity in the soma of mScarlet-positive D1R-MSN in each group (n= 4-6 mice per group). Two-way ANOVA followed by a Bonferroni post-hoc test: treatment effect, F (1,16) = 0.06339; virus effect: F (1,16) = 10.82, * P 0.05 saline NLS vs saline CaMBP4. Error bars denote s.e.m.
Supplementary materials

Animal and drug administration
C57BL/6 mice were purchased from Janvier (Le Genest, St. Isle, France). Six-week-old males were housed 4 per cage at maximum and maintained in a 12h light/dark cycle in stable conditions of temperature (22°C) and humidity (60%) with access to food and water ad libitum. Animal were placed at the animal facility for at least one week prior the experiments for habituation. Animal care was conducted in accordance with standard ethical guidelines (NIH publication no. 85-23, revised 1985 and European Community Guidelines on the Care and Use of Laboratory Animals (86/609/EEC) and the French National Committee (2010/63)).

Cocaine hypochloride (Sigma Adrich, St. Louis, MO) was administrated intraperitoneally (i.p) in a volume of 10 ml/kg dissolved in a 0.9% NaCl (w/v) aqueous solution (saline).

Viral constructions
For in vitro nuclear calcium imaging the recombinant AAV-CKII-GCaMP3.NLS was used, which encodes the calcium probe GCaMP3 fused to three nuclear localization signals (NLS) under the control of the CaMKII promoter. For ex-vivo and in vivo cell-type-specific nuclear calcium imaging, a floxed version of a similar construct was used (AAV-EF1a:DIO-GCaMP3.NLS.myc). This virus was injected in combination with the AAV-PPTA-Cre (PPTA-cre), which contains an expression cassette encoding the Cre recombinase driven by the promoter of the D1R-MSN-specific PPTA gene (preprotachykinin) (1)(2)(3). For cell-type-specific blockade of nuclear calcium-dependent signaling, we used AAV-hSyn:DIO-mScarlet.T2A.CaMBP4.HA, which allows a Cre recombinase-dependent bicistronic expression of the fluorescent reporter protein mScarlet and the CaMBP4 peptide fused to a hemagglutinin (HA) tag. The corresponding control virus (AAV-hSyn:FLEX-mScarlet.T2A.3XNLS.myc (myc tag) allows a Cre-recombinase-dependent bi-cistronic expression on mScarlet and three NLS fused to a Myc tag. These viruses were injected with either AAV-PPTA-cre (specific expression in D1R-MSN) or AAV-PPE-cre, which drives the expression of the Cre-recombinase under the control of the D2R-MSN-specific gene PPE (preproenkephalinin) (1). All viruses were diluted in PBS pluronic 0.001%.

Stereotaxic injections
Mice were anesthetized with ketamine (150 mg/kg) and xylazine (100 mg/kg) and placed on a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA). Craniotomies were realized using the following coordinates: 1.7 mm rostral to the bregma, 1.2 mm lateral to midline and 4.6 mm ventral to the skull surface to target the NAc. Viral injections were performed bilaterally at a rate of 0.15 μl/min using a 10 μl-syringe (Hamilton 1700 series, Phymep, Paris, France) with a 200μm gauge needle (Phymep, Paris, France) mounted on a microinfusion pump (Harvard Apparatus, Holliston, MA). After the injection, the needle was left in place for an additional 8 min to avoid backflow.

Nuclear calcium imaging
-In vitro: Striatal cultures
Cultures were obtained from striata dissected out from 14-day-old Swiss mice embryos (Janvier). Cells were resuspended in Neurobasal medium supplemented with B27 (Invitrogen), 500 nM L-Glutamine, 60 μg/ml penicillin G and 25 μM β-mercaptoethanol (Sigma Aldrich) and plated at a density of 1,000 cells per mm2 into Nunc 4-well plates (Dutscher, Brumath, France; for
immunocytochemistry) or 8-μwell plates (Biovalley; for live videomicroscopy) coated with 50 μg/ml poly-D-lysine (Sigma Aldrich). The striatal primary culture obtained is then plated and placed at 37°C in a humidified atmosphere of 95% air and 5% CO2. Cultured neurons were infected at DIV6 with the AAV-GCaMP3-NLS at 6x10⁷ particles/ml. After five days, pharmacological treatments and imaging were performed.

Striatal-cultured plates were incubated for 30 min at RT in the recording medium containing in (mM): 129 NaCl, 4 KCl, 1 MgCl2, 2 CaCl2, 10 Glucose, 10 Hepes and H2O in the presence or not of APV (25 μM; Sigma Aldrich), or TAT-GluN1C1/ TAT-GluN1C1Δ peptides at 5 μM (4). After 3 gentle washes with the same buffer warmed at 37°C, plates were transferred to the spinning disk microscope (Leica, Nussloch, Germany) previously warmed up at 36.4°C for imaging. Calcium images were acquired for 90 seconds total, where the first 30 corresponded to basal signal prior pharmacological stimulation with glutamate 0.3 μM (Sigma), (R)-(+) SKF-38393 3 μM (Sigma) or co-stimulation with both. KCl (300 mM) was then added at the end of the recording to confirm that all neurons were responsive. For acquisition, 3 Z-stacks of 1 μm were performed every second. Image analysis were performed from maximum intensity Z-projection images using ImageJ software.

- Ex vivo: Striatal slices
Seven-week-old C57bl6 male mice were injected in the NAc with a mixture of AAV-DIO-GCaMP3-NLS and the AAV-PPTA-cre. After three weeks of expression, the brains were rapidly excised from mice and chilled for 2 min in calcium-free ice-cold ACSF containing (in mM): 126 NaCl, 25 sucrose, 26 NaHCO3, 1.25 NaH2PO4, 5.9 MgCl2, 2.5 KCl, 2.5 glucose and 1.5 kynurenic acid, equilibrated with 95% O2/5% CO2 (pH 7.4). Coronal slices of 200-250 μm thickness were cut on a vibratome (Leica 1200 vts) and transferred to an incubation chamber filled with equilibrated ACSF consisting of (in mM) 119 NaCl, 26.2 NaHCO3, 1 NaH2PO4, 1.3 MgCl2 (6H2O), 2.5 KCl, 2.5 CaCl2 and 11 glucose at 32°C, pH=7.4 in which they are incubated for at least 1h before imaging.

For time lapse studies, slices were laid on the glass bottom of a submerged-type chamber, which was placed on a microscope stage and continuously infused with standard ACSF gassed with 95% O2/5% CO2 (32°C, pH 7.4) at a rate of 2 ml/min. Slices were stabilize for 15 min in the chamber prior image acquisition. Then, baseline GCaMP3-NLS fluorescence intensity was recorded during 5 min of ACSF infusion. Glutamate 3 μM (Sigma) or (R)-(+) SKF-38393 10 μM (Sigma) or both compounds (co-stimulation) were then added to ACSF for 20 min. The stimulation was washed out with 5-10 min of ACSF alone to finally end with 5 min stimulation with KCl (150 mM) diluted in ACSF. Imaging was performed with a two-photon upright microscope (SP5 MPII, Leica) equipped with water-immersion objective (25x, NA 0.95, Leica). GCaMP3-NLS was excited at 900 nm and with 35 mW of laser power (Ti Sapphire, Coherent). The emitted light was filtered using band-pass filter (500-550 nm for GCaMP3) and images were acquired with hybrid detector equipped with avalanche gain and GaAsP photocathode (Leica). Images were acquired every 20 seconds in a range of 7μm with a z-step of 2μm. Maximum projection images were analyzed using Suit2P automated ROIs detection (https://www.biorxiv.org/content/10.1101/061507v1).
-In vivo: Fiber photometry

Three weeks after stereotaxic injection of mixture AAV-DIO-GCaMP3-NLS and the AAV-PPTA-cre in the NAC, a chronically implantable cannula (Doric Lenses, Québec, Canada) composed of a bare optical fiber (400 µm core, 0.48 N.A.) and a fiber ferrule was implanted at the location of the viral injection site. The fiber was fixed onto the skull using dental cement (Super-Bond C&B, Sun Medical). Real time fluorescence emitted from GCaMP3-NLS-expressing neurons was recorded using fiber photometry as described (5). Fluorescence was collected using a single optical fiber for both delivery of excitation light streams and collection of emitted fluorescence. The fiber photometry setup used 2 light-emitting LEDs: 405 nm LED sinusoidally modulated at 330 Hz and a 465 nm LED sinusoidally modulated at 533 Hz (Doric Lenses) merged in a FMC4 MiniCube (Doric Lenses) that combines the 2 wavelengths excitation light streams and separate them from the emission light. The MiniCube was connected to a Fiberoptic rotary joint (Doric Lenses) connected to the cannula. A RZ5P lock-in digital processor controlled by the Synapse software (Tucker-Davis Technologies, TDT, USA), commanded the voltage signal sent to the emitting LEDs via the LED driver (Doric Lenses). The light power before entering the implanted cannula was measured with a power meter (PM100USB, Thorlabs) before the beginning of the recording. The irradiance was ~9 mW/cm². The fluorescence emitted by GCaMP3-NLS in response to light excitation was collected by a femtowatt photoreceiver module (Doric Lenses) through the same fiber patch cord. The signal was received by the RZ5P processor (TDT). Real time fluorescence due to 405-nm and 465-nm excitations was demodulated online by the Synapse software (TDT). A camera was synchronized with the recording using the Synapse software. Signals were exported to MATLAB R2016b (Mathworks) and analyzed offline after careful visual examination of all trials for artifacts. The timing of events was extracted from the video.

Mice were habituated to the open field arena. On the day of the test, the fiber was connected but not switched on for habituation to the open field for at least 30 min. Recordings started 10 min before the i.p. injection with cocaine (20mg/kg) or saline and lasted for 30-35 min post-injection. For each trial, signal analysis was performed from -5 to +20 min around the injection. To calculate ΔF/F, a linear least-squares fit was applied to the 405 nm signal to align it to the 465 nm signal, producing a fitted 405 nm signal. This was then used to normalize the 465 nm signal as follows: ΔF/F = (465 nm signal - fitted 405 nm signal)/fitted 405 nm signal (6) . An additional bleaching correction was applied using a linear fit. Signal for all the mice were averaged for the 2 conditions. The percentage of change of the AUC was calculated between 0- and 20-min post injection.

Tissue preparation and immunohistochemistry

Mice were anesthetized with an i.p injection of Euthasol (100 mg/kg; Le Vet, Oudewater, Netherlands) and perfused transcardially with 0.1 M Na2HPO4/Na2HPO4, pH 7.5 containing 4% PFA at 4°C delivered with a peristaltic pump at 20 ml/min for 5 min. Brains were then extracted, post-fixed overnight in 4% PFA, and stored at 4°C. 40 µm-thick coronal sections were performed with a vibratome (Leica) and kept at -20°C in a cryoprotective solution containing 30% ethylene glycol (v/v), 30% glycerol (v/v) and 0.1 M PBS. On day 1, free-floating sections were rinsed three times for 5 min in Tris-buffered saline (TBS, 0.9% NaCl, 0.1 M Tris base, pH 7.5). Sections were then incubated in blocking solution containing 3% normal goat serum and 0.2% triton X-100 (Sigma Aldrich) for 2 h at room temperature (RT) before overnight 4°C incubation with primary antibodies (see antibodies section) diluted in the blocking solution. For detection of phosphorylated proteins, 50mM NaF has to be added. On day 2, after three 10-min rinses in TBS, sections were incubated for 90 min with secondary antibodies. After three 5-min rinses in
TBS, sections were incubated for 10 min with Hoechst (Invitrogen) for nuclear counterstaining. Three 5-min TBS and two Tris buffer (0.1 M Tris base, pH 7.5) washes were performed before sections were mounted in Prolong Gold (Invitrogen).

Images were taken with a confocal laser scanning microscope (SP5, Leica) using a 40X or a 63X objective. The pinhole was set to 1 Airy unit, excitation wavelength and emission range were 495 and 500-550 nm for green signal, 488 and 500-550 nm for Alexa fluo 488 and 590-650 nm for mScarlet. Laser intensity and detector gain were constant for all image acquisitions.

**Primary and secondary antibodies**

Arc expression was detected by using a mouse monoclonal antibody raised against Arc (1/30; Santa Cruz, Arc C-7). Expression of the immediate early gene c-fos was analyzed using a rabbit polyclonal antibody (1/1000; Santa Cruz Biotechnology, Santa Cruz, CA; reference sc-52). Detection of pERK and total ERK was achieved with the rabbit monoclonal diphospho-Thr-202/Tyr-204-ERK1/2 (1/400; Cell Signaling Technology, ref: 4370S) and rabbit monoclonal anti-ERK (1:500, Cell Signaling Technology), respectively. Other antibodies used include monoclonal anti-c-Myc from mouse (1:2000, Sigma Aldrich ref. M4439) and rabbit anti-HA-Tag (1:500, Cell Signaling Technology, Ref. C29F4). The secondary antibodies used goat anti-rabbit Alexa fluor 488 (1:500; Invitrogen) and goat anti-mouse Alexa Fluor 488 (1:500; Invitrogen).

**Neuronal survival**

Neuronal survival was quantified manually based on Hoechst-counterstained nuclei. Survival was defined as the percentage of viable neurons exhibiting large, uniform nuclei and even distribution of Hoechst among mScarlet-positive MSN over mScarlet-positive MSN classified as apoptotic based on, at least, two of the following criteria: condensed nuclei, single chromatin clump, small nuclei size, non-circular nuclei shape and increased Hoechst intensity.

**Cocaine-induced conditioned place preference**

The CPP was performed in a two-compartment Plexiglas Y-maze apparatus (Imetronic). Each compartment contains different visual cues and floor textures for which mice did not show any preference on average before conditioning. All sessions lasted 20 min. On day 1, mice were placed in the center of the apparatus and allowed to freely explore both compartments. Time spent in each compartment was automatically recorded. Mice spending more than 70% of the time in one compartment were excluded. On day 2, to avoid any initial preference bias, mice were randomly assigned to one or the other compartment. Mice were injected with saline and placed immediately after in the assigned closed compartment for 20 min. After 1h, mice were injected with saline or cocaine and placed in the other closed compartment (conditioning day 1). This was repeated on day 3 (conditioning day 2). The test was performed on day 4, during which mice had free access to both chambers. The CPP score was calculated as the difference between the time spent in each compartment during day 4 minus the same time difference but on day 1. All mice were perfused with 4% (w/v) paraformaldehyde (PFA) 24h post-behavioral assessment to systematically verify the accuracy of stereotaxic injections and expression of the mScarlet reporter protein. Mice that did not meet quality criterion (i.e. non-bilateral expression, off-target diffusion, excessive backflow or low mScarlet expression) were discarded from the study.
**Locomotor activity**
Locomotor activity was measured during the pre-test session of the CPP paradigm in the Y-maze apparatus (Imetronic). Three pairs of infrared beams that are facing each other (AV, VV and AR) are present in each compartment. Locomotor activity was expressed as a cumulative counts of beam breaks during 20 min.

**Spine density analysis**
Mice brains were fixed by intracardiac perfusion of 1.5% paraformaldehyde in 0.1 M Na2HPO4 /NaH2PO4 (phosphate buffer), pH 7.4 delivered with a peristaltic pump at 30 ml/min for 3 min. Brains were and post-fixed in the same fixative solution overnight at 4°C. Sections of 100 µm thickness were cut in the frontal plane using a vibratome. The labeling, imaging and analysis workflow has been extensively described previously (Heck et al. 2012) Ref BrainStructureFunction2012). Briefly, 50 mg of tungsten beads (Biorad) was mixed with 3 mg of solid green DiO (3,3'-Dioctadecyloxycarbocyanine Perchlorate) dissolved in methylene chloride. DiI-coated beads were coated on the inner surface of a PolyVinylPyrrolidone (Sigma-Adrich) pretreated Teflon tube. Helium gas pressure (150 psi) applied through the geneun eject the beads out of the cartridge onto the brain slice. Beads were delivered through a 3-µm pore-size filter (Isopore polycarbonate, Millipore) to avoid clusters. After labeling, slices were kept in PBS at RT for at least 2 h and mounted in Prolong Gold.

Images stacks were taken with a Confocal Laser Scanning Microscope (SP5, Leica) equipped with a 1.4 NA objective (oil immersion, Leica) with pinhole aperture set to 1 Airy Unit, pixel size of 60 nm and z-step of 200 nm. Excitation wavelength and emission range were 488nm and 500-550nm, respectively. Laser intensity was set so that each image occupies the full dynamic range of the detector. Images were acquired with a low noise Hybrid detector combining avalanche gain and GaAsP detection (HyD Leica). Deconvolution with experimental PSF from fluorescent beads using Maximum Likelihood Estimation algorithm was performed with Huygens software (Scientific Volume Imaging). 150 iterations were applied in classical mode, and signal to noise ratio values were set to a value of 20.

For dendritic spine analysis, the software Neuronstudio (version 0.9.92) was used to reconstruct the dendrite and dendritic spines were manually spotted. For each striatal neuron, a dendritic segment of 50–70 µm in length and distant from at least 50 µm from the soma or after the first branching point was considered.

**Statistical analysis**
Results were analyzed with Graphpad Prism (version 8.0.1). Sample size was predetermined on the basis of published studies, pilot experiments and in-house expertise. All data are displayed as mean +/- SEM. Two-tailed Student’s test was used for the comparison of two independent groups. For more than two groups comparison, one-way or two-way repeated-measures ANOVA were performed followed by the Post-hoc Bonferroni comparisons. Data distribution was assumed to be normal and variances were assumed to be homogenous. The main effect and post-hoc statistical significances are given in the appropriate figure legend for each experiment.
References
Discussion

Article I

The main objective of this work was to determine the role of heteromers formed between dopamine and glutamate receptors in the long-term adaptations induced by chronic exposure to cocaine. The first part was dedicated to studying the impact of repeated cocaine exposure on the heteromerization of these receptors. Using the “proximity ligation assay” (PLA) technique, we showed that the development of locomotor sensitization is associated with an increase in DAR/NMDAR heteromers in the NAc, without any modification of the expression levels of partner receptors. In the second part, we validated a viral approach allowing the expression of interfering peptide sequences capable of dissociating D1R/GluN1 or D2R/GluN2B heteromers in a temporally controlled manner, without altering the individual functions of each receptor independently of their heteromerization. We were thus able to show a differential role of the D1R/GluN1 and D2R/GluN2B heteromers in different stages of cocaine-induced locomotor sensitization and conditioned place preference. Our results show that D1R/GluN1 heteromers are involved in the development of morphological, synaptic and behavioral adaptations induced by cocaine. In fact, the uncoupling of D1R from GluN1 selectively blocks the development, but not the maintenance, of locomotor sensitization and of cocaine-induced CPP. Conversely, D2R/GluN2B heteromers are necessary for the maintenance of these two behaviors as well as for the development of CPP. Interestingly, we were also able to show that blocking either type of heteromer does not affect food-induced conditioned place preference. From a translational point of view, the preferential role of D2R/GluN2B heteromers in the maintenance of behavioral alterations evoked by cocaine and their lack of effect on the reinforcing properties of a natural reward make them a privileged target. This led us to set up the detection of these heteromers by PLA in human caudate putamen from subjects with a psychostimulant dependence history and matched control subjects. This allowed us to show that despite a sharp decrease of D2R expression levels, the proportion of D2R forming heteromers with GluN2B was three times higher in dependent subjects.

Modulation of DAR/NMDAR heteromers by cocaine and underlying molecular mechanisms.

In agreement with previous studies, we confirmed the presence of the D1R/GluN1 heteromers and D2R/GluN2B in dorsal and ventral striatum using the PLA technique (Cahill, Pascoli, et al., 2014; Fiorentini et al., 2003; X.-Y. Liu et al., 2006). Moreover, we were able to show that locomotor sensitization was associated with an increase in D1R/GluN1 heteromers in all of the striatal sub-regions, which were analyzed 24 hours after the last injection, since several neurophysiological studies showed that changes in glutamatergic plasticity induced by repeated exposure to cocaine in the striatum are observed at this time point (Kourrich et al., 2007; Pascoli et al., 2012). Concerning D2R/GluN2B complexes, their induction is sharper in the NAc core and shell than in the dorsal striatum.

Our preliminary data shows that after the initial increase in D1R/GluN1 heteromers observed 24 hours after the last cocaine injections, these heteromers return to a basal level after 7 days of abstinence but increase again upon re-exposure to cocaine. The decrease in heteromerization
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during withdrawal appears to correlate with the decrease in basal DA and glutamate concentrations during abstinence previously described in the literature (Baker et al., 2003; Hotsenpiller et al., 2001; Parsons et al., 1991; Pierce et al., 1996; Robertson et al., 1991), therefore, suggesting that extracellular levels of these two neurotransmitters may regulate levels of D1R/GluN1 heteromers.

Increased heteromerization induced by cocaine can be explained by several hypotheses. First, we considered that repeated cocaine exposure could alter the expression of D1R, D2R and NMDAR subunits. Using western blot analysis in total striatal lysates, we found that receptor expression levels remained unchanged after cocaine administration. To our knowledge, no study using locomotor sensitization protocol similar to ours (5 days, 15 mg/kg, analysis 24 hours after the last injection) has studied protein level expression of these receptors in the striatum. However, one study reported a decrease in the binding of radiolabeled raclopride to D2R, suggesting a 50% decrease in its expression (Thompson et al., 2010). Conversely, despite the lack of change in overall receptors expression, cocaine-induced heteromer formation may rely on an increased expression at the membrane of these receptors. Other possibility includes silent synapses. In fact, it has been shown in rats that repeated injections of cocaine (15 mg/kg, 5 days, 24 hours post-cocaine) increase the surface expression of NMDARs expressing GluN2B (GluN2B-NMDAR) subunit in the NAc generating “silent synapses” (Y. H. Huang et al., 2009), which are nascent glutamatergic synapses, containing a very high proportion of GluN2B-NMDAR and little or no AMPAR (Groc et al., 2006). In this regard, it would be interesting to test whether the silent synapses formed in response to cocaine contain a larger proportion of DAR/GluN2B-NMDAR heteromers, or if the blocking of the heteromers impacts the formation of such silent synapses in the NAc. The membrane expression of DA receptors is also altered in response to cocaine. A study has indeed shown an increase D1R expression in the striatum and a decrease in D2R, 24 hours and up to 45 days after self-administration with cocaine (Conrad et al., 2010). Taken together, these data therefore suggest that changes in surface expression of DA and glutamate receptors may, at least in part, account for the modulation of D1R/GluN1 and D2R/GluN2B heteromerization we observed in the striatum in response to cocaine.

Previous studies concerning the role of DARs or NMDARs stimulation in the formation of heteromers have led to contradictory results (for review (Andrianarivelo et al., 2019)). We observed an increase in DAR/NMDAR heteromers following repeated cocaine injections resulting in a concomitant release of DA and glutamate in the NAc (Baker et al., 2002; Parsons et al., 1991; Pierce et al., 1996; Reid et al., 1997; Y. Zhang et al., 2001). This raises the question as to how DARs and NMDARs stimulation modulate their interactions. Previous work from the laboratory on primary cultures of striatal neurons has shown that a D1R agonist induces the formation of these heteromers, while a low dose of glutamate has no effect. In contrast, co-stimulation of striatal neurons with a D1R agonist in the presence of a low dose of glutamate leads to higher heteromerization levels than with D1R agonist alone (Cahill, Pascoli, et al., 2014). These results therefore suggest a preponderant role of D1R stimulation on D1R/GluN1 heteromerization but also a synergistic role of the co-stimulation of the two partner receptors. In line with the predominant role of D1R, we obtained preliminary data showing that a single injection of cocaine, which induces the release of DA only (Di Chiara & Imperato, 1988; D. K. Lee et al., 2008), is sufficient to trigger the heteromerization of D1R with GluN1 (data not shown). These observations therefore support an important role of D1R stimulation in D1R/GluN1 heteromerization. However, the density of these heteromers observed after a single injection of cocaine is much lower than
the one observed after repeated injections, which is in favor of a synergistic effect of the stimulation of the DA and glutamate receptors in D1R/GluN1 heteromerization in vivo in the NAc.

In the case of D2R/GluN2B interaction, a high dose of cocaine has been shown to induce a significant increase in the formation of these receptor complexes in the striatum 30 min after the injection. This increase is blocked if the animal is pretreated with eticlopide, a competitive D2R antagonist (X.-Y. Liu et al., 2006), which again suggests that stimulation of D2R by DA might be necessary to form D2R/NMDAR heteromers. In agreement with these results, we have shown that, as in the case of D1R/GluN1 heteromers, D2R/GluN2B interaction is increased 24 hours after a single injection of cocaine (data not shown), but at lower levels than those observed in the locomotor sensitization paradigm. Furthermore, we found that a pretreatment with a D2R antagonist was able to block cocaine-induced D2R/GluN2B heteromerization in the NAc, suggesting a critical role of D2R stimulation in the formation of D2R/GluN2B heteromers.

In order to conclude more directly and definitively on the respective implications of the stimulation of DAR and NMDAR in the formation of heteromers in response to cocaine, we will perform injections of competitive antagonists of the D1R, D2R and NMDAR receptors administered together or separately before cocaine injections in a locomotor sensitization paradigm to analyze by PLA the levels of D1R/GluN1 and D2R/GluN2B heteromers.

Together, these data suggest that alterations of surface expression of the DA and glutamate receptors could, at least in part, account for the modifications of D1R/GluN1 and D2R/GluN2B heteromers that we observe. The increase of DAR/NMDAR heteromers may also result from changes in the lateral diffusion dynamics and synaptic localization of these receptors induced in response to cocaine. Indeed, a study carried out on ex vivo acute striatal slices showed that D1R display a dynamic lateral diffusion (Scott et al., 2006) and NMDAR stimulation decreases D1R diffusion rate, which leads to an increased proportion of D1R localized in dendritic spines as a result of D1R interaction with NMDAR at synapses.

Additionally, we have shown, in agreement with previous work (Yao et al., 2004), a decrease in the expression of PSD-95 in the NAc following repeated exposure to cocaine. This decrease could contribute to heteromer formation because PSD-95 is the only known endogenous inhibitor of D1R/GluN1 interaction (S.-J. Zhang et al., 2009). In fact, PSD-95 interacts with the C-terminal parts of D1R and GluN2B and competitively prevents D1R/GluN1 interaction. On the other hand, it has been shown that PSD-95 also interacts with D2R (P. Sun et al., 2009) and could also antagonize D2R/GluN2B heteromers. The decreased expression of PSD-95 could therefore facilitate the formation of DAR/NMDAR heteromers in response to repeated cocaine exposures. An experiment we could do to verify if the decrease in PSD-95 expression is indeed involved in the formation of these heteromers requires blocking the expression of PSD-95 locally in the striatum (using shRNA approach for example), or by overexpressing PSD-95, and compare the levels of DAR/NMDAR heteromers in those mice and control animals.

Interestingly, these heteromers can be present in different cell types. Indeed, our study shows the presence of D1R/GluN1 and D2R/GluN2B heteromers in D1R-MSN and D2R-MSN, respectively. However, NMDAR and D2R, for example, are also expressed by all interneurons and glial cells (Tepper et al., 2010). Furthermore, a subpopulation of NAc MSN has been shown to co-express D1R and D2R (Bertran-Gonzalez et al., 2008). It would be interesting to know if DAR/NMDAR...
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heteromers are also present in this particular MSN subpopulation and determine their implication in downstream signaling. Another aspect that would be interesting to analyze is whether the levels of heteromers are increased specifically in cells that express markers of cellular activity, such as pERK or c-Fos, in response to cocaine. Unfortunately, the punctate pattern resulting from heteromer PLA detection make difficult to distinguish to which cellular body belongs each identified complex. Something that can be even harder when we want to characterize heteromerization in cell populations that represent less than 5 % of the striatum as is the case of interneurons (Tepper et al., 2018). One possibility is to use FACS sorting experiments to separate different cell types (neurons, astrocytes, glial cells) or neurons activated in response to cocaine (c-Fos positive cells for example) and then perform co-immunoprecipitation (co-IP) experiments for DAR and NMDAR. However, this alternative is not trivial to set up and it would require a high number of animals in order to obtain enough quantities of sorted cells to perform the co-IPs.

Also, the PLA technique limits our ability to detect the specific region where DAR/NMDAR heteromers are formed. Some studies suggest a synaptic localization since D1R/GluN1 complexes have been detected in PSD fractions of striatal neurons by co-IP (Fiorentini et al., 2003). In addition, a previous study conducted in the laboratory established the role of D1R/GluN1 heteromers in the modulation of NMDA postsynaptic currents by DA and LTP of glutamatergic synapses projecting onto D1R-MSN, suggesting their location at the synapse (Cahill, Pascoli, et al., 2014). Conversely, an in vitro study suggested that D1R and GluN1 are already coupled in the endoplasmic reticulum and cytoplasm, and that their assembly with GluN2B would allow their surface expression at the plasma membrane (Fiorentini et al., 2003). In addition, D2R/GluN2B heteromers have also been detected in PSD fractions of striatal, hippocampal and PFC neurons (X.-Y. Liu et al., 2006). However, their presence in the presynaptic compartment is also conceivable because the IL3 sequence of D2R, necessary for the interaction with GluN2B, is also present in the presynaptic short isoform of D2R, D2S, and an expression of presynaptic GluN2B has also been reported in the hippocampus, although it remains controversial (Berg et al., 2013). Yet, the presynaptic existence of these heteromers cannot be ruled out. We could answer to this question using the PLA technique on brain slices from DS2 KO mice (Neve et al., 2019).

Data from the literature, in particular those from the group of Dr Christian Lüscher, have made it possible to establish that the different phases of behavioral alterations induced by cocaine are based on the modulation of specific glutamatergic afferents projecting on D1R-MSN (Pascoli, Terrier, et al., 2014; Salery et al., 2020). Thus, it would also be interesting to determine if DAR/NMDAR heteromers are preferentially formed at the level of synapses resulting from specific glutamatergic afferents projecting onto MSNs and if the stimulation of a glutamatergic afference can be sufficient to form heteromers in target cells. Retrograde labeling of striatal afferents and optogenetic stimulation of these terminations would make it possible to see, by fluorescent PLA, whether these heteromers are formed at the level of particular synapses in response to cocaine and whether the activation of these synapses locally generates DAR/NMDAR heteromers.

Since our work has shown an increase of D1R/GluN1 and D2R/GluN2B heteromerization in the non-operative locomotor sensitization protocol, we thought it was crucial to extend this analysis to the operant conditioning model of cocaine self-administration. This work is part of an ongoing collaboration with the team of R. Maldonado from the University of Barcelona. Interestingly, the "yoked" control animals (passively receive cocaine) and the "master" animals (self-administered cocaine), display an increase in DAR/NMDAR heteromers compared to the animals who received
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saline. Beyond the pharmacological effect of cocaine, which can be evaluated in the "Yoked" mice group, we observed that the increase in D1R/GluN1 interactions is even greater in "master" mice (Fig. 30). This suggests an instrumental learning effect that correlates with studies showing that dopamine release in the NAc is higher in animals that self-administer cocaine compared to "yoked" animals (Wydra et al., 2013). This effect is not found for D2R/GluN2B heteromers which increase at the same level in "yoked" and "master". On the other hand, the increase of these heteromers is greater in the dorsal striatum than in the NAc, which suggests a greater involvement of the dorsal striatum in instrumental tasks and could perhaps underlie the progressive transfer of plasticity induced by cocaine from the NAc to the dorsal striatum in the cocaine self-administration task (Everitt & Robbins, 2013).

This study shows that regardless the modalities, exposure to cocaine promotes heteromerization of DARs with NMDARs in the striatum. Given the differential roles of D1R/GluN1 and D2R/GluN2B heteromers on developmental and maintenance phases in non-operative behavioral responses demonstrated in article 1, we will investigate the modulation of these heteromers after the extinction and relapse phases of self-administration. Furthermore, this analysis will be extended to other structures of the reward circuit in all phases of cocaine self-administration.
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Figure 29. Cocaine self-administration is associated with an increase of heteromer formation. (A) number of muzzle entries into the active hole in a session of 2 hours/day over 10 days, therefore 5 days in forced ratio of 1 (FR1) and 5 days in FR2. Detection by PLA and quantification of heteromers (B) D1R/GluN1 and (C) D2R/GluN2B, in animals self-administered with saline or cocaine (Master) or passively receiving cocaine (Coc yoked). The animals were sacrificed 24 hours later last exposure. The PLA signal is shown as an increase ratio over the salt group. ANOVA one way. ** P <.01, *** P <.001 master vs saline; ## P <.01, ### P <.001 coc yoked vs saline; °° P <.01, °°° P <.001 Master vs coc yoked.
Role of DAR / NMDAR heteromers in cocaine-induced long-term adaptations

In order to study the roles of DAR/NMDAR heteromers, we have developed a strategy based on the use of an adeno-associated virus of serotype 9 (AAV9), which allows a doxycycline-dependent bicistronic expression of the red fluorescent reporter protein (RFP) and the C1 cassette of the C-terminal part of GluN1, (AAV-Tet-On-GluN1C1 which will be abbreviated as “C1” for the following) to prevent interaction between D1R and GluN1. Previous studies carried out in the laboratory have shown that the C1 peptide makes it possible to block heteromers in vitro, while preserving the functions of D1R (production of cAMP and activation of PKA) or of NMDAR (calcium flux) independently of their heteromerization (Cahill, Pascoli, et al., 2014). The main advantages of this construct include the use of a promoter controlled by doxycycline which allows the expression of the interfering sequence in a precise time window during which the expression of the minigen is continuous to counteract the action of endogenous peptidases. Regarding the effectiveness of this viral approach, we observed by PLA a drastic decrease in interactions between D1R/GluN1 and D2R/GluN2B in NAc neurons infected with the Tet-On-C1 and Tet-On-IL3 viruses, respectively. In agreement with previous results from the laboratory, the disruption of D1R/GluN1 heteromers blocks the phosphorylation of GluN2B and ERK induction by an acute cocaine injection. We have also shown that the IL3 virus has no effect on the functions of D2R and NMDAR, independently of their heteromerization. Indeed, expression of the interfering IL3 sequence does not alter the decrease in cAMP induced by a D2R agonist nor the amplitude and kinetics of postsynaptic NMDA currents evoked by electrical stimulation of the cortex. Altogether, these results therefore show that the strategy we have developed allows an efficient blockade of D1R/GluN1 and D2R/GluN2B heteromers in vivo in a controlled manner over time, while preserving the individual functions of the partner receptors. In order to study the impact of DAR/NMDAR heteromers on alterations in glutamatergic transmission induced in the striatum by cocaine, we used a protocol developed in the laboratory of Dr. Christian Lüscher's group (Pascoli et al., 2012; Terrier et al., 2016).

In collaboration with Jacques Barik’s group, we observed, as expected, that D1R-MSN of the NAc (visualized using a viral approach) infected with the control virus (Tet-On-C1Δ) did indeed show an increase in the AMPA/NMDA (A/N) after 10 days of cocaine withdrawal (Terrier et al., 2016). Interestingly, we were able to show that blocking D1R/GluN1 heteromers with AAV Tet-On-C1 blocks the increase in the ratio of AMPAR/NMDAR currents induced by cocaine. This is in agreement with previous laboratory results which showed that the interaction between D1R and GluN1 is central for the induction of striatal LTP in D1R-MSN (Cahill, Pascoli, et al., 2014). Cocaine-induced LTP at glutamatergic synapses onto NAc D1R-MSNs has also been shown to depend on activation of ERK pathway (Pascoli et al., 2012). In this regard, the laboratory has shown that D1R/GluN1 heteromers control the potentiation of calcium fluxes by NMDAR induced by the stimulation of D1R which triggers the activation of ERK by cocaine. (Cahill, Pascoli, et al., 2014; Pascoli et al., 2011). Thus, inhibition of ERK pathway could, at least in part, account for the blockade of LTP induced in D1R-MSN by cocaine when D1R and NMDAR are decoupled.

Interestingly, the modulation of D1R/GluN1 heteromers appears to be correlated with changes in glutamatergic plasticity induced by cocaine. In fact, the D1R/GluN1 heteromers increase on the 1st day of abstinence then decrease during withdrawal and start to rise again after re-exposure to cocaine. Kourrich and his colleagues observed a decrease in the A/N ratios in the NAc on the
first day of abstinence, which the authors say may be due to an increase in the number of surface NMDARs or their function. An increase in D1R/GluN1 heteromers 24 hours after the last injection could explain this increase in NMDAR currents. Indeed, it has been shown that blocking the interaction between D1R and GluN1 blocked the phosphorylation of GluN2B on Tyr 1472 induced by cocaine (Cahill, Pascoli, et al., 2014; Pascoli et al., 2011), a post-translational modification which promotes the surface expression of NMDARs containing the GluN2B subunit (Hallett et al., 2006). During a prolonged withdrawal, Kourrich and his colleagues, as well as the group of Christian Lüscher, observed an increase in the A/N ratio which could be attributable to an increase in the number of surface AMPARs or a decrease in NMDAR currents and/or a decrease in the surface number of NMDARs (Terrier et al., 2016). Concerning NMDAR, the decrease in D1R/GluN1 heteromers observed after 7 days of withdrawal could reflect a reduction in NMDAR function. On the other hand, Marina Wolf's group detected an increase in calcium permeable AMPARs (CP-AMPAR) between 7 and 21 days post-cocaine (Boudreau & Wolf, 2005), an increase that takes place in D1R-MSNs (Terrier et al., 2016), which enhances the efficiency of AMPAR signaling. One hypothesis is that the initial increase in D1R/NMDAR heteromers may contribute to the increase in calcium permeable AMPAR during withdrawal. Indeed, a possible mechanism that may explain the link between D1R/GluN1 heteromers and the insertion of CP-AMPAR resides in the capacity of the these heteromers to control the facilitation of calcium fluxes dependent on NMDAR in D1R-MSNs during increases in DA levels. This increased calcium entry could activate CaMKII, a kinase capable of increasing synaptic membrane expression of GluA1 through phosphorylation on serine 831, a key event in the maintenance of NMDAR-dependent LTP (Barria et al., 1997; Robison, 2014). On the other hand, the increase in Ca$^{2+}$ dependent on D1R/GluN1 heteromers could promote the surface expression of AMPARs by activating the ERK pathway (Zhu et al., 2002), which in turn inhibits phosphodiesterase 4. Inhibition of phosphodiesterase 4 increases the production of cAMP, activate the PKA pathway targeting the GluA1 subunit of AMPAR, which leads to an increased surface expression of GluA1 (R. S. Song et al., 2013). We have already shown that the D1R/GluN1 heteromers control the activation of ERK. To test this hypothesis of a link between insertion of CP-AMPAR surface expression and D1R/GluN1 heteromers, CP-AMPARs should be detected in animals injected with the C1 or C1Δ virus, 10 days after locomotor sensitization.

D2R/GluN2B heteromers have been shown to be necessary for the inhibition of NMDAR currents following stimulation of D2R (X.-Y. Liu et al., 2006). In this study, we show under basal conditions that blocking this complex does not affect A/N ratios, nor the amplitude and kinetics of NMDAR currents. These results therefore show that the viral expression of the IL3 fragment chosen to block D2R/GluN2B interaction does not alter basal glutamate transmission at the level of the synapses formed by the cortical afferents which project onto D2R-MSNs. In response to repeated cocaine injections followed by a 10-day withdrawal, it has been shown that cocaine does not alter glutamate transmission at the level of glutamatergic synapses formed by cortical afferents projecting on D2R-MSN (Terrier et al., 2016). It is for this reason that we did not study the impact of blocking D2R/GluN2B interactions after cocaine treatment, but only under basal conditions. However, in the cocaine self-administration paradigm, when animals have prolonged access to cocaine, a large increase in AMPAR current rectification index suggests an increase in CP-AMPAR, which is observed specifically at the level of synapses from the basolateral amygdala (BLA) to D2R-MSN (Terrier et al., 2016). It would therefore be interesting to study the role of D2R/GluN2B heteromers on the plasticity induced at its synapses in this operant behavioral paradigm.
The observation of a central role of D1R/GluN1 heteromers in the development of behavioral responses induced by repeated cocaine injections is in agreement with previous results from the laboratory, showing a role of these heteromers in an "acute" model of locomotor sensitization protocol induced by two cocaine injections performed at one-week interval (Cahill, Pascoli, et al., 2014). Thus, by preventing the facilitation of NMDA currents by DA in D1R-MSN (Cahill, Pascoli, et al., 2014), the inhibition of D1R/GluN1 heteromers would block the activation of this subtype of MSN induced by cocaine that is required for the acquisition of locomotor sensitization and CPP (Calipari et al., 2016; Chandra et al., 2013; Ferguson et al., 2011; Lobo et al., 2010). D1R/GluN1 heteromers could also participate to the acquisition of these behaviors by modulating ERK pathway activation. Indeed, activation of ERK is necessary for the acquisition of locomotor sensitization (Ferguson et al., 2006; Valjent, Corbillé, et al., 2006) and CPP (Valjent et al., 2000).

We have also shown in collaboration with Nicolas Heck that blocking these heteromers during CPP blocks the morphological adaptations induced by cocaine. D1R/GluN1 heteromers may play a role in the generation of new spines by regulating ERK pathway activity. Indeed, the ERK pathway controls both the growth of new spines in the NAc in response to cocaine, but also their maintenance by a signaling pathway requiring ERK phosphorylation of the cytoplasmic protein MNK-1 (Dos Santos et al., 2017).

On the other hand, our results show that D1R/GluN1 heteromers formed in response to cocaine in the dorsal and ventral parts of the striatum are not necessary for the maintenance of locomotor sensitization and CPP. This could be explained by the fact that the levels of D1R/GluN1 heteromers are reduced during withdrawal. These results contrast with the chemogenetic inhibition of D1R-MSN in the dorsal striatum during a withdrawal period that impairs the maintenance of amphetamine-induced sensitization (Ferguson et al., 2011). This suggests, on the one hand, that the manipulation of the activity of D1R-MSN does not have the same consequences as the blocking of heteromers and, on the other hand, that D1R/GluN1 heteromers have a role restricted to the initial phases of cocaine-induced alterations.

In D2R-MSN, our results show that D2R/GluN2B heteromers are necessary for the development of CPP and also for the maintenance of locomotor sensitization and CPP. Numerous studies have shown that activation of D2R-MSN blocks behavioral alterations in response to repeated exposure to cocaine (Farrell et al., 2013; Lobo et al., 2010; S. S. Song et al., 2014). In our study, blocking D2R/GluN2B heteromers could lift the inhibition of NMDAR currents by DA in D2R-MSNs and thus increase D2R-MSN activity, which would act as a brake on responses induced by cocaine. Indeed, activation of D2R-MSN by optogenetics has been shown to decrease cocaine-induced CPP (Lobo et al., 2010). Consistent with the specific involvement of D2R/GluN2B heteromers in the maintenance of locomotor sensitization, optogenetic activation of D2R-MSN during cocaine injections has been shown to have no effect in the development of behavioral sensitization, while their inhibition during cocaine withdrawal alters the maintenance of this sensitization (S. S. Song et al., 2014). We indirectly verified whether blocking these heteromers could indeed influence the activity of D2R-MSN. To do this, 24 hours after re-exposure to cocaine we analyzed the expression of the transcription factor ΔFosB. ΔFosB was chosen here as an indirect indicator of neuronal activity. Indeed, it has been shown that ΔFosB accumulates specifically in NAc D1R-MSN in response to chronic administrations of cocaine (Hope et al., 1994; Lobo et al., 2013) downstream from CaMKII activation by calcium (Robison et al., 2013). We observed an "ectopic" induction of ΔFosB expression in D2R-MSN infected with IL3 virus, suggesting an increase in
calcium signaling in D2R-MSN in response to cocaine when D2R/GluN2B heteromers were blocked. This increase in D2R-MSN activity, following blocking of D2R/GluN2B heteromers, could counteract the increase in D1R-MSN activity in response to cocaine by increasing the strength of the indirect pathway and possibly also the strength of D2R-MSN collateral inhibitions projecting onto D1R-MSN (Burke et al., 2017; Cazorla et al., 2014; Dobbs et al., 2016). Indeed, if we quantified an increase of ΔFosB expression in D2R-MSN in response to cocaine (visualized by a viral approach) during the blockade of D2R/GluN2B heteromers, we also observed a decreasing trend of its expression in non-D2R-MSN neurons (GFP negative). This could suggest that inhibition of D2R/GluN2B heteromers could be accompanied by a decreased activity of D1R-MSN in the NAc. However, these hypotheses remain to be verified, in particular by measuring the activity of D2R-MSN infected with the IL3 or scramble virus, using calcium imaging or electrophysiology, in response to cocaine.

We cannot exclude that the behavioral effects observed during the blocking of D2R/GluN2B interaction are not due to an effect of the IL3 peptide on the interaction between D2R and β-arrestin, which could for example facilitate the internalization of D2R following its stimulation by dopamine. Indeed, β-arrestin interacts with D2R at the level of the IL3 loop but the key residues for this interaction are upstream from the D2R interaction domain that mediates its interaction with GluN2B (Clayton et al., 2014). In fact, in mice KO for the drd2 gene (coding for D2R), which does not present hyperlocomotion in response to cocaine, the overexpression of a mutant form of D2R capable of recruiting arrestin, but not the G protein, restores the acute locomotor response to cocaine, therefore showing that the coupling of D2R to arrestin signaling is necessary for this response (Donthamsetti et al., 2020). But in our case, blocking D2R/GluN2B does not alter the inhibition of D2R agonist-induced cAMP production suggesting that D2R signaling via G proteins is intact. We could perform additional checks, for example by performing PLA to see if blocking D2R/GluN2B heteromers can disrupt D2R/β-arrestin interaction in baseline condition and in response to cocaine. Altogether, our results obtained by manipulating D1R/GluN1 and D2R/GluN2B heteromers formed in the D1R-MSN and D2R-MSN, respectively, demonstrate a complementary role of these interactions in these two MSN populations in different phases of cocaine-induced adaptations.

Because the behavioral effects we observed in response to cocaine could result from deficits in other functions such as natural reward processing or spatial memory, we studied whether blocking DAR/NMDAR heteromers could alter CPP for food. We did not observe any alteration of this behavior, thus suggesting a preferential role of D1R/GluN1 and D2R/GluN2B heteromers in drug-induced responses. These results also show that blocking these heteromers does not induce a deficit in associative memory and processing of natural rewards.

Modulation of D2R/GluN2B heteromers in human cocaine addicts.

Individuals suffering from cocaine addiction, when entering a process of abstinence, frequently experience episodes of relapse. This high rate of relapse has disastrous consequences on the lives of these individuals (Melemis, 2015). Therefore, the discovery of a therapeutic strategy that can decrease the incidence of relapse would be a big step for the development of innovative treatment of cocaine addiction.
Our results show a preferential role of D2R/GluN2B heteromers in the maintenance of behavioral responses to cocaine, without having an effect in the case of natural reward. This suggests that they could be a therapeutic targets to consider. Therefore, we were interested in studying whether these heteromers could be detected in human caudate putamen samples and whether they were eventually modulated in individuals with psychostimulant use disorder. These subjects, although poly addicts, were selected because they were mainly dependent to psychostimulants and had traces of psychostimulants in the blood at the time of death. Despite a sharp decrease in D2R expression in dependent individuals, we showed that the proportion of D2R forming heteromers with GluN2B was three times greater in dependent subjects compared to controls.

The decrease in protein expression of D2R is in agreement with imaging studies in humans showing a decrease in the bioavailability of D2R in individuals addicted to psychostimulants (Martinez et al., 2004; Trifilieff et al., 2017; Volkow et al., 1990, 1993, 1997, 2009). The increase in D2R/GluN2B heteromers despite this decrease in D2R expression suggests that the remaining pool of D2Rs preferentially heteromerizes with GluN2B. This could be a compensation mechanism that would take place in order to, despite the decline in D2R expression, maintain functional D2R signaling in MSN. Indeed, we surprisingly found a negative correlation between D2R expression and D2R/GluN2B heteromer levels. Patients with the lowest D2R expressions have the highest D2R/GluN2B levels. In view of the links between susceptibility to cocaine addiction and polymorphisms in the DRD2 gene encoding D2R (Levran et al., 2015; Sullivan et al., 2013), we can wonder whether these patients had DRD2 allelic traits favoring the formation of D2R/GluN2B heteromers.

On the other hand, one information that we lack in this part of the study is the history of addiction of these patients, in fact we do not know the duration of their addiction or the time of the last drug intake of these individuals. The very probable high variability of this parameter seems to indicate that the increase in D2R/GluN2B heteromers is relatively stable. However, we do not have a large enough sample of individuals to correlate the increase in these heteromers with the nature of the addictive substances preferentially consumed by these subjects. The subjects with the lowest heteromeric levels were the three individuals who regularly consume either cocaine and an antidepressant, or cocaine and opioids, or cocaine with cannabinoids. In contrast, individuals who had cocaine and ethanol in their blood all showed a large increase in heteromers. It is questionable whether this may be related to exposure to cocaethylene, a cocaine homologue that is formed during co-administration of these two substances and which has higher euphoric effects than cocaine alone (Landry, 1992; Maia et al., 2017). Finally, suicide is the predominant cause of death in our sample and interestingly, individuals who died from suicide tend to have higher levels of D2R/GluN2B heteromers. It would have been interesting to know if any of these individuals died of an overdose suffered from of addiction and a comorbid depression. Indeed, preliminary data show that DAR/NMDAR heteromers could be increased in animals after chronic social defeat. A project is currently carried out in the laboratory to study the role of these heteromers in animal models of social defeat and in the context of exposure to chronic stress and cocaine. This project will also be interested in the modulation of these heteromers from human extracts from subjects having presented depressive episodes with or without comorbidity with addiction to psychostimulants.

Furthermore, our study focused on the nucleus accumbens, but we could also study the levels of these heteromers in the putamen and the caudate nucleus. Indeed, these regions are involved in
habit formation and initiation of actions and they are believed to be involved in drug seeking behavior in dependent patients (Volkow et al., 2006). It is also important to know if there are any gender differences in the regulation of these heteromers.

Taken together, these results highlight the dynamic nature and central role of dopamine and glutamate receptors interaction in distinct phases of cocaine-evoked long-lasting adaptations. In this study we also developed tools to dissect the role of these interactions. These tools could subsequently be used to study the functions of these heteromers both under physiological conditions but also in the context of other neurological and psychiatric pathologies involving a disturbance of the balance between DA and glutamate transmission. Finally, this study shows that targeting heteromers can be a promising therapeutic approach. Unlike strategies using individual receptor antagonists that often cause serious side effects, targeting the interaction between receptors may offer a more selective alternative as it spares the function of individual receptors, which may limit the occurrence of deleterious side effects.

Altogether our work therefore provides the first demonstration of a modulation of the interactions between endogenous dopamine and glutamate receptors in the striatum by chronic exposure to cocaine. It also shows that these heteromers are detectable in humans and that their formation is increased in subjects with a history of psychostimulant addiction. This work also provides evidence for a distinct role of these heteromers in the different phases of long-term responses induced by cocaine. This study also helps to strengthen the hypothesis that targeting the interaction between dopamine and glutamate receptors could be a possible therapeutic strategy to relieve symptoms associated with addiction but also potentially for other psychiatric illnesses linked to an imbalance of glutamate and DA transmissions.
Article II

Multiple evidences suggest that nuclear calcium-dependent signaling is one of the main mechanisms for synapse-to-nucleus communication, playing a central role in different forms of long-term neuronal adaptations. Because striatal signaling is a key driver of pathological drug-adaptive behavior, a main objective of the work carried out during this thesis was to investigate the yet unknown dynamics and roles of nuclear calcium signals in striatal neurons in cocaine-evoked cellular, molecular and behavioral responses.

Nuclear Ca\(^{2+}\) dynamics in response to cocaine

Nuclear calcium signals were first imaged in vitro in cultured striatal neurons incubated with a D1R agonist and a low dose of glutamate, a molecular model that mimics acute cocaine-induced responses in D1R-MSN in vivo (reviewed in section 2.5) (Cahill, Pascoli, et al., 2014; Pascoli et al., 2011). These responses include D1R-mediated potentiation of NMDAR-dependent Ca\(^{2+}\) influx and ERK activation, which are critical for the development of sensitization and rewarding effects in response to psychostimulants in vivo (Pascoli et al., 2011). We demonstrate for the first time that the crosstalk between D1R- and NMDAR-dependent signaling, not only triggers ERK activation but also generates fast NMDAR-dependent nuclear calcium transients. Furthermore, disrupting D1R-GluN1 heteromers, which blocks the development of cocaine-evoked sensitization and conditioned place preference in vivo ((Cahill, Pascoli, et al., 2014), article1), also blocked nuclear calcium rises induced downstream of D1R and NMDAR, therefore suggesting that nuclear calcium signals in D1R-MSN may play an important role in drug-evoked responses in vivo.

To assess the dynamics of nuclear calcium in mature D1R-MSN, we have used an ex vivo model of acute cocaine exposure previously validated in the lab ((Dos Santos et al., 2017), supplementary information). Acute slices were stimulated with D1R and NMDAR agonists used separately or in combination. Using this model, we observed the same synergistic effect on nuclear calcium signals when comparing the treatment of agonists alone to the co-stimulation paradigm. Surprisingly, we observe a gradual and sustained increase of nuclear Ca\(^{2+}\) concentration, which reflects different kinetics when comparing to results obtained with cultured cells. Finally, we found that a single injection of cocaine caused a progressive and sustained nuclear Ca\(^{2+}\) rise in NAc D1R-MSN of freely-moving mice.

Distinct nuclear Ca\(^{2+}\) dynamics observed in vitro in cultured striatal neurons and ex vivo or in vivo experiments can be explained by different physiological conditions. First, cultured striatal neurons come from embryonic striatum (E14), containing immature neurons. For instance, to our knowledge these neurons are not fully segregated in the two main populations, since most of them express D1R (>80%) and around 60% express the D2R. Additionally, these neurons go through a digestion process that damage dendritic projections and disorganize circuit connections that are never restored, leading to isolated cultured MSN. Furthermore, in vitro cultures are differentiated only in the presence of artificial medium (NBC), lacking important glutamatergic inputs, specially from the cortex. This, combined with the fact that there are other important cells for neuronal function that are also absent, such as astrocytes, microglia, interneurons, etc. (Falk
Discussion: Article II

et al., 2006; Penrod et al., 2011, 2015), highlights the limitations of primary cultured striatal neurons.

Previous work, using the same in vitro model with another Ca\(^{2+}\) sensor, Fluo-4, which is distributed in the whole neuron (\(K_d=345\) nM; \(F_{\text{max}}/F_{\text{min}} >100\); thermofisher), show similar Ca\(^{2+}\) dynamics (Fig 29). These results, evidence a fast increase and a subsequent decrease of intracellular Ca\(^{2+}\) levels in response to co-stimulation of D1R and NMDAR (Cahill, Pascoli, et al., 2014).

![Graph showing calcium profiles](#)

**Figure 30.** Dopamine D1 receptor (D1R)/GluN1 complexes control D1R-mediated potentiation of NMDAR signalling.

(a) Calcium profiles (\(\Delta F/F \pm \text{s.e.m.}\)) from medium spiny neurons (MSNs) treated with glutamate or SKF38393 or both (co-stim) in the absence or presence of ifenprodil (10 μM). (b) Images of calcium signals under basal conditions (left) and at the peak response induced by the co-stimulation in the absence (Control, top row), or presence of TAT-GluN1C1 (middle) or TAT-GluN1C1Δ (bottom). (c) Calcium profiles and (d) corresponding area under the curves (AUC) from neurons treated co-stimulated in the absence or presence of TAT-GluN1C1 (black) or TAT-GluN1C1Δ (grey); N=3–5, n=72–176 cells. One-way analysis of variance (ANOVA), Bonferroni post hoc test, **P<0.01; (Control); ***P<0.001; (TAT-GluN1C1Δ). Modified from (Cahill, Pascoli, et al., 2014)
However, since we have used the same sensor (GCaMP3-NLS) in all three models, the difference in nuclear calcium signal dynamics in D1R-MSN observed in vitro and ex vivo/in vivo likely relies on distinct Ca\(^{2+}\) homeostasis regulation of MSNs in cultured neurons and in mature MSN. As we review in section 3.1 and 3.2, Ca\(^{2+}\) buffering processes depend on the expression of many Ca\(^{2+}\) binding proteins, as well as the expression and activity of different Ca\(^{2+}\) channels, ATPases, transporters and receptors involved in Ca\(^{2+}\) signaling and release from intracellular stores. We hypothesized that differences in the expression pattern of these proteins, occurring as a consequence of distinct levels of maturity of MSN, may be responsible for the different kinetics of nuclear calcium signals observed in vitro and ex vivo/in vivo.

For our experiments, we used the calcium probe GCaMP3, one of a series of Ca\(^{2+}\) sensors that started to be developed in early 2000. They are in continuous renewal to improve their features, such as better dynamic range, or changes in Ca\(^{2+}\) affinity to measure different types of signals. Some of these parameters are summarized in table 7. It would be interesting for future experiments to image nuclear calcium with a newer version of GCaMP with distinct properties to explore whether there are nuclear calcium fluctuations that we cannot detect when using the GCaMP3 sensor, which has a low dynamic range (T.-W. Chen et al., 2013; Pérez Koldenkova & Nagai, 2013).

<table>
<thead>
<tr>
<th>Sensor</th>
<th>Dynamic range (F(<em>{\text{max}})/F(</em>{\text{min}}))</th>
<th>K(_d) (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GCaMP3</td>
<td>13.5 ± 0.7</td>
<td>345 ± 17</td>
</tr>
<tr>
<td>GCaMP5G</td>
<td>45.4 ± 0.9</td>
<td>447 ± 10</td>
</tr>
<tr>
<td>GCaMP6s</td>
<td>63.2 ± 3.1</td>
<td>144 ± 4</td>
</tr>
<tr>
<td>GCaMP6m</td>
<td>38.1 ± 1.8</td>
<td>167 ± 3</td>
</tr>
<tr>
<td>GCaMP6f</td>
<td>51.8 ± 2.8</td>
<td>375 ± 14</td>
</tr>
</tbody>
</table>

Table 7. Dynamic range and dissociation constant (K\(_d\)) of different GECIs. (Modified from (T.-W. Chen et al., 2013), supplementary information).

Interestingly, the kinetics observed in ex vivo and in vivo experiments correlate to the ones reported for whole-cell calcium imaging performed in D1R-MSN after acute cocaine administration (see Fig. 28) (Luo et al., 2011). Luo and colleagues used another fluorophore, Rhod2/AM, to measure intracellular Ca\(^{2+}\) transients, and they performed these experiments on anesthetized mice. By contrast, in D2R-MSN, cocaine and cocaine-associated cues have been shown to decrease Ca\(^{2+}\) levels (Calipari et al., 2016; Luo et al., 2011).

Conditions where nuclear Ca\(^{2+}\) rise independently of cytosolic Ca\(^{2+}\) increase have not been identified so far (Bading, 2013), thus it is unlikely that cocaine exposure could increase nuclear Ca\(^{2+}\) in D2R-MSN. Accordingly, our results show that inhibiting nuclear calcium-dependent signaling in D2R-MSN did not have an impact on any of the cocaine-evoked adaptations tested (see below), thus supporting that nuclear Ca\(^{2+}\) rise does not occur in D2R-MSN in response to cocaine or, alternatively, that they are not functionally relevant for the cocaine-induced responses studied. However, imaging of nuclear Ca\(^{2+}\) transients in D2R-MSN is currently being performed to test our hypothesis.
Altogether, these data indicate that nuclear Ca\textsuperscript{2+} increases in D1R-MSN may be generated at glutamatergic synapses; where upon stimulation, NMDARs are activated triggering intracellular Ca\textsuperscript{2+} influx, which is rapidly propagated through the neuron towards the nucleus by VGCCs and/or RyRs, as already proposed (Bading, 2013).

**Nuclear Ca\textsuperscript{2+}- dependent signaling blockade in response to cocaine**

In order to investigate the role of nuclear calcium-dependent signaling in NAc MSN, we used a viral-based strategy to express the CaMBP4 protein in either D1R- or D2R-MSN. CaMBP4 has been shown to efficiently block nuclear Ca\textsuperscript{2+}/CaM complexes and inhibits subsequent downstream nuclear signaling both *in vitro* and *in vivo* (Mauceri *et al.*, 2011; Simonetti *et al.*, 2013; Weislogel *et al.*, 2013; S.-J. Zhang *et al.*, 2009). One of the limitations of this approach is that it preserves calmodulin-independent functions of the nuclear calcium pool. One example is, the transcription repressor DREAM, which contains EF motifs that directly bind nuclear Ca\textsuperscript{2+} ions. When nuclear Ca\textsuperscript{2+} rises, the direct binding of Ca\textsuperscript{2+} to DREAM disrupts its binding to DNA and promote a nuclear calcium-dependent DRE-driven transcription independently on nuclear Ca\textsuperscript{2+}/CaM complexes (Carrión *et al.*, 1999; Mellström & Naranjo, 2001).

Nuclear calcium has been causally linked to acquired neuroprotection in cultured hippocampal neurons (S.-J. Zhang *et al.*, 2009). We therefore wanted to evaluate whether nuclear calcium blockade *in vivo* could alter MSN survival in mice expressing CaMBP4 in either NAc D1R- or D2R-MSN. We found that the long-term expression of CaMBP4 was not noxious for either type of MSN. This may be explained by the low basal activity of MSN that did not generate spontaneous nuclear calcium transients in freely-moving naïve mice (see Fig 2, article 2), contrary to cultured hippocampal neurons displaying spontaneous bursts of activity (Arnold *et al.*, 2005) that may trigger spontaneous nuclear calcium transients.

Because D1R-MSN and D2R-MSN display distinct but complementary roles in cocaine behavioral responses ((Salery *et al.*, 2020), Article 1), we chose to block nuclear calcium-dependent signaling in D1R-MSN or D2R-MSN and evaluate the consequences of these manipulations at the behavioral level. First, we evaluated basal locomotor activity prior to cocaine exposure which was similar in every group. Conversely, blocking cocaine-induced nuclear calcium-dependent signaling in D1R-MSN impaired the development of cocaine-induced CPP, whereas the same procedure in D2R-MSN did not impact on the rewarding effects of cocaine. This supports that inhibiting nuclear calcium signaling in NAc D1R-MSN, alters the association between the rewarding effects of the drug and the drug-paired context, supporting the idea of a central role of nuclear calcium in associative learning and memory (Mauceri *et al.*, 2011; Weislogel *et al.*, 2013), but here in the context of drug reward processing.

Dendritic spine density has been shown to be increased in response to acute and chronic cocaine in D1R-MSN (Dos Santos *et al.*, 2017; J. Kim *et al.*, 2011). This is associated with an augmentation of glutamate synapse formation onto D1R-MSN (Dos Santos *et al.*, 2017), which is likely linked to the long-lasting effects of cocaine (Cahill, Pascoli, *et al.*, 2014; Pascoli *et al.*, 2011). In our experiments, the inhibition of nuclear calcium-dependent signaling in either D1R-MSN or D2R-MSN did not impact on the dendritic spine density in saline-treated mice. By contrast, the augmentation in dendritic spine density induced by cocaine in D1R-MSN was impaired when
nuclear calcium signaling was blocked in those neurons. Hence, morphological changes in D1R-MSN observed in response to cocaine require the activation of nuclear calcium signaling in this MSN subtype. This is in agreement with the observation that a constitutive activation of nuclear calcium-CaMKIV signaling increases spine density in hippocampal neurons (Marie et al., 2005).

At the molecular level, the blockade of nuclear calcium-CaMKIV signaling fully prevented cocaine induction of the IEGs c-Fos and Arc, two genes involved in neuronal-plasticity regulation and in drug-induced adaptations (Salery et al., 2017; S.-J. Zhang et al., 2009).

Bilbao and colleagues, using a transgenic mouse line (Camk4D1RCre mice), ablated Camk4 gene in the striatum and other brain regions expressing the D1R, such as cortex. They showed that CaMKIV ablation produced an enhancement of cocaine-induced CPP and acute locomotor response, while gene expression profile remained similar, except for FosB, which was found to be up-regulated after cocaine challenge (Bilbao et al., 2008). Moreover, using a viral approach to express a kinase-dead mutant of CaMKIV in NAc-MSN, they showed increased cocaine-induced CPP. The discrepancies between these findings and ours may come from different technical approaches. We employed a viral-based strategy to locally alter nuclear Ca\textsuperscript{2+}/CaM-dependent signaling specifically in NAc D1R-MSN, whereas they evaluated CaMKIV function using either CAMKIV gene ablation in all D1R-expressing brain cells or viral expression of a non-functional CaMKIV indistinctly in NAc neuronal populations. With the first strategy, it is possible that developmental adaptations and potential compensation mechanisms may occur, while using the second approach they are decreasing nuclear Ca\textsuperscript{2+}-dependent signaling in both MSN populations as well as in interneurons, which could have completely different effects within the striatal micro-circuitry than the cell-type specific approach we have used. Nonetheless, this work highlights the complex roles achieved by nuclear-calcium dependent signaling in striatal cocaine-induced adaptations.

In previous studies, including from our group, it was shown that an ERK inhibitor was able to completely abolish the induction of these IEGs induced by cocaine (Salery et al., 2017; Valjent et al., 2000). This led us to evaluate a possible convergence between nuclear calcium signaling and the ERK pathway in D1R-MSN. Interestingly, overexpression of CaMBP4 in D1R-MSN blocked the activation of ERK induced by acute cocaine in the NAc, therefore unraveling a crosstalk between nuclear calcium signaling and the ERK pathway in response to acute cocaine exposure. A direct transcriptional regulation of ERK downstream nuclear calcium signaling activation is unlikely since total ERK protein levels was not decreased in D1R-MSN expressing CaMBP4.

Since ERK is locally activated in dendrites in a calcium-controlled manner prior its nuclear translocation (Pascoli et al., 2011; Trifilieff et al., 2009; Valjent et al., 2000), we first considered that the inhibitory effect of CaMBP4 on ERK activation could be due to an artefactual inhibition of dendritic and cytosolic calcium resulting from a leakage of CaMBP4 outside the nucleus. For this reason, we performed HA immunostaining, which is the tag fused to the CaMBP4, confirming its absence from dendrites and its nuclear expression. As an alternative, nuclear calcium-dependent signaling could control the expression of several proteins involved in ERK activation induced by a single cocaine administration. However, ERK activation by cocaine is controlled by a complex signaling network, which makes the identification of molecular mechanisms linking nuclear calcium to the modulation of ERK activation rather difficult (Fig. 31).
Previous work indeed showed that cocaine triggers a calcium-dependent activation of ERK relying on a D1R-mediated potentiation of calcium influx through GluN2B-containing NMDAR, via the activation the tyrosine kinase Fyn (Pascoli et al., 2011). An alteration of D1R expression levels, NMDAR subunits or Fyn induced by nuclear calcium signaling could therefore explain the inhibition of ERK activation by cocaine upon CaMBP4 overexpression. Other actors involved in cocaine-induced activation of ERK are the protein Ras-GRF1 (Fasano et al., 2009), which is activated downstream GluN2B-containing NMDAR (Krapivinsky et al., 2003), the cAMP-regulated phosphoprotein of 32 kDa (DARPP32), protein phosphatase 1 (PP1) and the phosphatase STEP (Valjent et al., 2005). Elucidating a possible transcriptional control of these signaling proteins by nuclear calcium could unravel new mechanisms involved in cocaine-induced ERK activation (Fig. 31).

The next future experiment would be to analyze by western blot the levels of expression of D1R, GluN2B, pGluN2B, GluN1, DARPP32, PP1 and STEP in mice overexpressing CaMBP4 in NAc D1R-MSN and treated or not with cocaine for 10 minutes, which corresponds to the peak of ERK activation by cocaine. This could elucidate if CaMBP4 expression prior cocaine exposure induces long-lasting transcriptional changes that may alter ERK activation. Another more challenging and non-biased strategy will be to perform a global transcriptional analysis to identify genes which expression is modulated in response to cocaine in a nuclear-calcium dependent manner.

D1R/GluN1 heteromers mediate the development of CPP and behavioral sensitization (Article 1), as well as ERK activation (Cahill, Pascoli, et al., 2014). In order to unravel the link between nuclear calcium dependent signaling and decreased behavioral responses and ERK activation, we will analyze D1R/GluN1 heteromers by Proximity Ligation Assay in mice overexpressing CaMBP4, 24h after the last injection of a chronic cocaine treatment.

Although overexpressing CaMBP4 allowed us to highlight that nuclear calcium is key for the nuclear calcium-dependent signaling in cocaine-evoked adaptations, we may have underestimated the full impact of nuclear calcium signaling by using this interfering approach that does not fully block calcium signals. Further studies are needed in order to understand the complexity of this signaling pathway in the context of drugs of abuse.
Figure 31. Schematic representation of the mechanistic question on how nuclear calcium signaling modulates ERK activation.

In conclusion, our study reveals an important and central role of cocaine-induced nuclear calcium-dependent signaling in D1R-MSN. Nuclear calcium increases in NAc D1R-MSN control molecular, cellular and behavioral adaptations to cocaine, and our study represents a promising discovery as it could contribute to the development of future innovatory therapeutic approaches to alleviate addiction symptoms.
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Anexes
Unraveling the Functions of Endogenous Receptor Oligomers in the Brain Using Interfering Peptide: The Example of D1R/NMDAR Heteromers

Andry Andrianarivelo, Estefani Saint-Jour, Pierre Trifilieff, and Peter Vanhoutte

Abstract

Decoding signaling pathways in different brain structures is crucial to develop pharmacological strategies for neurological diseases. In this perspective, the targeting of receptors by selective ligands is one of the classical therapeutic strategies. Nonetheless, this approach often results in a decrease of efficiency over time and deleterious side effects because physiological functions can be affected. An emerging concept has been to target mechanisms that fine-tune receptor signaling, such as heteromerization, the process by which physical receptor–receptor interaction at the membrane allows the reciprocal modulation of receptors’ signaling. Because of the central role of the synergistic transmission mediated by dopamine (DA) and glutamate (Glu) in brain physiology and pathophysiology, heteromerization between DA and Glu receptors has received a lot of attention. However, the study of endogenous heteromers has been challenging because of the lack of appropriate tools. Over the last years, progress has been made in the development of techniques to study their expression in the brain, regulation and function. In this chapter, we provide a methodological framework for the design and use of interfering peptides to study endogenous receptor oligomers through the example of the dopamine type 1 receptor (D1R) and the GluN1 subunit of NMDA receptor heteromers.

Key words Oligomers, Protein–protein interaction, Interfering peptide, Heteromerization, Dopamine receptor, NMDA receptor

1 Introduction

In mammals, many brain structures involved in fundamental physiological functions, ranging from motor coordination, cognition, learning and memory to goal-directed behavior, are the targets of imbricated and convergent dopamine (DA) and glutamate projections. As a witness of the crucial role played by dopamine-mediated modulation of excitatory glutamate transmission in these brain regions, an imbalance of dopamine and glutamate transmissions has been incriminated in a vast array of neurological or psychiatric disorders, such as Parkinson’s and Huntington’s diseases, schizo-
phrenia, depression, obsessive compulsive disorders, or addiction [1–6]. Based on these observations, current treatments for the aforementioned pathological conditions primarily aim at targeting cognate neurotransmitter receptors but the loss of therapeutic efficacy over time as well as the appearance of severe deleterious side effects calls for alternative strategies. Intracellular signaling pathways recruited downstream from individual DA and Glu receptors stimulation are imperative for physiological cell responses and normal brain functions. Therefore, the targeting of molecular mechanisms specifically involved in dopamine–glutamate interactions, rather than the targeting of the receptors themselves, appears as an alternative strategy. This approach holds great potential to pave the way for new therapeutic avenues [7–9].

DA and glutamate receptors of the NMDA (N-methyl-D-aspartic acid) subtype mutually modulate their functions. This synergy takes place through mechanisms involving the recruitment of specific signaling cascades but their direct physical interactions (i.e., heteromerization or oligomerization) progressively appear as a complementary mechanism by which these receptors can reciprocally finely tune their functions and trafficking [10–18].

Receptor oligomers in general possess pharmacological and functional properties that are distinct from individual component receptors and allow a spatiotemporal modulation of the receptors’ functions. For these reasons, heteromers have been the subject of intense research and, in particular, a number of studies showed the critical role of the physical interaction between DA and NMDA receptor in vitro for the modulation of their subcellular distribution and functions [7, 11, 19–21].

Until the past few years, the existence and roles of endogenous heteromers in the brain were still under debate, mostly due to a lack of mechanistic understanding of the heteromerization process and appropriate tools. The emergence of techniques such as the proximity ligation assay (see [22, 23]) now allows the detection of endogenous receptor heteromers in their native environment from fixed tissues and confirmed their presence in the brain [7, 24–26]. To study the functional roles of receptor heteromers, one option that emerged was to develop bivalent compounds that are capable of preferentially targeting a receptor when it forms an heteromer, rather than the receptor on its own, as validated for the heteromers formed by DA type 2 (D2R) and adenosine A2 (A2R) receptors [27]. As an alternative, the precise characterization of protein–protein interaction domains required for the formation of heteromers offers the possibility of using peptidic sequences designed to interfere with endogenous receptor heteromer formation though a competition mechanism. This is particularly applicable to DA and NMDA receptors, thanks to the seminal work from Dr. Fang Liu and others, which led to the characterization of short sequences in DAR and NMDAR subunits that are essential for the interaction of
D1R with either GluN1 or GluN2A or D2R with GluN2B subunits [10, 11, 14].

Based on the knowledge of the interaction domains involved in the binding of D1R to the obligatory subunit of NMDAR GluN1 [10, 13], we developed an interfering peptide-based strategy to alter D1R/GluN1 heteromerization, while preserving the functions of individual component receptors. Using this strategy, we demonstrated that D1R/GluN1 heteromers appear as a molecular bridge linking DA to the facilitation of NMDAR-mediated currents and NMDAR-dependent targeting of the Extracellular signal-regulated kinase (ERK) downstream from D1R and NMDAR. We also demonstrate that the recruitment of D1R to NMDAR was mandatory for long-term potentiation in the striatum as well as for some adaptations induced by exposure to cocaine [18].

Through the example of D1R/GluN1 heteromers, we propose in this chapter to provide a comprehensive and generally applicable methodological framework for the use of interfering peptides, and appropriate controls, to study the specific roles of endogenous receptor oligomers in the brain.

### 2 Methods

#### 2.1 Design of Cell-Penetrating Interfering Peptide Sequence to Disrupt D1R/GluN1 Heteromers, While Preserving the Functions of Individual D1R and NMDAR

As a general statement, when the interaction domains between two proteins have been characterized in both partners, two peptides can potentially disrupt their interaction. A peptide corresponding to the region of the protein A that binds a protein B or the region of protein B interacting with protein A (Fig. 1a). A priori, these two strategies should yield a disruption of the binding of both partners; however, the functional consequences on downstream signaling may be different, and even opposite.

The interaction between D1R and the GluN1 subunit of NMDAR involves identified domains located within the intracellular c-terminus ends of both receptors: the t2 domain (L387-L416) of D1R and the C1 cassette (D864-T900) of GluN1 [10, 11] (Fig. 1b). Synthetic peptides corresponding to the t2 domain of D1R [15, 17] (Fig. 1c) or the C1 cassette of GluN1 [18] (Fig. 1d) have thus been used to study D1R/GluN1 functions in the brain.

These two strategies led to an efficient disruption of D1R/GluN1 complexes [10, 11, 18]. When using the peptide corresponding to the C1 cassette of GluN1, we observed a slight decrease of basal ERK activity and a complete blockade of ERK activation downstream from D1R and NMDAR (Fig. 2a). By contrast, we found that the interfering peptide corresponding to the t2 domain of D1R induced per se an increase of NMDAR-mediated activation of the ERK pathway and was not able to significantly diminish the increase of ERK activity induced by a co-stimulation of D1R and NMDAR of cultured neurons (Fig. 2b). These discrepancies
between the two strategies could be attributed to the fact that the t2 peptide, and not the C1 mirror peptide, may compete with the binding of the protein Calcium Calmodulin, which constitutively inhibits NMDAR through interaction domains comprising a portion of the C1 cassette [28] (see Note 1). It is also possible that the binding of the t2 peptide at the receptor by itself mimics heteromerization with D1R, exerting an effect as an allosteric modulator of the NMDAR. This could explain why studies using these two strategies yielded opposing results regarding the role of D1R/ GluN1 on (ERK-dependent) long-term neuronal plasticity [15, 17, 18]. Since we verified that the C1 cassette was able to disrupt heteromer formation without interfering with functions of individual D1R and NMDAR (see below), we pursued our work using the peptide GluN1C1 to selectively interfere with D1R/GluN1 formation and functions.

To achieve intracellular delivery of the GluN1C1 peptide we fused it to the trans-activating transcriptional activator sequence (TAT; sequence: GRKKRRQRRR) (see Note 2). A “spacer,” which is composed of two proline residues, has been added

Fig. 1 Interfering peptides used to disrupt D1R/GluN1 heteromer formation (a) General diagram depicting the two strategies that can be used to disrupt the interaction between proteins A and B when the interaction domains (dark blue/red) are identified within both proteins. Panels (1) and (2) illustrate the mechanisms of action of both possible synthetic interfering peptides (*). Schematic representation of the mechanisms of action of the synthetic peptide corresponding to (c) the C1 cassette (GluN1C1*) that binds to D1R (c) [18] or (d) the t2 domain of D1R (t2*), which interacts with GluN1 (c) used in [15, 17]
between the TAT and the GluN1C1 sequence to increase flexibility between these two parts of the peptide.

The design of a control peptide has been inspired by the work of Woods and coworkers who established that the C1 cassette of GluN1 was containing a stretch of 9 arginine-rich amino acids ($^{890\text{SFKRRRSSK}^{908}}$), which plays a critical role for the electrostatic interactions mediating the interactions between D1R with GluN1 [29]. A peptide corresponding to the C1 sequence deleted of these nine amino acids (TAT-GluN1C1Δ) has thus been used as a control and we were able to confirm its inability to alter D1R/GluN1 heteromer formation (Fig. 3a). However, alternative options should be considered to design control peptides (see Note 3).

**Fig. 2** Impact of D1R/GluN1 heteromer blockade with two different interfering peptides on downstream ERK signaling. Cultured striatal neurons were incubated or not with 5 μM of TAT-GluN1C1 (a) or TAT-t2 (b) prior to a co-stimulation (co-stim) of D1R and NMDAR with 3 μM of SKF38393 together with 0.3 μM of glutamate for 10 min (+) or a vehicle solution (−). The activity of ERK downstream from D1R and NMDAR was assessed by western blot using an antibody that specifically recognizes the phosphorylated (i.e., activated) form of ERK1/2. Quantifications show that TAT-GluN1C1 fully blocks ERK activation induced by the co-stimulation (a) whereas the TAT-t2 increases by itself basal ERK activity levels and does not inhibit ERK activation induced by the co-stimulation. Two-way ANOVA; Bonferroni post-hoc test; $^{**}p < 0.01$; $^{***}p < 0.0001$ (compared to control); $^{°°°}p < 0.0001$ (compared to co-stim); n.s not significant. Adapted from [18]
1. Preparation and storage of peptides:

TAT-GluN1C1 and TAT-GluN1C1Δ, synthesized with a purity >98%, were diluted in sterile deionized water as stock solutions of 1 mM. Since peptides are unstable molecules by nature (see Note 4), it is critical to avoid freeze-thaw cycle(s). The stock solution should be aliquoted in a volume corresponding to the need for one experiment and stored at −80 °C. One aliquot should be defrosted slowly on ice and used for a single experiment.

2.2 Guideline on Storage and Use of Interfering Peptides Through the Example of D1R/GluN1 Disruption Using TAT-GluN1C1

![Detection of D1R/GluN1 by PLA](image)

**Fig. 3** The TAT-GluN1C1 peptide blocks D1R/GluN1 heteromer formation, while preserving individual D1R- and NMDAR-dependent signaling. Neurons were pre-incubated with TAT-GluN1C1Δ (top panels) or TAT-GluN1C1 (bottom panels) prior to and during an incubation with 3 μM of SKF38393 together with 0.3 μM of glutamate (co-stim) or a vehicle solution (cont) for 10 min. (a) Representative confocal images illustrating the punctate pattern of the PLA signal corresponding to D1R/GluN1 heteromers. Note that the co-stimulation favors D1R/GluN1 heteromer in the presence of the control peptide, whereas this phenomenon is fully blocked in neurons pre-incubated with the TAT-GluN1C1 peptide. (b) cAMP production (fold increase) in control condition and after 10 min of the co-stimulation paradigm. Note the lack of effect of TAT-GluN1C1 on D1R-mediated cAMP production. One-Way ANOVA, Newman Keuls post-hoc test; *p < 0.05 when compared to the control group. (c) Illustration of calcium profiles obtained from cultured neurons pre-treated with either a vehicle solution, the TAT-GluN1C1 or the TAT-GluN1C1Δ peptides and loaded with the calcium probe Fluo-4. Calcium influx is induced by a stimulation with glutamate 10 μM added at the indicate time. (d) Diagram summarizing the results shown in (a–c). Adapted from [18]
2. **Incubation of cultured neurons with TAT-interfering peptides.**

The incubation of primary cultured neurons with the TAT-GluN1C1 to D1R/GluN1 heteromers requires the following steps (see **Note 5**):

(A) Primary cultures of striatal neurons were performed from striata dissected out from 14-day-old embryos from pregnant Swiss mice as described in [30]. Neurons were then plated at a density of 1000 cells/mm² and maintained for 6 days in vitro (DIV) in a Neurobasal A culture medium supplemented with B27, 500 nM l-glutamine, 60 μg/ml penicillin G, and 25 μM β-mercaptoethanol in a humidified atmosphere of 95% air and 5% CO₂.

(B) At DIV 6, the culture medium was replaced by fresh medium and put back to the incubator in order to avoid the presence of endogenous glutamate that may alter the response of the cell to low doses of exogenous glutamate (see steps 2–4).

(C) At DIV 7, an aliquot of TAT-GluN1C1 and TAT-GluN1C1Δ stock solutions were defrosted on ice and added directly to the culture medium containing the neurons to reach a final concentration of 5 μM (see **Note 6**). Neurons were then put back at 37 °C in the incubator for 1 h.

(D) Neurons incubated in the presence of interfering peptides were then stimulated with 3 μM of the D1R agonist SKF 38393 together with 0.3 μM of glutamate to stimulate concomitantly D1R and NMDAR for 10 min at 37 °C in the incubator.

(E) At the end of the treatments, when immunostaining or PLA experiments were performed (Fig. 3a), the culture medium was removed and neurons were fixed for 20 min at room temperature with a solution of Phosphate Saline Buffer (PBS) containing 4% paraformaldehyde (PFA) and 4% sucrose and washed three times with PBS (see **Note 7**). To perform immunoblotting (see Fig. 2), the culture medium was removed and the culture dishes containing the neurons were placed on dry ice. Neurons were then homogenized by sonication in a boiling solution of 1% sodium dodecylsulfate (SDS) and 1 mM of the phosphatase inhibitor sodium orthovanadate diluted in water, and then placed for 5 min at 100 °C before being stored at −80 °C.

3. **Infusion of TAT-interfering peptides into the brain.**

To evaluate the functional roles of D1R/GluN1 heteromers in the brain, we performed intracerebral injection of the TAT-GluN1C1 peptide although other routes of administrations can be considered (see **Note 8**).
To deliver the TAT-GluN1C1 in the ventral part of the striatum (i.e., the nucleus accumbens), bilateral injections were performed on mice anesthetized with isoflurane. Stainless-steel guide cannulae of 22 gauge were implanted in the nucleus accumbens by using the following stereotaxic coordinates: antero-posterior: +1.5; medio-lateral: ±1.6; dorso-ventral: 4.1 with an angle of 13° and then fixed to the skull with dental cement. After 1 week of recovery, 5 pmol of peptide diluted in water were infused 1 h prior to any further pharmacological treatment. At the end of the experiments, the mice were perfused by intracardiac perfusion of 4% PFA in 0.1 M Na$_2$HPO$_4$-NaH$_2$PO$_4$ buffer, pH 7.5 delivered with a peristaltic pump. Brains were then removed and post-fixed overnight in the aforementioned fixative solution. The brains were then sliced in 30 μm-thick sections kept in a solution of 30% ethylene glycol, 30% glycerol, 0.1 M phosphate buffer. These sections can be used to detect receptor heteromers (and their disruption by an interfering peptide) by using techniques such as PLA [18, 22, 23]. When biochemical analyses were performed, mouse heads were immediately frozen in liquid nitrogen and punches were prepared from both hemispheres of the brain and homogenized as described in Sect. 2.4 for cultured neurons.

3 Testing of the Efficacy and Specificity of an Interfering Peptide-Based Strategy

To validate the specificity of an interfering peptide, the first step is to show the ability of the peptide to alter the interaction between the receptors of interest and confirm the absence of effect of the control peptide. In the case of D1R/GluN1 heteromers, cultured striatal neurons, which co-express both D1R and NMDAR [18], were pre-incubated with TAT-GluN1C1 or TAT-GluN1C1Δ and co-stimulated or not with a D1R agonist together with glutamate as described in Sects. 2-3 and 2-4. After 10 min of co-stimulation, neurons were fixed as described in Sect. 2-5. Endogenous D1R/GluN1 heteromers were detected by performing a PLA according to the manufacturer’s instructions. For details on the PLA procedures, see [18, 22, 31]. As illustrated in Fig. 3a, D1R/GluN1 proximity visualized by PLA appears as a punctate pattern and a concomitant stimulation of both D1R and NMDAR for 10 min triggers an increase of the PLA signal in the presence of the control TAT-GluN1C1Δ. By contrast, in the presence of the TAT-GluN1C1, basal levels of D1R/GluN1 are decreased and their increased formation induced by the co-stimulation of both receptors is fully blocked, thus illustrating the efficacy and selectivity of the TAT-GluN1C1 as an inhibitor of D1R/GluN1 heteromers.

On a functional standpoint, the second validation step is to verify that the TAT-GluN1C1 peptide does not interfere nonspe-
specifically with the functions of D1R and NMDAR independently on heteromerization.

The D1R is a G protein-coupled receptor that is positively coupled to adenylyl cyclase and cAMP production through the \( G_{olf} \) protein [32]. As shown in Fig. 3b, an Elisa-based assay was used to measure cAMP production in cultured neurons that have been co-stimulated with a D1R agonist and glutamate to confirm that the presence of TAT-GluN1C1, which binds to the t2 domain of D1R, did not alter cognate pathway downstream from D1R (see Note 9). On the other side, the impact of TAT-GluNC1 has also been tested on NMDAR-mediated calcium influx triggered by a purely glutamatergic stimulation with 10 \( \mu \)M of glutamate. Live calcium imaging on cultured striatal neurons loaded with the calcium probe Fluo-4 demonstrated that the calcium dynamics and amplitude were similar when glutamate was applied on neuron pre-treated with TAT-GluN1C1, TAT-GluN1C1\( \Delta \), or a vehicle solution (Fig. 3c).

Altogether, this series of controls showed that the TAT-GluN1C1 peptide blocks the binding of D1R to GluN1 subunits of NMDAR, while preserving the normal functioning of individual D1R and NMDAR (Fig. 3d). This interfering peptide-based strategy allowed the demonstration that D1R/GluN1 heteromers can be considered as molecular bridges by which DA facilitates both NMDAR-dependent signaling in D1R-expressing neurons and neuronal plasticity in the striatum [18].

### Notes

1. The tethering of receptors at synaptic sites involves protein–protein interactions with numerous partners through interaction sites that may be contiguous or overlapping with the docking domains responsible for heteromer formation. Prior to the design of an interfering peptide sequence, an exhaustive literature screening is necessary to identify the location of known interaction domains of all binding partners of a given receptor of interest. This preliminary step is important in order to increase the chances to disrupt the receptor heteromer without altering the interaction between the receptors of interest and other protein partners, as it may change the function and trafficking of this receptor independently of heteromerization.

2. Alternatives to the cell-penetrating TAT sequence to deliver interfering peptides into the cell:

   (a) The penetrating sequence can be composed of a stretch of positively charged amino acids, such as arginines, or other known plasma membrane-permeant peptide sequence such as penetratin [33].
(b) For electrophysiological studies, the interfering peptide does not necessarily need to be coupled to a penetrating sequence as it can be filled in the patch pipet. The peptide will diffuse in the cytoplasm of the recorded cell and reach its target receptor \[18\].

c) Virus-mediated expression of interfering peptides can be used and offers several advantages when compared to the use of synthetic peptides. The use of strong promotors such as CMV, CAG, and PKG to drive the expression the interfering sequence allows a continuous production of the peptide that can overcome the degradation of the peptide by endonucleases and exonucleases. Virus-mediated expression should be privileged in studies where a long-lasting blockade of receptor heteromer needs to be achieved. Furthermore, the injection of viruses expressing a floxed construct encoding the interfering sequence into the brain of a mouse line that expresses the enzyme cre recombinase driven by a cell-type specific promotor enables the blockade of receptor heteromers in specific cell subpopulations of interest.

3. The most common way to test for the specificity of an interfering peptide is to use a control peptide composed of the same amino acids placed in a random order (i.e., “scramble peptide”). However, as shown for several heteromers including D1R/GluN1 [29] or D2R/A2R [34], clusters of charged amino acids are critical for the formation of heteromers through electrostatic interactions. Hence, it is important to be aware that the randomized control peptide should display a distribution of charged amino acids that has to be as much as possible different from the one of the active interfering peptide.

4. To overcome the low stability of peptides against proteolysis, several chemical modifications of amino acids can be used. For instance, retro-inverso amino acids in which the N- and C-terminus are reversed and the natural L-conformation of the peptide is changed to the D-enantiomer form. Other modification such as introduction of N-alkylated amino acids or cyclization can also be considered [35].

5. The experimental procedures described in this section relate to the disruption of D1R/GluN1 heteromers with TAT-GluN1C1 striatal cultured neurons. However, the global procedure may be generalizable to the use of any TAT-coupled peptide and any cell type [36].

6. A dose–response curve has to be performed since the concentration of interfering peptide yielding a maximum inhibitory effect on the disruption of a receptor heteromer of interest may depend on the accessibility of the targeted receptor, its localization within the cell, and its expression level.
7. The penetration of the peptide can be confirmed by coupling the peptide to a fluorophore [36] or a molecule of biotin [18] or any kind of tag that can be detected by immunofluorescence. However, such labeled peptides may not be suitable for functional studies since the presence of the fluorophore may alter the ability of the peptide to reach the target receptor.

8. TAT peptides have the characteristic to cross the plasma membrane but they also cross the blood–brain barrier. As such, they can be injected intraperitoneally in order to reach neurons of the central nervous system [37].

9. The absence of effect of TAT-GluN1C1 on D1R-mediated cAMP production is in agreement with the fact that this peptide binds to the t2 domain of D1R, which does not overlap with the third intracellular loop of D1R that is involved in the coupling of D1R to the G protein [38].

References

Invited review

Modulation and functions of dopamine receptor heteromers in drugs of abuse-induced adaptations

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HIGHLIGHTS

• Many DAR heteromers have been identified but their biological significance is only emerging.
• Targeting DAR heteromers has a strong therapeutic potential the field of addiction.
• The selective inhibition of receptor heteromers is a major limitation to uncover their roles in vivo.

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ABSTRACT

Drug addiction is a chronic and relapsing disorder that leads to compulsive drug intake despite deleterious consequences. By increasing dopamine (DA) in the mesolimbic system, drugs of abuse hijack the brain reward circuitry, which is critical for the development of enduring behavioral alterations. DA mainly acts onto DA D1 (D1R) and D2 (D2R) receptor subtypes, which are positively and negatively coupled to adenylyl cyclase, respectively. Extensive research has aimed at targeting these receptors for the treatment of addiction, however this often results in unwanted side-effects due to the implication of DA receptors in numerous physiological functions. A growing body of evidence indicates that the physical interaction of DA receptors with other receptors can finely tune their function, making DA receptor heteromers promising targets for more specific treatment strategies. An increasing number of articles highlighted the ability of both D1R and D2R to form heteromers, however, most studies carried out to date stem from observations in heterologous systems and the biological significance of DA receptor heteromers in vivo is only emerging. We focused this review on studies that were able to provide insights into functions on D1R and D2R heteromers in drug-evoked adaptations and discuss the limitations of current approaches to study receptor heteromers in vivo.

This article is part of the Special Issue entitled ‘Receptor heteromers and their allosteric receptor-receptor interactions’.

1. Introduction

Drug addiction is a chronic and relapsing disorder that results from protracted drug consumption by vulnerable individuals, leading to compulsive drug intake despite deleterious consequences. Drug-evoked neuronal activity changes within the so-called brain reward circuit are fundamental for the instatement of the enduring behavioral alterations that are characteristic of addiction (Lüscher and Malenka, 2011). Such

Abbreviations: A2AR, adenosine A2A receptor; BRET, bioluminescence resonance energy transfer; CaMKII, Calcium Calmodulin-dependent protein kinase II; CTD, C-terminal domain; DA, dopamine; D1R, dopamine receptor type 1; D2R, dopamine receptor type 2; ERK1/2, extracellular signal-regulated kinase 1/2; FRET, fluorescence resonance energy transfer; GPCR, G protein-coupled receptor; IL, intracellular loop; MSN, medium sized-spiny neurons; NAcc, nucleus accumbens; NMDAR, N-methyl-D-aspartate glutamate receptor; PET, positron emission tomography; PKC, protein kinase C; PLA, proximity ligation assay; PSD, post synaptic density; TM, transmembrane domain

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modulations of synaptic efficacy are translated into specific patterns of signaling pathway activation and gene expression driving a long-lasting remodeling of neural circuits, likely involved in the transition from casual to compulsive drug intake and addiction (Nestler, 2001, 2014). Despite their distinct targets, all drugs of abuse hijack the natural reward system by increasing dopamine (DA) concentration in the mesolimbic system, especially in the striatum (Di Chiara and Imperato, 1988), resulting in alterations in glutamate transmission-dependent plasticity (Lüscher and Malenka, 2011). The striatum is considered a key target structure of drugs of abuse within the reward circuit because it is at the crossroad of converging glutamate signals arising from limbic, thalamic and cortical regions, which encode components of the NAcc and dStr.

Within the NAcc, this functional dichotomy also applies to reward-dependent learning since the activation of the direct pathway neurons promotes reward whereas the stimulation of indirect pathway neurons inhibit locomotion. Based on these observations, it has been proposed that the imbalance between the activity of dMSN and iMSN evoked by drugs of abuse may drive towards compulsive drug intake and addiction (Lobo et al., 2010; Kravitz et al., 2012).

The surge of DA induced by drugs of abuse thus triggers a stimulation of D1R that activates dMSN and iMSN, which receive glutamate axon terminals and DA afferences converging on MSN dendritic spines (Moss and Bolam, 2008; Doig et al., 2010). MSN form glutamate axon terminals and DA afferences converging on MSN den-
functions (Wang et al., 2012; Cahill et al., 2014a). Instead, a selective targeting of the molecular mechanisms responsible for the modulation of excitatory transmission by DA appears as a promising strategy.

Beside local intracellular cascades downstream from DA receptors, a growing body of evidence supports that the physical interaction between receptors is a powerful mechanism by which receptors can mutually modify their functions through allosteric modulations. The formation of such receptor oligomers can engage two, or more, identical (homomers) or distinct (heteromers) receptors. These receptor complexes have been the subject of intense investigation because they can fine-tune downstream signaling and binding affinity for the component receptors in a spatio-temporal manner, which makes them attractive for the development of more selective pharmacological treatments for numerous neurological and psychiatric diseases (Missale et al., 2006, 2013; Borroto-Escuela et al., 2017a, 2017b). DA receptors have been shown – mostly in heterologous systems – to form many heteromers with other GPCR, ionotropic channels and transmembrane proteins, resulting in functional changes of partner receptors, modification of binding affinity for ligands and biased signaling. The diversity and biophysical properties of DA receptor heteromers have been exhaustively reviewed elsewhere (Wang et al., 2012; Ferré et al., 2016). Moreover, numerous studies showed the critical role of the physical interaction between DA receptors and NMDAR for their reciprocal modulation (Lee et al., 2002; Pei et al., 2004; Cepeda and Levine, 2006; Wang et al., 2012; Ladepeche et al., 2013), which makes them particularly relevant for drug addiction.

The current review focuses on reports that were able to provide proof of concept that endogenous DA receptor heteromers in the brain can be modulated in response to exposure to drugs of abuse and eventually participate to drug-evoked neuronal adaptations (Table 1). For each DA receptor heteromer, we start from its historical discovery in heterologous systems, the identification of the protein-protein interaction domains and associated changes in signaling, to finally discuss their modulation in vivo and the strategies used to establish their potential roles in drug-evoked adaptations.

2. D2R heteromers and addiction

Notably because alterations of D2R-mediated signaling have been associated with numerous pathologies, there has been extensive work to identify putative heteromers formed by the D2R. Most of them were characterized in heterologous systems by bioluminescence or fluorescence resonance transfer (BRET/FRET) analyses, co-immunoprecipitation, or indirectly through modulation of ligand binding affinity and signaling effectors. These studies led to the identification of numerous and diverse receptors that are able to form complexes with the D2R and alter downstream signaling through allosteric modulations, as extensively reviewed (see Fuxe et al., 2014; Borroto-Escuela and Fuxe, 2017). Herein, we focus on D2R heteromers for which a functional impact has been characterized in vivo. Later on, the first indirect evidence for the existence of D2R/A2AR heteromers came from the observation that the A2AR agonist CGS 21680 was able to decrease D2R affinity for the agonist apomorphine in native membranes prepared from the rat striatum, suggesting allosteric modulations within D2R/A2AR complexes (Ferré et al., 1991). Since this discovery, extensive work, mainly by Ferré’s and Fuxe’s groups, characterized the biophysical and pharmacological properties of D2R/A2AR heteromers, which have become a model study of allosteric modulation between GPCR (Ferré et al., 2018; Borroto-Escuela et al., 2018a). The extensive description of the biophysical properties of D2R/A2AR heteromers is out of the scope of the current article but remarkable recent reviews can be recommended (Ferré et al., 2016, 2018). A current model developed by Ferré and collaborators propose that A2AR/A2AR and D2R/D2R homodimers, coupled to their respective G protein – Gi/α and Gs/olf and Gs/o, constitute an heterotetrameric structure forming a macromolecular complex with type 5 adenylate cyclase (AC5) (Navarro et al., 2018; Ferré et al., 2018). Electrostatic interactions between transmembrane domains (TM) 6 form the homodimer interface, while heteromeric interactions involve TM 4 and 5 of one receptor within each homodimer (see Navarro et al., 2018). D2R/A2AR heteromerization results in negative allosteric interactions by which A2AR ligands – agonists and antagonists - decrease the affinity and efficacy of D2R ligands (Azzad et al., 2009; Bonaventura et al., 2015) and, reciprocally, D2R agonists inhibit A2AR-dependent activation of AC5 (Navarro et al., 2018). In terms of signaling, this antagonistic interaction would result in a switch from cyclic AMP- to PLC- and arrestin-dependent pathways, whether stimulation of A2AR or D2R, respectively, is dominant (Borroto-Escuela et al., 2011; see Ferré et al., 2018 for review).

These unique properties of D2R/A2AR heteromers have opened new routes for pharmacological strategies for pathologies related to alterations in DA transmission. For instance, even though the direct dependence to D2R/A2AR heteromers has not been demonstrated, preclinical evidence support some efficacy of A2AR antagonists to alleviate symptoms in Parkinson’s disease (Salamone et al., 2013; Podurgiel et al., 2016) or apathy – i.e. loss of motivation (Mott et al., 2009; Salamone et al., 2009; Pardo et al., 2012; Nunes et al., 2013; Randall et al., 2014; Lopez-Cruz et al., 2018). Recently, Volkow and colleagues showed that the psychostimulant cocaine, a potent A2AR antagonist, enhances D2R availability in healthy control subjects measure by Positron Emission Tomography (PET) (Volkow et al., 2015). It is unclear whether this effect results from upregulation of membrane expression of D2R or change in their binding affinity for the radioligand (3H)-Raclopride, but Ferré and colleagues proposed that it could directly result from the “ability of cocaine to antagonize the effect of endogenous adenosine on the binding of exogenous D2R antagonist” (Ferré et al., 2018). This hypothesis remains to be tested but these observations highlight the potential interest of D2R/A2AR heteromerization in the modulation of D2R activity and binding affinity. Yet, a decrease in striatal D2R availability in subjects suffering from drug addiction is one of the most consistent findings in PET imaging (Koob and Volkow, 2016; Triffilieff et al., 2017). The causes for such decrease are still unclear and could be both a consequence of drug exposure and/ or a vulnerability factor for drug abuse. Nevertheless, this neurobiological feature is likely to constitute a central mechanism for the development of addiction (Triffilieff and Martinez, 2014). Because the use of selective D2R ligands would impact crucial physiological functions and lead to severe unwanted side-effects, compounds that could act as allosteric modulators of D2R activity are a promising strategy in the field of addiction. In this context, D2R/A2AR heteromerization should be considered as a potential target.

Accordingly, preclinical data suggest that D2R/A2AR heteromerization could be involved in some aspects of addiction for psychostimulants. Extended cocaine self-administration resulted in an increased binding for (3H)-2M 24138S – an A2AR antagonist – in the NAcc of rats, which was reversed after extinction or withdrawal...
have been associated with numerous developmental, neurological and psychiatric disorders, including addiction (Lau and Zukin, 2007). NMDAR are heteromeric structures composed of two obligatory GluN1 subunits (formerly named NR1) and two regulatory GluN2 (GluN2A, GluN2B, GluN2C, GluN2D) and/or GluN3 subunits (GluN3A, GluN3B). Depending on the subunit composition, NMDAR fall into two main categories, the di-heteromeric (GluN1 with identical GluN2) or the tri-heteromeric ones, composed of GluN1 associated to a mixture of GluN2 and/or GluN3 subunits. Most differences between these subunits mainly reside in their intracellular C-terminal domains (CTD), which are subjected to post-translational modifications controlling NMDAR trafficking, protein interactions, Ca²⁺ dynamics andcoupling to specific pathways (Paolotti et al., 2013). Among other post-translational modifications, Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) (Omukumar et al., 1996) and protein kinase C (PKC) (Liao et al., 2001) can phosphorylate the GluN2B CTD on Ser1303, which enhances NMDA currents (Lieberman and Mody, 1994; Liao et al., 2001), notably by favoring NMDAR surface expression (Jin et al., 2015).

In an attempt to identify the molecular mechanisms underlying the integration of DA and NMDA-mediated signals in response to drugs of abuse, Liu and coworkers highlighted a direct physical interaction between D2R and GluN2B subunits in post synaptic density (PSD) fractions of the rat hippocampus, prefrontal cortex and striatum (Liu et al., 2006). They observed that a single cocaine injection triggers an increased D2R/GluN2B heteromerization in the NAcc and dorsal striatum, paralleled by a decrease of phospho-GluN2B-Ser1303. This was strictly dependent on D2R signaling since the D2R antagonist Eticlopride blocked both cocaine-mediated inhibition of phospho-GluN2B-Ser1303 and D2R/GluN2B interaction, while a D2R agonist had opposite effects. The protein domains involved in D2R/GluN2B interaction were then characterized and the residues R220, A234 within third intracellular loop of D2R (D2R-IL3) appeared as critical for the binding of D2R to GluN2B (Fig. 1a). By using a penetrating peptide corresponding this R²²⁰A²³⁴-D2R-IL3 fragment, which disrupts D2R/GluN2B heteromers (Fig. 1b), it was shown that the cocaine-induced stimulation of D2R facilitates the D2R/GluN2B interaction, leading to a decrease of NMDAR currents in iMSN. This inhibition of NMDAR relies on a competition mechanism whereby D2R/GluN2B heteromers induced by cocaine disrupt the binding of CaMKII to GluN2B and reduces CaMKII activity (Fig. 1c). At the behavioral level, disruption of D2R/GluN2B interaction inhibits the acute hyperlocomotion and stereotypies induced by a single high dose of cocaine (Liu et al., 2006). This pioneer study strongly supports the hypothesis that D2R/GluN2B heteromers...
could mediate the inhibitory effects of psychostimulants on iMSNs. However, it raises several important questions that we are currently pursuing. With regard to the chronic nature of addiction-related disorders, it would be important to determine how D2R/GluN2B heteromers are modulated in response to repeated exposure cocaine and if disrupting D2R/GluN2B interaction could overcome cocaine-evoked long-lasting responses. Furthermore, the R220A224 -D2R-IL3 peptide used to alter D2R/GluN2B interaction comprises a part of the Arginine-rich domain of D2R that is critical for its binding to A2AR (Ciruela et al., 2004; see section 2.1). Using a shorter D2R-IL3 peptide that selectively alters D2R/GluN2B interaction would definitely precise the specific role of D2R/GluN2B in long-term effects of drugs of abuse. This is particularly relevant in light of recent evidence showing that D2R-mediated inhibition of NMDAR also involves D2R/A2AR interaction (Azdad et al., 2009).

2.3. D1R/D2R heteromers

Another DA receptor heteromer that has been extensively studied, mostly by the group of Susan R. George, is the one formed by the D1R and D2R. Co-expression of both receptors in the striatum has been reported mostly in the shell of the NAcc (Deng et al., 2006; Hasbi et al., 2009; Perreault et al., 2010; Gangarossa et al., 2013; Frederick et al., 2015; Gagnon et al., 2017) and D1R/D2R complexes were detected by PLA in situ in the striatum of rodents and non-human primates (Perreault et al., 2016; Rico et al., 2017; Hasbi et al., 2018), even though there has been some controversy since the work form Frederick and co-workers showed D1R/D2R complexes were not detectable by using the same approach in vivo in mice within the fraction of MSN expressing both D1R and D2R receptors (Frederick et al., 2015). In heterologous systems, the formation of D1R/D2R complexes could lead to atypical signaling: instead of the canonical G<sub>i</sub> or G<sub>q</sub> proteins, D1R/D2R heteromers would be able to recruit G<sub>i</sub> protein, leading to PLC-mediated intracellular Ca<sup>2+</sup> release (Perreault et al., 2014). It was argued that the D1R ligand SKF83959 preferentially recruits D1R/D2R heteromer-dependent Ca<sup>2+</sup> signaling, due to alterations of the D1R binding pocket when in the heteromeric complex, supporting the idea of an allosteric modulation within the D1R/D2R heteromers (Hasbi et al., 2009, 2010; Verma et al., 2010; Perreault et al., 2014).

To target D1R/D2R heteromers, Hasbi et al. (2014) designed and interfering peptide, based on putative interaction sites between both receptors involving 2 adjacent Arginine in the D2R-IL3 and 2 adjacent Glutamic acid of the D1R CTD (O’Dowd et al., 2012). This “Tat-D1” peptide, corresponding to amino acid 396–413 of the D1R CTD, was shown to perturb i) D1R/D2R − but not D2R-D5R - interaction, ii) SKF83959-induced Ca<sup>2+</sup> transients in vitro and iii) co-immunoprecipitation from membrane extracts, while a scrambled peptide had no effect (Hasbi et al., 2014).

In the context of addiction, the Tat-D1 peptide was shown to reverse the SKF83959-induced inhibition of amphetamine sensitization (Shen et al., 2015a). The same group showed that the Tat-D1 peptide alone enhanced cocaine-induced locomotor sensitization and place-preference, as well as the reinstatement after self-administration (Perreault et al., 2016; Hasbi et al., 2018). It should be noted that the Tat-D1 peptide displayed, by itself, pro-locomotor effects (Shen et al., 2015a) and induced place-preference (Perreault et al., 2016; Hasbi et al., 2018). Importantly, the Tat-D1 peptide used to disrupt D1R/D2R interaction strongly overlaps with the so-called “Tat-D1-t2 peptide” originally designed to block D1R/NMDAR heteromers (Nai et al., 2010; Ladepeche et al., 2013; see section 3.1). Furthermore, the Tat-D1-t2 was shown to induce, by itself, ERK activation (Cahill et al., 2014b) and to potentiate glutamatergic synapses (Ladepeche et al., 2013), suggesting that this peptide could mimic D1R/NMDA interaction (see section 3.1), which could explain the pro-rewarding effect of the Tat-D1 peptide used by the George’s group. The specificity of the Tat-D1 peptide in selectively impairing D1R/D2R interaction has therefore to be further validated. Moreover, most of the in vivo effects of the Tat-D1 peptide were assessed through its ability to abolish/attenuate the behavioral impact of SKF83959 (Hasbi et al., 2014, 2018; Shen et al., 2015a,b). Yet, the properties of SKF83959 as a preferential ligand for D1R/D2R heteromers capable of recruiting G<sub>i</sub>-dependent mechanisms has been recently challenged (Chun et al., 2013; Lee et al., 2014a,b; Frederick et al., 2015). Further work is needed to unravel the unique properties of this atypical subpopulation of MSN co-expressing both D1R and D2R and their potential regulation by putative D1R/D2R heteromers.

3. D1R/D2R heteromers and addiction

3.1. D1R/NMDA heteromers

As opposed to the inhibitory role of D2R on NMDAR currents (see section 2.3), there is a general consensus toward a facilitating effect of D1R stimulation on NMDAR functions (Flores-Hernandez et al., 2002; Wittmann et al., 2005; but see Lee et al., 2002). This positive feedback is bi-directional since NMDAR stimulation also favors D1R surface expression and downstream cAMP production (Pei et al., 2004). The mechanisms underlying the interplay between D1R and NMDAR signals have been intensely investigated, notably because targeting these processes offers the prospect of restoring the imbalance in D1R and NMDAR functions described in numerous psychiatric diseases (Cepeda and Levine, 2006; Missale et al., 2006; Yao et al., 2008; Wang et al., 2012).

The synergy between D1R and NMDAR plays functional roles in drug of abuse-mediated responses. We observed that psychostimulant-mediated striatal activation of ERK1/2, which controls long-term neuronal adaptations, relied on a concomitant stimulation of D1R and NMDAR (Valjent et al., 2000, 2005; Pascoli et al., 2011a). This convergence of DA and glutamate signals onto ERK1/2 involves a D1R-mediated phosphorylation of GluN2B-containing NMDAR on Tyr<sup>1472</sup> via the tyrosine kinase Fyn, which triggers a Ca<sup>2+</sup>-dependent activation of ERK1/2 that launches cocaine-induced epigenetic, genic, morphological and behavioral changes (Fig. 1d; Pascoli et al., 2011a). In addition to signaling cascades linking D1R stimulation to a facilitation of GluN2B-NMDAR, the potential role D1R/NMDAR heteromers in drug of abuse-evoked plasticity is only emerging.

The seminal work of Fang Liu and colleagues led to the identification of a direct binding of D1R, but not D5R, to the GluN1 and GluN2A subunits of NMDAR in cultured cells overexpressing the receptors, and hippocampal tissues (Lee et al., 2002; Pei et al., 2004). These D1R-GluN1 complexes were also detected in the mouse striatum (Cahill et al., 2014b), including in PSD fractions (Fiorentini et al., 2003), as well as in human post-mortem caudate putamen tissues (AA, ES, RW, PT, PV unpublished observations). In vitro assays showed that the D1R-t2 (i<sup>387</sup>-L<sup>416</sup>) and D1R-t3 (S<sup>417</sup>-T<sup>446</sup>) domains of D1R CTD bind to the CTD of GluN1 and GluN2 subunits, respectively (Fig. 1e–f; Lee et al., 2002). The D1R-t2 domain binds to the C1 cassette of GluN1 (GluN1-C1; T<sup>464</sup>-T<sup>490</sup>; Lee et al., 2002; Fiorentini et al., 2003; Pei et al., 2004), which comprises multiple PKA and PKC phosphorylation sites (Tingley et al., 1997; Wang et al., 2014) and controls NMDAR trafficking (Ehlers et al., 1995; Scott et al., 2003). Pull-down assays coupled to mass spectrometry established that electrostatic interactions between the Arginine-rich epitope localized in GluN1-C1 and the acidic residues in the D1R-t2 region mediate D1R/GluN1 interaction (Woods et al., 2005). These findings highlight the importance of epitope-epitope electrostatic interactions for both D1R/NMDA and D2R/A2AR (see section 2.1), suggesting that they might represent a general mechanism for receptor heteromerization. Of note, the D1R-t2 and GluN1-C1 amino acid sequences are conserved across species, supporting the hypothesis that D1R/NMDAR heteromers might be involved in physiological functions that have been preserved through evolution (Woods et al., 2005).
Studies on the modulation of D1R/GluN1 interaction by receptor agonists or antagonists led to controversial findings, with authors reporting a D1R agonist-dependent decrease of endogenous D1R/GluN1 heteromers in cultured hippocampal neurons (Lee et al., 2002), whereas BRET experiments performed on cell lines overexpressing the receptors showed no effect of D1R or NMDAR agonists and antagonists (Fiorentini et al., 2003).

To study whether cocaine could modulate endogenous D1R/GluN1 heteromerization, we first used cultured striatal neurons co-stimulated with a D1R agonist together with a low dose of glutamate, a model that we validated as instrumental to study cocaine-evoked signaling in vivo (Pascoli et al., 2011a; Cahill et al., 2014b). Notably, this co-stimulation paradigm, which induces a D1R-mediated facilitation of NMDAR as observed in vivo in response to cocaine, led to a significant increase of D1R/GluN1 heteromers (Cahill et al., 2014b). Their potential role in the convergence of D1R and NMDAR signals onto ERK1/2 activity was assessed with a cell-penetrating peptide corresponding to the C1 cassette of GluN1 (TAT-GluN1-C1; Fig. 1g). This peptide efficiently disrupted D1R/GluN1 heteromerization induced by the co-stimulation, as well as the D1R-mediated potentiation of Ca2+ transients through GluN2B-NMDAR and downstream ERK activation. Importantly, the TAT-GluN1-C1 peptide was able to block the D1R/NMDAR-dependent signaling, while preserving the functions of individual D1R and NMDAR (Cahill et al., 2014b). The inhibition of D1R/GluN2A interaction with a TAT-D1R-t3 peptide had no effect on ERK1/2 activation downstream of D1R and NMDAR. These observations posit D1R/GluN1 heteromers as the molecular bridge linking DA to the facilitation of NMDAR and ERK activation in an in vitro model of cocaine exposure.

Since drug-induced adaptive behavior has been causally linked to long-term synaptic plasticity of glutamate synapses onto dMSN (Pascoli et al., 2011b, 2014b), it seems critical to determine whether D1R/NMDAR heteromers are present at synaptic sites and can control synaptic plasticity.

On organotypic striatal cultures the lateral diffusion of D1R at the plasma membrane is greatly reduced upon NMDAR stimulation, which favors confined D1R expression within dendritic spines. This recruitment of D1R in spines depends on D1R/GluN1 heteromer formation, leading to the model of "diffusion trap system" whereby agonist-induced allosteric changes of NMDAR enhances D1R/NMDAR interaction at synaptic sites (Scott et al., 2006). This model stipulates that upon glutamate release, D1R/NMDAR heteromers are preferentially formed within, or at the close vicinity of, synapses. Accordingly, the CTD of both GluN2 subunits of NMDAR and D1R are known to bind the synaptic protein PSD-95, which stabilizes their localization at synapses (Kornau et al., 1995; Niethammer et al., 1996; Kim and Sheng, 2004; Zhang et al., 2009; Ladepeche et al., 2013). The expression of both receptors is thus confined at synaptic sites owing to their interaction with PSD-95. However, Zhang and colleagues demonstrated that GluN1 and PSD-95 bind to the D1R CTD through docking sites that overlap at the level of the D1R-t2 domain (Zhang et al., 2009). Through a competition mechanism, PSD-95 is thus a limiting factor for D1R/GluN1 heteromerization at synapses, even though these receptor complexes are detected in striatal PSD fractions (Fiorentini et al., 2003).

To assess the role of D1R/GluN1 interaction in the modulation of synaptic transmission, electrophysiological recordings were performed from cortico-striatal slices prepared from the drd1a-egfp reporter mice to visualize dMSN (Gong et al., 2003). Inhibition of D1R/NMDAR interaction with the GluN1-C1 peptide fully blocked D1R-mediated facilitation of NMDA-mediated excitatory post-synaptic currents, while sparing basal synaptic transmission (Cahill et al., 2014b). A GluN2B antagonist was also able to abrogate the D1R-mediated facilitation of NMDA currents, whereas disruption of D1R/GluN2A heteromers with the D1R-t3 peptide had no effect. This suggests that D1R/GluN2A heteromers are not primarily involved in the modulation of NMDAR currents by DA in dMSN. It also shows that D1R/GluN1 interaction preferentially modulates GluN2B-NMDAR, even though the underlying mechanism is still unknown.

To study the role of D1R/GluN1 interaction in striatal plasticity, high frequency electrical stimulations (HFS) of the cortex triggering long-term potentiation (LTP) of striatal synapses in both dMSN and iMSN were applied to cortico-striatal slices (Pascoli et al., 2011b). This allowed us to demonstrate the mandatory role of D1R/NMDAR heteromers for LTP in dMSN, but not iMSN (Cahill et al., 2014b). Accordingly, the facilitating effect of D1R on NMDAR-mediated LTP also required D1R/NMDAR interaction in the hippocampus (Nai et al., 2010). In contrast, Ladepeche and co-workers found that blocking D1R/GluN1 interaction with the TAT-D1R-t2 peptide increased the number of potentiated synapses in a chemically-induced LTP in cultured hippocampal neurons (Ladepeche et al., 2013). The apparent discrepancy between these results is likely due to differences in experimental models (cortico-striatal slices vs. hippocampal neurons) and/or to the strategies used to block D1R/GluN1 heteromers. In fact, we found that the D1R-t2 peptide was able to activate NMDAR and ERK by itself (Cahill et al., 2014b), which could explain its permissive effect on chemical LTP. By contrast, when using the GluN1-C1 peptide, which disrupts D1R binding to NMDAR without affecting the functions of individual D1R and NMDAR, we observed a blockade of HFS-induced striatal LTP (see Andrianarivelolo et al., 2018). Regarding cocaine-evoked responses in vivo, the infusion of the TAT-GluN1-C1 peptide into the NAcc was able to attenuate cocaine-induced ERK1/2 activation. Inhibiting D1R/GluN1 heteromers in the NAcc did not impact neither the basal locomotion nor the acute hyperlocomotor response to a first injection. By contrast, the disruption of D1R/GluN1 interaction prior and during the first injection of cocaine altered the locomotor sensitization induced by a second injection of cocaine administered a week later (Cahill et al., 2014b). This supports a role for D1R/GluN1 heteromers in the NAcc in the development of persistent cocaine-induced adaptations in acute models of cocaine exposure. To date, the impact of acute or chronic exposure on endogenous D1R/GluN1 heteromerization in vivo is still unknown. Whether or not these heteromers are involved in the development and maintenance of long-lasting structural and behavioral adaptation remains to be established. We are currently tackling these issues, which are critical because they will unravel whether of D1R/GluN1 and D2R/GluN2 heteromers are mediators of the excitatory and inhibitory actions of drugs of abuse on dMSN and iMSN, respectively.

4. Conclusions and perspectives

Numerous studies focused on the mechanisms underlying allosteric changes induced receptor heteromerization and associated modification of downstream signaling but only few reports described roles of endogenous heteromers in vivo (Table 1). This is likely due to limitations of current approaches to detect heteromers in situ. In fact, class C GPCRs form stable oligomers but class A GPCRs oligomerization seems highly dynamic, and its existence is still a matter of debate. Furthermore, most discoveries on receptor oligomers originate from studies in heterologous systems lacking the dense synaptic macromolecular complexes where receptors have multiple and dynamic interactors. These interactions, sometimes involving overlapping sites, represent a major challenge to selectively alter heteromer formation without impacting on the component receptor’s interaction with other partners. This implies a thorough characterization of potential off-target effects and caution when interpreting the results. Such strategy design could benefit from crystal structures of the receptors to model the interface of heteromers, as recently performed for D2R/A2R (Borroto-Escuela et al., 2018c). This could facilitate the identification of the minimal amino-acid residues necessary for receptor heteromerization, the design of non-peptidic interfering molecules or bi-valent compounds selectively targeting receptor oligomers (Soriano et al., 2009).

Several DA receptors heteromers with a therapeutic potential have been described, including D1R/D3R (Fiorentini et al., 2010), but their
roles remain to be established. Moreover, most studies on receptor heteromers in addiction focused on the striatum because of its key role in the pathology and its enrichment in DA receptors. However, a thorough characterization of heteromer expression within the entire brain reward circuit would be of importance considering the implication of various brain regions in distinct components of addiction. Moreover, the implication of DA receptor heteromers has been mainly studied in the initial phases of drug exposure. Addressing their roles in each phases of drug addiction (maintenance, craving and relapse) seems of crucial importance as it could open new routes for the development of selective strategies targeting these heteromers for the treatment of drug addiction.

Declaration of interest
The authors report not biomedical financial interest or conflicts of interest.

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References


SWI/SNF chromatin remodeler complex within the reward pathway is required for behavioral adaptations to stress

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Abstract
Stress exposure is a cardinal risk factor for most psychiatric diseases. Yet, the underlying mechanisms are far from being understood. Both preclinical and clinical studies point to changes in gene expression involving epigenetic modifications within mesocorticolimbic brain circuits. Besides chemical chromatin modifications, chromatin reorganization is an essential step that promotes enduring plastic changes in gene expression. So far, the role of chromatin remodeling in stress-related mental disorders has been largely understudied. Brahma (BRM) and Brahma-Related Gene 1 (BRG1) are ATPase subunits of the SWI/SNF chromatin remodeling complexes, which are known to potentiate nuclear receptors transcriptional activity such as the glucocorticoid receptor. Here, we show that social defeat stress induces enduring changes in BRG1 nuclear distribution. The inactivation of Brg1 gene within dopamine-innervated regions, and the constitutive inactivation of Brm gene lead to a virtual resilience to chronic social defeat stress and strongly decreases the behavioral responses to cocaine. These mutations do not impact midbrain dopamine neurons activity. In contrast, they lead to increased levels of heterochromatin and markedly reduce stress- and cocaine-induced immediate early genes and phosphorylation of histone H3S10 within striatal medium spiny neurons. These data highlight the importance of SWI/SNF chromatin remodeler complexes in the transcriptional and behavioral adaptations to salient environmental challenges.

Introduction
In the past decade, intensive efforts have been made to understand the mechanisms through which stress exposure can lead to life-long behavioral impairments. These persistent behavioral changes are underpinned by long-lasting neuroadaptations which are thought to arise from maladaptive gene expression. In this context, the focus has naturally been brought onto epigenetic mechanisms and compelling evidence shows that transcription factors\(^1\), but also histone modifications\(^2\)-\(^4\), and DNA methylations\(^5\) within the meso-cortico-limbic system regulate the vulnerability to chronic stress exposure and the development of depressive-like behaviors in mice.

Stress exposure elicits a rapid rise of glucocorticoid hormones within the blood flow. Through their binding to glucocorticoid receptors (GR), a ubiquitously expressed transcription factor, they play a key role in orchestrating the transcriptional response
to stress exposure thereby facilitating one’s ability to cope with environmental challenges. However, if the stress response is too frequently solicited, it may lead to psychiatric diseases such as depression or addiction\(^6\) that are often associated with dysregulated glucocorticoids homeostasis. Our previous work shows that pathological stress-responses triggered by exposure to chronic social stress involve GR in dopamine-innervated brain areas, which include the striatum, the nucleus accumbens (NAc), and deep-layers of the prefrontal cortex (PFC)\(^7\). Similarly, we have also demonstrated that GR gene inactivation in these same regions decreases behavioral responses to psychostimulants\(^8\)\(^-\)^\(^10\).

GR modulates gene expression through distinct mechanisms. It can either interact with components of the basal transcriptional machinery or recruit histone modifiers or chromatin remodeler complexes such as the mating-type switching/sucrose non-fermenting (SWI/SNF) complex\(^11\)\(^-\)^\(^12\). These complexes use the energy of ATP to disrupt nucleosome DNA contacts, making possible the shift of nucleosomes along DNA and the removal or the exchange of nucleosomes. Consequently, they facilitate the accessibility of DNA or chromatin to proteins that can shape transcriptional responses\(^13\). Mammalian SWI/SNF are multi-subunit complexes that contain either Brahma (BRM) or Brahma-related gene 1 (BRG1) as central ATPase subunits, associated with 10-12 BRM/BRG1 associated factors\(^14\).

While there is compelling evidence for the involvement of DNA- and histone-modifying enzymes in behavioral impairment induced by stress and drug exposure, very little is known regarding the potential role of chromatin remodeling complexes in such responses. Recently, the upregulation within the NAc of ATP-utilizing chromatin assembly and remodeling factor (ACF), a distinct chromatin remodeler, has been shown to be key for the development of stress-induced depressive-like behavior and for cocaine’s reinforcing properties\(^15\)\(^,\)^\(^16\). In addition, striatal BRG1 has been recently involved in cocaine relapse\(^17\). Here, we show that stress exposure induces a stable reorganization of GR and BRG1 nuclear distribution within striatal medium spiny neurons (MSNs). We further show that BRG1 in dopaminoceptive neurons and BRM are essential for the development of social avoidance and pathological anxiety after repeated social defeat in mice, a well-validated preclinical model of depression. Mice lacking either BRG1 or BRM also exhibit decreased responses to cocaine in locomotor
sensitization and conditioned place preference paradigms. Stress- and cocaine-induced behaviors are known to rely on dopamine neurons activity within the ventral tegmental area, however, the absence of BRG1 or BRM in our models does not alter this neuronal population. Instead, we found that BRG1 is necessary for stress-induced transcriptional response in striatal MSNs. Indeed, while the integration of glutamate and dopamine signals and the ensuing activation of the ERK signaling pathway within the MSNs remained unchanged in absence of BRG1, the induction of immediate early genes along with the Serine 10 phosphorylation of the histone 3 by stress exposure were strongly reduced and the surface of transcriptionally silent heterochromatin was increased.

Altogether, these results posit the SWI/SNF complex within the mesocorticolimbic system as a key player in maladaptive transcriptional responses to stress and drugs of abuse and the ensuing pathological behaviors.

Material and Methods
Animal Breeding
Animals were bred and raised under standard animal housing conditions, at 22°C, 55% to 65% humidity, with a 12-h light/dark cycle (7 AM/7 PM) and had free access to water and a rodent diet. All experiments were performed in accordance with the European Directive 2010/63/UE and the recommendation 2007/526/EC for care of laboratory animals (decision #7074 2016091623299400). Mice were 2 to 4-month-old males. Experiments were carried out during the light phase.

Mice carrying an inactivated Smarca2 gene, generated by homologous recombination, have been previously described\(^1\). Briefly, an internal exon of Smarca2 gene (thereafter denominated Brm), corresponding to amino acids 120-276, was disrupted by the insertion of a neomycin cassette. This led to the absence, in Brm\(^{-}\) mice, of any functional BRM protein able to assemble in SWI/SNF complexes. To inactivate Smarca4 (thereafter denominated Brg1), gene in dopaminergic neurons, we crossed mice carrying a conditional allele of Brg1 gene, sensitive to the Cre recombinase \((Brg1^{L2})\), with TgYAC-D1aCre mouse line\(^2\). The Brg1\(^{L2}\) allele contains DNA LoxP sites flanking two internal exons. Their excision results in the deletion of the sequence encoding amino acid residues 814 (threonine) to 872 (lysine) and the creation of a frameshift in exon 4 with a stop codon at amino acid position 819.
The remaining protein produced from the disrupted gene will encode a C-terminally 
truncated protein lacking the ATP hydrolysis site.

DNA from mice tails was extracted and analyzed by PCR. For BrmWT and Brm- alleles 
the primers were CCTGAGTCATTTGCTATAGCCTGTG (sense strand), 
CTGGACTGCCAGCTGCAGAG (reverse strand) and CATCGCCTTCTATCGCCTTC 
(reverse strand in the neomycin cassette). The amplified bands corresponding to the 
wild-type and the mutated allele are 310 and 700 bp in length, respectively. Brg1+ and 
Brg1L2 alleles were detected using GATCAGCTCATGCCCTAAGG (sense strand) and 
GCCTTGTCCTCAAACTGATAAG (reverse strand), primers giving rise to 241bp and 
313bp DNA segments, respectively. To detect the D1Cre transgene, primers were 
GCCTGCATTACCGGTCGATGCAACGA (sense strand) and 
GTGGCAGATGGCGCGGCAACATT (reverse strand). The amplified band was 
700 bp long.

Animals were bred on a mix genetic background derived of 129SvE and C57BL/6 
mouse strains. For experiments in which Brm, Brg1 and compound mutants were 
compared (essentially cocaine sensitization), animals were bred following a strict 
breeding scheme. Brm+/+:Brg1+/L2: TgYAC1aCre males and females, issued from a 
mating between Brm−/−:Brg1L2/L2: TgYAC1aCre males and C57BL/6 females, were 
mated to obtain Brm+/+:Brg1+/L2/L2 females and Brm+/+:+Brg1+/L2/L2:TgYAC1aCre males, 
as well as Brm−/−:Brg1L2/L2 females and Brm−/−:Brg1L2/L2: TgYAC1aCre males. These 
individuals were then mated to generate experimental males that were first cousins: 
control mice (Brm+/+:Brg1L2/L2, denominated Brg1LoxP thereafter), single mutants (Brm− 
/l:Brg1L2/L2 and Brm+/+:Brg1L2/L2: TgYAC1aCre, denominated Brm−/- and Brg1D1Cre 
respectively thereafter) and compound mutants (denominated Brm+/+:Brg1D1Cre 
thereafter). For the other experiments, each model was compared to its own control 
littermates. The GRLoxP/loxP and GRD1Cre mice used for the co-immunoprecipitation 
experiment have been previously described8.

Co-immunoprecipitation assays and western blots
Whole striata were dissected and homogenized in 200 μl of radio-immunoprecipitation 
assay (RIPA) buffer (50 mM Tris- HCl, pH 7.6, 150 mM NaCl, 1% Nonidet P-40, 0.1% 
SDS, 0.5% sodium deoxycholate, 2 mM EDTA, 0.2 mM sodium orthovanadate and 1X 
protease inhibitor complete cocktail (EDTA-Free, Roche, 11873580001). Samples 
were centrifuged for 30 minutes at 13,000 rpm and cell lysates were subjected to a
second centrifugation for 20 minutes. 150 μl of lysate from $GR^{loxP/loxP}$ male striata were precleared with 20 μl of protein-A sepharose beads (Sigma, 82506) by a 4 h incubation at 4°C. Immunoprecipitation was performed by incubating overnight 50 μl of the supernatants each containing the following antibodies: an anti-GR (a rabbit polyclonal M20 antibody raised against the N-terminal region, 1:1000 dilution, Santa Cruz Biotechnology), an anti-BRG1 (mouse monoclonal, 1:1000, Santa Cruz, sc-17796 #2012) and an anti-BRM (rabbit polyclonal 1:500, abcam, ab15597 #GR49552-3). The following day, the supernatants were washed three times with 1 ml PBS/Tween and each precipitate was resuspended in 60 μl of 2X Laemmli Buffer. 20 μl of the immunoprecipitated fractions and 20 μg of striata lysates from $GR^{D1Cre}$ and $GR^{loxP/loxP}$ mice were denatured at 95°C for 5 minutes. The samples were then separated on a 10% SDS-PAGE gel and transferred onto a nitrocellulose membrane (Bio-Rad, 1620112). The membranes were incubated in a blocking solution (5% milk in TBS-T: 20mM Tris-HCl, pH 7.6, 137 mM NaCl, and 0.1% Tween 20) for 1h at room temperature and then incubated with anti-GR antibody (1:1000 M20 #0315) in a 5% BSA in TBS-T overnight at 4°C. Membranes were washed 3 times with TBS-T, then incubated with peroxidase-conjugated donkey anti-rabbit secondary antibody (1:1000, Jackson Laboratories, 711-035-152) diluted in a milk solution 5% BSA in TBS-T for 1 h at room temperature and rinsed 3 times in TBS-T. The proteins were detected using enhanced chemiluminescence (ECL) detection kit (GE Healthcare, RPN2235).

**Histology**

Mice were deeply anaesthetized with pentobarbital (Centravet, France) and transcardially perfused with cold phosphate buffer (PB: 0.1 M Na$_2$HPO$_4$/NaH$_2$PO$_4$, pH 7.4), followed by 4% PFA. Brains were post-fixed overnight in 4% PFA-PBS. Free-floating vibratome sections (30 μm) were rinsed three times with PBS (10 minutes) and incubated (2 hours) in PBS-BT (PBS-Triton 0.1%, 0.5% BSA, $\gamma$) with 10% normal goat serum (NGS). Sections were incubated overnight (4°C) in PBS-BT containing 1% NGS with primary antibodies. Sections were then rinsed in PBS and incubated (2 h) with secondary antibodies anti-rabbit or anti-mouse Alexa488 (1:1000, Invitrogen), and/or anti-rabbit or anti-mouse CY3 (1:1000, Invitrogen). Sections were then rinsed with PBS (3x10 min) and mounted using Vectashield Mounting Medium with DAPI (Vector Laboratories). Images were acquired using a fluorescence microscope equipped with an Apotome module (Zeiss, Axiovert 200M) and a confocal microscope (Leica TCS...
The primary antibodies used include a rabbit polyclonal Cre antibody (dilution 1:3000, homemade), a rabbit polyclonal NeuN (1:200, Millipore, MABN140 #24120257), a mouse monoclonal BRG1 (1:1000, Santa Cruz, sc-17796), a rabbit polyclonal BRM (1:500, Abcam, ab15597), a mouse monoclonal c-FOS antibody (1:1000, Santa Cruz, sc-166940), a rabbit polyclonal EGR1 (1:500, Abcam, ab133695), a rabbit polyclonal phospho-ERK (1/400, Cell Signaling Technology, 4370), a mouse monoclonal H3S10P (1:1000, Millipore, 05-598 #2517825), and a mouse monoclonal Lamin B antibodies (1/1000, Abcam, ab16048).

Social defeat and ensuing sociability, anxiety and depressive like behaviors
For all tests, animals were daily transferred for habituation in a dedicated room one to two hours before the tests. For each test, chambers of the apparatus were cleaned with alcohol 10% and dried with paper towels between each trial.

Repeated social defeat and social interaction
Social defeat was performed as previously described. Six-month-old CD1 breeder male mice were screened for their aggressiveness. Brm and Brg1 gene mutants, as well as their respective control littermates were subjected to 10 consecutive days of social defeat. Each defeat consisted of 5 minutes of physical interactions between the resident mouse (CD1) and the intruder (experimental mouse). The rotation schedule was set to exclude a repeated defeat by an already encountered resident. Following this, the intruder remained for another 24 h in the resident’s home cage that was partitioned in half by a perforated transparent polycarbonate. It allowed visual, auditory, and olfactory communication whilst preventing direct contact. Undefeated mice were handled and rotated daily and housed 2 per cage with the polycarbonate partition separating the cage in 2 halves.

Social interaction was studied 24 h after the last defeat (day 11) in a low luminosity environment (30 lux). Undefeated and defeated mice were introduced for 150 s in a corner of an open-field (40x40x25 cm) containing in one side an empty perforated polycarbonate box (“no target” condition). Immediately after this, mice were rapidly removed and an unfamiliar CD1 mouse was placed in the box (“target” condition) and mice were re-exposed to the open-field for another 150 s. The time spent in the interaction zone surrounding the polycarbonate box was recorded and used as an index of social interaction.

Three chambers test
Sociability was also measured on day 12-14 after the last defeat in a three-chambered box (30x15x15 cm per compartment) with opening (5x5 cm) in each delimiting wall. The test was divided in 2 phases, each lasting 5 minutes. During the habituation phase, the challenged mouse was restrained to the central chamber, with the two doorways closed. Doors were then opened and the challenged individual could either visit the compartment with an unfamiliar adult male mouse (C57BL/6J) placed in a perforated transparent plastic box or the other compartment containing a similar empty box. The amount of time mice spent in direct interaction with each box was scored.

**Elevated zero-maze**
Elevated zero-maze was performed on day 15-18 after the last defeat. The maze was a circular track (width 7 cm, diameter outer 47 cm) elevated 80 cm from the floor and made of grey polyvinyl chloride (PVC). It was divided in four sections of equal lengths: two opposite open portions and two closed portions with PVC walls, 15 cm in height. At the start, mice were placed in front of a closed portion. Their movement was recorded for 10 minutes. The latency to enter into the open portion, the number of entries and the time spent in the open parts were quantified. A mouse was considered in the open portion when the 4 paws were introduced. The intensity of the light was set to 30 lux in the open parts.

**Dark-light test**
The dark-light test was performed on day 18 after the last defeat. The dark-light box was 45x20x25 cm, separated into two compartments connected by a central aperture (5x5 cm). The dark compartment (black PVC, 15 cm) was covered. The lit compartment (white PVC, 30 cm) was illuminated at an intensity of 30 lux. At the start, individuals were placed in the dark compartment and the latency to enter and time spent in the lit compartment were measured for 10 minutes. A mouse was considered to be in the lit area when its 4 paws were inside.

**Fear conditioning**
For context conditioning, animals were placed into a conditioning chamber and allowed to habituate for 3 minutes, followed by three consecutive tone and foot shocks (85 dB 30 s, 0.4 mA shock at the last 2 s) separated by 30 s. Animals remained in the chamber for 1 minute after the delivery of the last foot shock. Contextual fear conditioning was measured 24 h after in the same chamber, and freezing was scored for a total of 6 minutes. Sessions were recorded and freezing was automatically scored. Freezing
was defined as complete absence of movement, except for respiration. Cued fear conditioning was measured 24 h after contextual fear conditioning. After changing the context of the chamber (ground, walls, shape, odor and light color) animals were placed into a new chamber and allowed to habituate for 3 minutes, followed by tone for 3 minutes, and freezing was scored for the first 3 minutes and compared with the last 3 minutes.

**Motor coordination and learning**

Motor coordination was assessed using a rotarod apparatus (Roarod/LSI Letica, Biological Instruments). Mice were placed on the rotating rod with an accelerating speed levels (4 to 40 rpm within 2 minutes) mode. Four trials per day with an intertrial interval of 30 minutes were performed during 6 consecutive days. The time of the fall was noted for each mouse. The maximum duration of each trial was set to 1 minute 30.

**T-maze**

We used the methods previously described\textsuperscript{23}. Briefly, this task was performed in a T-Maze with a starting arm and two goal arms. Mice were tested on 10 trials per day, each trial consisting of two runs, a forced run (in which one of the two goal arms is blocked) and a choice run (where both goal arms are open). To obtain a reward, mice were required during the choice run to enter the goal arm not visited during the forced run. The number of correct choices were scored daily and the task ended when all the mice reached the learning criterion of 7 correct choices out of 10 for 3 consecutive days.

**Behavioral responses to cocaine**

*Spontaneous locomotor activity and drug sensitization*

Locomotor activity was assessed in circular chamber (4.5 cm width, 17 cm external diameter) crossed by four infrared captors (1.5 cm above the base) placed at every 90° (Imetronic, Pessac, France). Locomotor activity was scored automatically when animals interrupted two successive beams, having traveled a quarter of the circular corridor. The spontaneous locomotor activity was measured during 180 minutes following a first introduction into the locomotor apparatus. For locomotor sensitization to cocaine (Sigma-Aldrich, Saint-Quentin Fallavier, France), mice were habituated to
the apparatus for 3 hours for 3 consecutive days and received a saline injection on Days 2 and 3 after 90 minutes of recording. On days 4 to 7 animals were daily injected with either cocaine (10 mg/kg, i.p.) or saline. Following 6 days of withdrawal, all mice received an acute challenge injection of cocaine (10 mg/kg, i.p.).

**Conditioned Place Preference**

The conditioned place preference apparatus consisted of two chambers (10x25x20 cm) with distinct visual and tactile cues connected by a neutral area. On Day 1 (preconditioning), mice were placed in the neutral area allowed to explore the apparatus freely for 18 minutes. The time spent in each chamber was measured. On Days 2, 4, and 6, cocaine-paired mice received a cocaine injection (10 mg/kg, i.p.) and were confined to one chamber for 20 minutes. On days 3, 5, and 7, cocaine-paired individuals received saline in the opposite chamber and were also confined for 20 minutes. Saline-paired animals received saline in both chambers. During the post-conditioning (Day 8), mice (in a drug free state) were allowed to explore both chambers freely for 18 minutes. The CPP scores were expressed as the increase of time spent in the paired chamber between the post- and the preconditioning sessions.

**In vivo electrophysiological recordings**

Adult male mice (6-12 weeks) were anesthetized with chloral hydrate (8%), 400 mg/kg i.p. supplemented as required to maintain optimal anesthesia throughout the experiment, and positioned in a stereotaxic frame. A hole was drilled in the skull above midbrain dopaminergic nuclei (coordinates: 3 ± 0.3 mm posterior to bregma, 0.5 ± 0.1 mm [VTA] lateral to the midline)24. Recording electrodes were pulled from borosilicate glass capillaries (with outer and inner diameters of 1.50 and 1.17 mm, respectively) with a Narishige electrode puller. The tips were broken under microscope control and filled with 0.5% sodium acetate. Electrodes had tip diameters of 1-2 µm and impedances of 20–50 MΩ. A reference electrode was placed in the subcutaneous tissue. The recording electrodes were lowered vertically through the hole with a micro drive. Electrical signals were amplified by a high-impedance amplifier and monitored with an oscilloscope and an audio monitor. The unit activity was digitized at 25 kHz and stored in Spike2 program. The electrophysiological characteristics of dopamine neurons were analysed in the active cells encountered when systematically passing the microelectrode in a stereotaxically defined block of brain tissue including the VTA (1). Its margins ranged from -2.9 to -3.5 mm posterior to bregma (AP), 0.3 to 0.6 mm mediolateral (ML) and 3.9 to 5 mm ventral (DV). Sampling was initiated on the right
side and then on the left side. Extracellular identification of dopamine neurons was based on their location as well as on the set of unique electrophysiological properties that distinguish dopamine from non-dopamine neurons in vivo: (i) a typical triphasic action potential with a marked negative deflection; (ii) a long duration (>2.0 ms); (iii) an action potential width from start to negative trough > 1.1 ms; (iv) a slow firing rate (<10 Hz and >1 Hz).

Electrophysiological recordings were analysed using the R software (https://www.r-project.org). Dopamine cell firing was analysed with respect to the average firing rate and the percentage of spikes within bursts (%SWB, number of spikes within burst divided by total number of spikes). Bursts were identified as discrete events consisting of a sequence of spikes such that: their onset is defined by two consecutive spikes within an interval <80 ms whenever and they terminate with an inter-spike interval >160 ms. Firing rate and %SWB were measured on successive windows of 60 s, with a 45 s overlapping period on a total period of 300 s.

**Quantification of IEGs and pERK in response to cocaine and acute social defeat.**
Immediate early genes expression (c-fos and egr1) were quantified as previously described. Mice either received an acute injection of 10 mg/kg cocaine or were exposed for 5 minutes to social defeat. The respective controls received either a saline injection or were similarly handled without aggression. The mice were deeply anesthetized with pentobarbital 1 hour after and went through transcardiac perfusion. The immunostainings were performed on 4 free-floating sections equally spaced along the rostro-caudal axis and encompassing the striatum and the NAc. The images were acquired using a fluorescence microscope equipped with an Apotome module (Zeiss, Axiovert 200M) and the number of c-FOS and EGR1 positive nuclei were quantified using Image J. Phospho-ERK was quantified the same way, except that the mice were anesthetized and their brain sampled 10 minutes after the acute social defeat.

**Quantification of nuclear H3S10P, GR and BRG1 distribution in response to acute social defeat.**
Nuclear levels and distribution of H3S10P, GR and BRG1 were quantified 1 hour after a 5 minutes social defeat (acute condition) or one day after 10 repeated daily social defeats (chronic condition). Control mice were handled in parallel, without aggression.
The images were acquired using a confocal microscope. Whole nucleus quantification has been performed automatically on 3D stacks using Image J.

**Primary striatal cultures**

Striata were dissected out from E14 (Brg1D1cre or control littermates). Mouse embryos and striatal cultures were prepared as previously described\(^\text{25}\), separately for each individual. Briefly, after trypsinisation of the tissue (Gibco), for 15 minutes at room temperature (RT), cells were incubated for 5 minutes at RT in fetal calf serum complemented with and DNase I (Worthington), followed by mechanical dissociation. Cells were then suspended in neurobasal medium supplemented with B27 (Invitrogen), 500 nM L-glutamine, 60 µg/ml penicillin G and 25 µM β-mercaptoethanol (Sigma Aldrich) and plated at a density of 1,000 cells per mm\(^2\) into 8-µwell plates (Biovalley) coated with 50 µg/ml poly-D-lysine (Sigma Aldrich). Cultures were kept in at 37°C in a humidified atmosphere with 5% CO\(_2\). Culture medium was changed on the sixth day and live calcium imaging was performed the day after.

**Calcium imaging**

On the seventh day, cultured neurons were loaded for 35 minutes at RT with a calcium probe Fluo-4 and pluronic F 127 (v/v; 50%; Invitrogen) diluted to 1/500 in a glutamate-free recording medium (129 mM NaCl, 4 mM KCl, 1 mM MgCl\(_2\), 2 mM CaCl\(_2\), 10 mM Glucose, 10 mM HEPES). Neurons were washed three times in this recording medium before imaging. Acquisitions were performed at 36.2°C every second, prior to and during treatments with the indicated doses of (R)-(+)SKF38393 (Sigma Aldrich) and/or L-glutamic acid (Calbiochem), with a spinning disk confocal microscope (Leica) using a 40X oil immersion objective. Fluorescence intensity was analyzed in the soma of all neurons present in a given field by using the ImageJ software. Viability of the neurons was tested after stimulation with a high dose of glutamate (10 µM), which yields high and sustained calcium responses (not shown), at the end of image acquisition. Data are presented as means ± s.e.m. of \(\Delta F/F\), \( ([Ft-F0]/F0) \) where Ft and F0 are the fluorescence intensities after and before stimulation, respectively. Representative pictures of the \(\Delta F/F\) signal at the pic of the responses were prepared with Matlab. Calcium imaging experiments were independently performed on 5 littermates and 5 to 23 µ-wells were analyzed for each pharmacological treatment.
Statistical analyzes

Data are presented as means ± s.e.m. Statistical analysis was carried out using either one-way or two-way ANOVA followed by Bonferroni’s multiple comparisons. Unpaired Student’s t-test were used for the nuclear shape quantification, fear-conditioning data, for the frequency of dopamine firing rates and for the calcium imaging analysis. Wilcoxon test was used for the spike in burst analysis.

Results

GR and BRG1 interact in NAc MSNs

BRG1/BRM containing SWI/SNF complexes interact with GR in various tissues and cell lines. We indeed observed an interaction between BRG1/BRM and GR in the brain by performing co-immunoprecipitation from striatal protein extracts (Figure 1a). GR were detected in striatal lysates from control GRloxP/loxP mice and their levels were reduced in striatal lysates prepared from GRD1Cre mice in which GR is depleted from most projecting medium spiny neurons (MSNs) (two first lanes respectively). We detected GR in protein extracts immunoprecipitated by GR antibodies (Figure 1a lane 3, control) as well as in extracts immunoprecipitated with anti-BRG1 (lane 4) and anti-BRM (lane 5) antibodies. To complement this biochemical approach, we next sought to examine whether BRG1 and GR could co-localize within MSNs nuclei. Using co-immunostainings, we showed that BRG1 and GR proteins are present in cells nuclei within most brain regions including MSNs from dorsal striatum and NAc (Figure 1b). To study the topological distribution of GR and BRG1 within MSNs nuclei and to evaluate the proportion of GR possibly interacting with BRG1 containing complexes, we analyzed GR-BRG1 co-localization using confocal imaging. BRG1 and GR proteins are not distributed homogenously but in nuclear foci. Under basal conditions, we observed an average of 80 ± 9 foci containing BRG1 (green) per MSN nucleus of the NAc, 247 ± 24 foci containing GR (red) and a limited number of foci (2.6 ± 0.5) containing both (yellow, Figure 1c, basal condition and Figure 1d). An acute social defeat, which elicits glucocorticoids release and GR activation, triggered a 1.7-fold increase in the number of GR foci per nucleus one hour post defeat (Figure 1c, acute social defeat and Figure 1d left panel) and a 2.2-fold increase in the number of BRG1 foci (Figure 1d middle panel). By contrast, twenty-four hours following ten days of daily social defeat, the numbers of foci for both GR and BRG1 were not significantly different
from naive basal conditions although a trend toward an increase was observed for GR (Figure 1c, repeated social defeat, and Figure 1d left and middle panels respectively). We observed that the number of foci containing both proteins was higher after both an acute social defeat (3.5 folds) and repeated social defeats (3 folds, figure 1d). The latter result suggests a stable reorganization of GR and BRG1 nuclear distributions and points to a potential involvement of GR and BRG1 interaction in the long-term behavioral effects mediated by protracted stress exposure.

**Brg1 gene ablation in dopamine-innervated areas prevents behavioral adaptation induced by repeated social defeat**

Ten days of repeated social defeat induces enduring social aversion and anxiety. This paradigm is widely used as a pre-clinical model of depression\(^2^9\) (Figure 2a and 2b). We previously showed that GR gene inactivation in dopamine-innervated areas (GR\(^{D1Cre}\) mice) prevents the appearance of social aversion after chronic social defeats\(^7\). We thus assessed the involvement of chromatin remodeling, through BRG1 and BRM containing complexes, in the appearance of long-term behavioral changes induced by stress exposure. We generated mice deprived of BRG1 in the same cell populations as GR\(^{D1Cre}\) mice (i.e. dopamine-targeted neurons; Brg1\(^{D1Cre}\) mice). Due to the simingly functional redundancy of BRG1 and BRM functions, we also used constitutive Brm mutant mice (Brm\(^{−/−}\)) to assess whether both factors act independently or if they can compensate each other. With that same aim in mind we also generated compound mutants bearing both mutations (Brg1\(^{D1Cre}\): Brm\(^{−/−}\)). In control mice, both BRG1 and BRM proteins are present within MSNs nuclei (Figure S1a, upper row) and are generally co-expressed in the whole brain (data not shown). Brg1\(^{D1Cre}\) mice show a similar profile of gene recombination to that of GR\(^{D1Cre}\) mice with a deletion of Brg1 in most MSNs of the dorsal striatum and the NAc (Figure S1a, middle row), as well as in pyramidal neurons of deep cortical layers (data not shown), a profile coherent with that expected when using the YAC-D1aCre transgenic line\(^2^1\). In Brm\(^{−/−}\) mice, the staining for BRM was totally absent from all cells (Figure S1a, lower row). Although SWI/SNF complexes are known to control the proliferation rate and the differentiation process of various cell types\(^1^4\), we observed no gross cytoarchitectural alteration and no difference in the number of neurons within the striatum and the cortex in any of our models (Figure S1a, Nissl stainings and S1b).
In absence of social defeat, Brg1\textsuperscript{D1Cre} and their control littermates exhibited significant social interaction as they spent more time interacting with an unfamiliar mouse than in the vicinity of an empty box. As expected, repeated social defeats abolished this social preference in control mice (Figure 2c, left panel). Strikingly, mice deprived of BRG1 in dopamine-innervated areas kept interacting normally with the unfamiliar mouse despite a history of social defeats (Figure 2b, right column, 2c left panel). To avoid any potential bias due to the use of CD1 background mice (same background as the aggressors) in our social interaction test, we also performed a similar task, the 3 chambers test, using C57Bl/6 mice as social targets and found a similar result (Figure S2a).

Interestingly, mice constitutively deprived of BRM showed a similar phenotype to that of Brg1\textsuperscript{D1Cre} mice, meaning that they also exhibited a normal social interaction despite the repeated social defeats (Figure 2c right panel). As previously observed\textsuperscript{7}, only a fraction of control mice (44%) exhibited social avoidance (interaction time with an empty box equal or higher than with a congener). This percentage was drastically lower in both Brg1\textsuperscript{D1Cre} and Brm\textsuperscript{-/-} mice with respectively 4% and 11% animals that displayed social aversion after repeated defeats (Figure 2d). In other words, the inactivation of either chromatin remodeler genes clearly favors a state of resilience.

Repeated social defeat increases anxiety-like behavior, as observed in defeated control mice when compared to undefeated control (Figure 2e and S2b). In the elevated O-maze test, Brg1\textsuperscript{D1Cre} mice showed a trend toward more anxiety under basal conditions but did not exhibit that increase after repeated social defeat (Figure 2e, left panel). The same result was found using the dark-light box test (Figure S2b). Brm\textsuperscript{-/-} mice displayed a different phenotype. Indeed, while repeated defeats significantly increased anxiety levels, no difference was observed between Brm\textsuperscript{-/-} mice and their control littermates (Figure 2e, right panel). Also, we did not observe any difference for locomotor activity in any group of mice (Figure S2c). Finally, social defeat is known to impact hormonal stress response. Thus, we measured the weight of adrenal glands as a proxy for average glucocorticoid production. As previously observed\textsuperscript{7, 29}, repeated social defeat induced a significant increase of adrenal gland weight and that increase was identical in both control and Brg1\textsuperscript{D1Cre} mice (Figure S3, left panel). In a similar way, general body weight increased in stressed individuals, but no difference was observed between Brg1\textsuperscript{D1Cre} mice and their control littermates (Figure S3, right panel).
Altogether, these data demonstrate that BRG1 in dopaminoceptive neurons is key for the long-term behavioral adaptation induced by chronic stress-exposure. Of note, these results were not due to altered fear learning since both controls and \(Brg1^{D1Cre}\) mice showed similar contextual and cued fear memory (Figure S4a). Finally, since \(Brg1^{D1Cre}\) mice are recombined in the whole striatum and in the cortex, we also assessed other types of learning and memory relying on these structures. In a motor learning task, the rotarod, both control and \(Brg1^{D1Cre}\) mice showed normal and identical learning and performance (Figure S4b). The same result was observed in a prefrontal-dependent T-maze non-matching to sample working memory task (Figure S4c).

**BRG1 in dopamine-targeted neurons modulates responses to cocaine**

In humans, stress-exposure is a well-known risk factor for drug addiction and many studies in animal models have shown that behavioral responses to drugs are sensitive to stress exposure and glucocorticoids\(^{30, 31}\). Since SWI/SNF complexes are key for behavioral adaptation to social defeat, and that aversive and rewarding events share overlapping mechanisms of action, we next investigated their role in cocaine responses. We first examined the locomotor sensitization that occurs following repeated injections of cocaine. We showed that the locomotor response to an acute injection of 10 mg/kg of cocaine in saline pre-treated animals was similar between controls and \(Brg1^{D1Cre}\) mice (Figure 3a and 3c). Daily injection of 10mg/kg cocaine induced a significant locomotor sensitization in control mice since they showed an enhanced activity after a challenge injection of cocaine compared to saline pre-treated animals. However, the same treatment failed to induce any locomotor sensitization in \(Brg1^{D1Cre}\) mice, which displayed locomotor responses similar to the one elicited by an acute challenge (Figure 3b and 3c). Although to a lesser extent, a trend toward a decreased sensitization was also found in \(Brm^{-}\) mutant mice compared to their control littermates while mice carrying both mutations showed an absence of locomotor response to either acute or chronic cocaine treatment (Figure S5a-c).

To further investigate the involvement of SWI/SNF complexes into behavioral responses to cocaine, we next assessed cocaine rewarding properties in a conditioned place preference paradigm. During pre-conditioning, mice showed no preference for any of the compartment of the place preference box (Figure 3d, left panel) confirming an unbiased design. After pairing one compartment to cocaine and the other to saline, control mice developed a classical place-preference for the compartment associated
with cocaine compared to the one associated with saline (score of -4 ± 41 s for saline-paired compartment and 355 ± 13 s for cocaine-paired compartment). Although $Brg1^{D1Cre}$ mice also showed a place preference for the compartment associated with cocaine injections, it was significantly lower than the one of control mice (score of 68 ± 33 s for saline-paired compartment vs 229 ± 19 s vs for cocaine-paired compartment, Figure 3d, right panel). Overall, these results show that SWI/SNF complexes in dopamine-innervated areas are required for the behavioral changes induced by drugs of abuse. In addition, as observed for the social aversion following repeated social defeat, BRG1 and BRM appear to modulate cocaine responses in a distinct manner.

Dopaminergic firing in response to chronic social defeat in $Brg1^{D1Cre}$ mice

Repeated social defeat induces a long-lasting increase of firing and bursting activities of dopamine neurons in the ventral tegmental area\textsuperscript{32}. We and others previously showed that this increase is causally linked with the appearance of social aversion\textsuperscript{7,33}. We thus examined whether the inactivation of BRG1 within dopaminoceptive neurons could also impact VTA cellular adaptations observed after social defeat. We thus measured dopamine neurons activity within the VTA of control and $Brg1^{D1Cre}$ mice in basal conditions or after chronic social defeat. In control mice, social defeat induced a significant increase of dopamine neurons firing rate (from 3.5 ± 0.2 Hz in unstressed animals to 4.3 ± 0.3 Hz after social defeat) and a significant increase of bursting activity (13.8 ± 2.8% of spike in burst in unstressed vs 27.0 ± 4.2 % in stressed animals) (Figure 4a). Surprisingly, a similar basal activity and the same increase of dopamine neurons activity was observed in $Brg1^{D1Cre}$ mice with a firing rate going from 3.2 ± 0.2 Hz to 4.8 ± 0.3 Hz after and a percentage of spike in burst going from 12 % to 28 % after social defeat (Figure 4b and S6a). Basal firing of dopamine neurons was also unchanged in both $Brg1^{D1Cre}$ and $Brm^{−/−}$ mice compared to respective control animals (Figure S6a and S6b). However, compound mutant mice deprived of both BRG1 in dopaminoceptive neurons and BRM exhibited a marked reduction of dopamine neurons firing rate and bursting activity compared to controls similar to that observed in GR mutant animals (Figure S6c)\textsuperscript{8}. These results suggest that the behavioral phenotypes observed in absence of BRG1 within dopaminoceptive neurons are unlikely due to changes of dopamine neurons activity. It is noteworthy however that compensatory mechanisms between BRM and BRG1 may be in play for the regulation of dopamine cells firing.
Changes of nuclear architecture in absence of BRG1

Responses to social defeat and cocaine are both relying on the NAc in which BRG1 is highly deleted in our mouse model. We thus asked whether the absence of BRG1 in NAc MSNs could lead to structural changes of the nucleus and to altered chromatin in this cell population. Recent report has shown that BRG1 may control nuclear shape in non-tumorigenic epithelia cell line due to its impact on chromatin dynamics\textsuperscript{34}. Using nuclear envelope Lamin B staining and confocal imaging, we found that $Brg1^{D1Cre}$ mice exhibited a significant increased percentage of abnormally shaped nuclei with an increased number of grooved (about 6\% in controls and 11\% $Brg1^{D1Cre}$ mice) and multi-lobed nuclei (about 2\% in controls and 10\% in $Brg1^{D1Cre}$ mice) compared to control mice (Figure 5a). Using DAPI staining, we also assessed the surface of heterochromatin within controls and $Brg1^{D1Cre}$ mice MSNs nuclei, which reflects the amount of transcriptionally silent regions. Indeed, BRG1 has been associated with transcriptional activation especially in the context of its interaction with nuclear receptors\textsuperscript{28}. In control mice, an acute social defeat tended to increase the surface of heterochromatin one hour after the stress while a significant decrease was observed after repeated social defeat when compared to the acute social defeat individuals. This modulation of heterochromatin surface by stress-exposure was absent in $Brg1^{D1Cre}$ mice. However, these mice showed significantly increased levels of heterochromatin in both basal and chronically stressed conditions (Figure 5b).

BRG1 in dopamine-targeted neurons is essential for striatal transcriptional responses to repeated social defeat and cocaine treatment

Since nuclear organization is impacted by the absence of BRG1, we next sought for a potential impact of $Brg1$ inactivation on gene expression. Stress exposure, as cocaine injections, activate the Extracellular Signal-Regulated Kinase (ERK) pathway, leading to the induction of immediate early genes expression, such as $c$-fos and $egr1$\textsuperscript{35-38}. These molecular responses are key for the establishment of long-term behavioral changes. We tested whether immediate early genes induction by social defeat could be altered in absence of BRG1. In control mice, an acute social defeat strongly enhanced the expression of both $c$-fos and $egr1$ genes within the dorsomedial striatum, the NAc core and the NAc shell. In $Brg1^{D1Cre}$ mice, $c$-fos induction in defeated mice was significantly reduced within the dorsomedial striatum and the NAc shell (Figure 6a, upper row), whereas $egr1$ induction was fully blocked in any structure tested.
(striatun, NAc core and shell, Figure 6a, lower row). In control mice, an acute injection of cocaine also induced the expression of c-fos gene and this induction was absent in mice deprived of BRG1 (Brg1D1Cre mice, Figure S7). These defects in immediate early genes expression are unlikely due to an ineffective ERK pathway activation. Indeed, an acute social defeat was able to induce similar levels of ERK1/2 phosphorylation in the NAc of both controls and Brg1D1Cre mice (Figure 6b). In response to cocaine, we have previously shown that the synergistic action of dopamine and glutamate, through D1 and NMDA receptors complexes, triggers a calcium-dependent activation of ERK that launches subsequent cocaine-evoked epigenetic, transcriptional and behavioral long-term adaptations. To study whether this integration of D1R and NMDAR signaling converging onto ERK was altered in Brg1D1Cre mice, we used an in vitro model of cultured striatal neurons co-stimulated with a low dose of glutamate together with a D1R agonist, which reproduces the main feature of cocaine-induced signaling. As a first step, we measured calcium signal in response to increasing glutamate concentrations, which are mediated by NMDAR stimulation in this model. We did not observe any difference in NMDAR-dependent calcium signal in cultured neurons from control and Brg1D1Cre mice (Figure S8a-e), suggesting that Brg1 deletion did not impact NMDAR-dependent signaling. By applying the co-stimulation paradigm, we could also show that the D1R-mediated facilitation of calcium entries through NMDAR that is responsible for cocaine-induced ERK activation, was not altered in Brg1D1Cre mice (Figure S8f and S8g). Altogether, these results suggest that the absence of BRG1 dampen the induction of immediate early genes without altering signal integration in MSNs. One key nuclear step before immediate early genes transcription is the phosphorylation of the serine 10 of histone H3 (H3S10P), which is catalyzed by the nuclear mitogen and stress-activated protein kinase 1 upon activation by ERK. Whereas acute social defeat triggered a significant increase of H3S10P positive foci within NAc MSNs nuclei of control mice, this was abolished in absence of BRG1, suggesting an early defect of transcriptional response at the chromatin level (Figure 6c). Altogether, these results indicate that in Brg1D1Cre mice, mechanisms controlling ERK activation in the NAc in response to stress or drugs of abuse are preserved but downstream ERK-dependent nuclear events are blunted.
Discussion

In this study, we showed that SWI/SNF chromatin remodelers complexes are essential for the behavioral and transcriptional responses to stress exposure using mouse models with genetic inactivation of their two central ATPase subunits, BRG1 and BRM. Indeed, the selective ablation of either Brg1 gene within dopaminergic neurons or the constitutive deletion of Brm gene blocked the social aversion induced by repeated social defeat, a classical model of depression. In addition, the sensitizing and hedonic properties of cocaine were also deeply reduced in our models. Importantly, these behavioral changes were not due to a general impairment of learning and memory functions since Brg1<sup>D1Cre</sup> mice showed normal performances in tasks assessing fear memory, working memory and motor learning. These persistent behavioral changes potentially involved a defect of transcriptional response as we observed in dopaminergic neurons within the striatum and the NAc but were clearly independent from any changes in dopamine neurons activity within the ventral tegmental area. In MSNs of the dorsomedial striatum and the NAc, the absence of BRG1 reduced the induction of c-fos and egr1 by a single defeat or an acute cocaine injection while the activation of the ERK signaling pathway was unaltered. At the chromatin level, in absence of BRG1, we observed a blunted induction of Serine 10 phosphorylation of H3 histones after social defeat and a global increase of heterochromatin surface within NAc MSNs suggesting a large-scale lack of genomic plasticity.

Repeated social defeat induces enduring anxiety and social avoidance, normalized by antidepressant treatment<sup>29</sup>. Epigenetic mechanisms are potentially involved to convert repeated acute stress responses into such long-term behavioral changes<sup>43</sup>. Studies focusing at DNA and histones modifications suggest that it is indeed the case. Chronic social defeat increases the expression of the DNA methyltransferase 3a in the NAc and its overexpression enhances depression-like behavior, while local inhibition of this enzyme with an inhibitor has the opposite effect<sup>5</sup>. Perinatal stress in rodents alters DNA methylation in specific genes promoters such as the one coding for the arginine vasopressine in the paraventricular nucleus, the GR and the BDNF in the hippocampus and PFC<sup>44, 45</sup>. In Human, changes in DNA methylation in post-mortem brain samples from suicide completers correlates with severe child abuse<sup>46, 47</sup>. At the level of histones, repeated social defeat also leads to changes of acetylation levels in several brain
regions including the NAc and local injections of histone deacetylase (HDAC) inhibitors within the NAc or the PFC, ultimately leading to increased acetylation, reverse the social avoidance triggered by repeated defeats. In general, systemic inhibition of histone deacetylation produces antidepressive-like effects comparable to that of regular antidepressant such as fluoxetine. On the opposite, pharmacological activation of HDACs enhances the anxiety and depressive-like effects of early life stress. Histone methylation has also been shown to be involved in long-term behavioral changes induced by stress and drug-exposure. Both repeated social defeat and cocaine have been shown to downregulate the expression of G9a methyltransferases in the NAc which catalyzes Lysine 9 histone3 dimethylation (H3K9me2), a major transcription repression mark. G9a overexpression within the NAc not only made mice resilient to repeated social defeat but also counteracted the additive effect of cocaine exposure potentially through an inhibition of the ERK signaling pathway and of CREB activation.

Epigenetic marks participate to the elaboration of a transcriptional response to environmental challenges and stress exposure may under certain circumstances push that response toward a pathological path. However, chromatin by itself shows limited mobility and the adaptation of genome expression requires the action of chromatin remodeling complexes which can specifically recognize histone modifications and through ATP hydrolysis unwrap, mobilize, exchange or eject the nucleosome, to subsequently recruit the transcriptional apparatus to nucleosomal DNA. So far, only few studies investigated the role of chromatin remodeling complexes in behavioral adaptations and in the pathophysiology of psychiatric disorders. Human exome sequencing and genome-wide association studies have linked mutations in genes encoding subunits of the SWI/SNF complex, including Brm and Brg1 to neurodevelopmental disorders such as Coffin-Siris syndrome, Nicolaides-Baraitser syndrome, autism spectrum disorder, and schizophrenia. In addition, meta-analyses of GWAS studies pointed at BRG1 as one of the central player. At the preclinical level, the expression of BRG1 and complex formation with SMAD3 within the NAc has been shown to increase in rats following withdrawal from cocaine self-administration. Intra-accumbal pharmacological inhibition of BRG1 attenuated cocaine-relapse while virally-mediated NAc overexpression enhanced this behavior. Our results are complementary since the inactivation of Brg1 gene in dopaminoceptive
neurons including the NAc decreases the locomotor sensitization and the conditioned place preference to cocaine. Also, Brm gene inactivation in mice have been previously reported to have decreased pre-pulse inhibition and social interaction. This last finding is not consistent with what we found since Brm mutant mice showed a similar social behavior than their control littermates in an open field and further showed enhanced social interaction after social defeat compared to defeated controls.

ISWI, another chromatin remodeler complex also affects stress-related behaviors. Mice susceptible to repeated defeat and patients with depression display, within the NAc, an upregulation of ACF1, a subunit of ISWI. Its overexpression in the same structure, with that of the ISWI ATPase subunit (SMARCA5) made mice more sensitive to social defeat whereas knocking it down made them more resilient. In addition, inactivation of BAZ1B, a subunit of ACF complexes, decreases cocaine self-administration. Increased ACF was associated with altered nucleosome positioning and a repressed transcription of key genes involved in the sensitivity to social defeat. Our findings are similar in that the inactivation of Brg1 or of Brm genes, the two ATPase subunits of the SWI/SNF complexes, made mice resilient to repeated social defeat and decreased behavioral responses to cocaine. However, whereas ACF seems to have a repressive impact on gene transcription, BRG1 has been mainly associated with transcriptional activation among others through its interaction with nuclear receptors. In line with this, we observed in absence of BRG1 a general increase of heterochromatin surface in MSNs nuclei, and a decreased induction of H3S10P in response to social defeat. A related effect has been observed in tumor cells in which the activation of BRG1, due to the inhibition of CDK9 that phosphorylates and inactivates BRG1, prevent its recruitment to heterochromatin and lead to a broad opening of chromatin and to the reactivation of transcription of repressed genes. The change in heterochromatin organization could also be responsible for the change of shape we observed in MSNs nuclei. Indeed, increased heterochromatin could translate to enhanced lamina-associated domains that directly shapes the nuclear envelope.

The absence of BRG1 leads to a defect of induction of c-fos and egr1 genes expression by social defeat despite an intact cell signaling transduction (assessed by the MAPK pathway activation). This could partly explain the behavioral phenotype of BrgiD1Cre
mice. Indeed, the induction of both immediate early genes have been shown to be crucial for behavioral responses to cocaine\textsuperscript{36,61}. Also, c-fos gene expression is under the control of CREB whose inactivation in the NAc has been shown to reduce social avoidance after social defeat\textsuperscript{4}. On the other side, \textit{egr1} is under the control of Elk1 activation and the systemic injection of a blood-brain-barrier permeable peptide that inhibits ERK-mediated activation of Elk1 has been recently shown to induce resilience to social defeat in mice\textsuperscript{62}. In primary cortical neuronal culture, BRG1 has been shown to directly control c-fos gene induction but with a dual action depending on cells activity. Through its interaction with CREST (Calcium Responsive Transactivator), a neuron specific member of SWI/SNF complexes, BRG1 is key for CBP (CREB binding protein) recruitment and for ensuing c-fos gene transcription upon calcium influx\textsuperscript{63}. The same mechanism could be at play within the striatum and the NAc, explaining the decreased c-fos gene induction we observed. In neurons at rest, however, BRG1-CREST complex rather inhibits c-fos gene transcription by the recruitment of a phospho-Retinoblastoma-HDAC repressor complex\textsuperscript{63}. This could explain why we observed a trend toward higher levels of c-FOS in saline injected mice deprived of BRG1. Since BRG1 and CREST also bind \textit{egr1} gene promoter, the same mechanism could also explain the lack of \textit{egr1} gene induction we observed. It is of note that here we do not distinguish between the D1- and D2-receptor expressing MSNs and, although beyond the scope of our study, manipulating BRG1 independently in one or the other neuronal population could yield distinct results as it has been shown for other factors\textsuperscript{64,65}.

We previously reported the essential role of GR in dopaminoceptive neurons for behavioral adaptations to stress\textsuperscript{7-10}. The fact that SWI/SNF complex containing either BRM or BRG1 are essential interaction partners of nuclear receptors, among which the GR, led us to address their role in this function. The interaction between GR and BRM or BRG1 is not direct but relies on connecting BAF proteins including BAF250, BAF60a, BAF57, and BAF53a\textsuperscript{26-28}. In cell culture, both BRM and BRG1 potentiate GR transcriptional activity\textsuperscript{13,66-68} and studies at a genome scale have shown that in the fibroblastic 3134 cell line, around 40% of GR-stimulated genes and 11% of GR-repressed genes require the function of BRG1-containing SWI/SNF complexes\textsuperscript{68}. Surprisingly, considering the large number of genes supposed to be co-regulated, only a small fraction of GR and BRG1 foci co-localized or were in close vicinity. Obviously, the identification of the genes present within the foci will be of great interest. We
followed the evolution of GR/BRG1 distributions after an acute social defeat stress and 24h after the last defeat of 10 daily successive defeats. An acute stress elicits a burst of glucocorticoids reaching its maximum one hour after its initiation and returning to basal levels 3-4 hours later. These high levels of hormones are most probably responsible for the increased number of GR foci we observed after a single defeat. Surprisingly, acute stress exposure also strongly increased the number of BRG1 foci. This cannot be explained just by a recruitment of BRG1 by GR. Indeed, although the fraction of co-localized GR-BRG1 foci was markedly increased, it still represents only a small fraction of GR foci. Interestingly, 24 h after 10 days of social stress, while the number of GR and BRG1 foci returned to basal levels, the number of foci containing both proteins remained enhanced. This change in nuclear distribution of GR and BRG1 may therefore constitute a remaining molecular trace of stress-exposure. Unfortunately, the lack of antibodies prevented us to perform similar analysis of the relative GR and BRM distributions.

The similarity of the behavioral phenotypes we reported here with the ones previously observed in GR<sup>D1Cre</sup> mice<sup>7, 9</sup>, suggest a functional role for the interaction between GR and BRG1/BRM containing SWI/SNF remodeler complexes in the modulation of stress-related behaviors. However, some data in the present study do not match what was previously shown in GR<sup>D1Cre</sup> mice. Indeed, the inactivation of GR gene in dopaminoceptive neurons leads to a decrease of dopamine neurons spontaneous firing under basal conditions, as well as after repeated social defeats. In contrast, the inactivation of Brg1 and Brm genes in the same cell population does not. Several hypotheses could explain this difference. GR and BRG1/BRM have functions that are independent from each other, and the control of presynaptic dopamine by GR could require a mechanism that does not involve BRG1 or BRM. This could be exerted at the level of gene expression control but could also involve non-transcriptional functions of GR such as interferences with signaling cascades or with synaptic activity via plasma membrane-bound GR<sup>60, 69</sup>. An alternate possibility would be that BRM and BRG1 may compensate for each other gene inactivation. This would be in line with our observation that double gene inactivation leads to a decrease of dopamine firing rate and number of spikes in bursts in Brg1<sup>D1Cre</sup>Brm<sup>−/−</sup> mice. However, the fact that both BRG1 and BRM deletions in our models lead to the same behavioral output exclude such compensatory mechanisms at this level. More work will be needed in order to clearly know which
genes and which functions are under the control of GR-SWI/SNF complexes. However, the present results along with previous work on GR mutants bring one more piece to the understanding of the molecular mechanisms underlying stress-induced behavioral adaptations and could be relevant for the pathophysiology of depression and drug addiction.

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Authors contributions
AZ, CB, SK, RDC, ESJ, SB, FM, and SP performed experiments; AZ, RDC, ESJ, FM, PV, JB and SP analyzed the data; FM, PV, PF, JB, FT and SP supervised the experiments, FT and SP designed the study; AZ, FT and SP wrote the manuscript.

Conflict of interest
The authors declare no conflict of interest.
References


Figure Legends

Figure 1: Colocalization of BRG1 and GR within the nucleus accumbens under basal conditions or after stress exposure. (a) Co-immunoprecipitation of GR, BRG1 and BRM in whole striatum extracts. Lane 1 and 2 correspond to GR detection in whole striatum lysates from GR<sup>loxP/loxP</sup> control mice and GR<sup>D1Cre</sup> mice respectively. Lane 3, 4 and 5 correspond to GR detection after immunoprecipitation with GR, BRG1 and BRM antibodies, respectively. Scale bar = 20µm (b) Co-staining of GR (in red), BRG1 (in green) and DNA (in blue, DAPI) in nuclei within the NAc. (c) High magnification of GR (red), BRG1 (green) and DNA (blue) co-staining in a NAc MSN nucleus in an undefeated mouse (basal condition), 1 hour after an acute social defeat or 24 hours after 10 days of repeated social defeats. The three first columns correspond to one focal for GR-DAPI, BRG1-DAPI and overlay stainings with the yellow bars pointing at foci in which BRG1 and GR colocalize. The last column corresponds to the nuclei 3D images. Scale bar = 2 µm (d) Quantification of GR (left chart; treatment effect F(2,29)=8.91, p<0.01), BRG1 (middle chart; treatment effect F(2,29)=10.30, p<0.001) and both GR and BRG1 colocalization foci (right chart; treatment effect F(2,29)=32.07, p<0.001) in MSNs of the NAc from undefeated mice (basal), 1 hour after an acute social defeat, or 24 hours after repeated (10 days) social defeat. n=10 nuclei in 4 mice per condition. One-way ANOVA followed by Bonferroni correction, **p<0.01, ***p<0.001. Data are represented as mean ± s.e.m.

Figure 2: Behavioral effects of repeated social defeat in control, Brgr<sup>D1Cre</sup> and Brm<sup>−/−</sup> mice. (a) Schematic view of the repeated social defeat paradigm. (b) Representative hitmaps (red color means increased spent time) of social interaction in undefeated controls (left), defeated controls (middle) and defeated Brgr<sup>D1Cre</sup> (right). (c) Effect of repeated social defeat on social interaction time in control and Brgr<sup>D1Cre</sup> mice (left) and in control and Brm<sup>−/−</sup> (right) mice. For Brgr1 experiment: undefeated control n=25, defeated control n=24, undefeated Brgr<sup>D1Cre</sup> n=24, defeated Brgr<sup>D1Cre</sup> n=22. Interaction social target x genotype x stress F(1,91)=10.25; p<0.01. Three-way ANOVA with repeated measure followed by Bonferroni correction ***p<0.001. NS=not significant. For Brm experiment: undefeated control n=17, defeated control n=18, undefeated Brm<sup>−/−</sup> n=9, defeated Brm<sup>−/−</sup> n=17. Interaction social target x genotype F(1,57)=7.82; p<0.01. Interaction social target x stress F(1,54)=11.38; p<0.01; three-
way ANOVA with repeated measure followed by Bonferroni correction ***p<0.001. NS=not significant. Individual results for defeated animals are indicated to assess individual resilience (green dots and lines) and susceptibility (gray dots and lines). (d) Percentage of resilient (in green) and susceptible mice (in orange) in defeated controls (n=42), defeated Brg1<sup>D1Cre</sup> (n=22) and defeated Brm<sup>-/-</sup> mice (n=17). (e) Anxiety levels in the elevated O maze for Brg1<sup>D1Cre</sup> (left chart), Brm<sup>-/-</sup> (right chart) and respective control mice. For Brg1: undefeated control n=25, defeated control n=24, undefeated Brg1<sup>D1Cre</sup> n=25, defeated Brg1D1Cre n=22. Main stress effect F(1,92)=11.67; p<0.001. Interaction genotype x stress F(1,92)=6.69; p<0.05. Two-way ANOVA followed by Bonferroni correction ***p<0.001, NS=not significant. For Brm: undefeated control n=8, defeated control n=10, undefeated Brm<sup>-/-</sup> n=6, defeated Brm<sup>-/-</sup> n=9. Main stress effect F(1,29)=7.37; *p<0.05. Data are represented as mean ± s.e.m.

Figure 3: Behavioral responses to cocaine in control and Brg1<sup>D1Cre</sup> mice. (a) Locomotor response (by 5 min blocks) to an acute cocaine injection (10mg/kg) in saline pre-treated controls (n=4) and Brg1<sup>D1Cre</sup> mice (n=7). (b) Locomotor response to a cocaine injection (10 mg/kg) in cocaine pre-treated control (n=8) and Brg1<sup>D1Cre</sup> mice (n=8). (c) Locomotor sensitization (cumulated activity during 15 min after injection) to cocaine in controls and Brg1<sup>D1Cre</sup> mice. This experiment has been performed simultaneously with the locomotor sensitization of Brm<sup>-/-</sup> and Brg1<sup>D1Cre</sup>:Brm<sup>-/-</sup> shown in Figure S5 hence statistical analysis includes all the groups. Main drug effect F(3,49)=8.19; p<0.01. Interaction genotype x drug F(3,49)=6.07; p<0.01. Two-way ANOVA followed by Bonferroni correction acute vs chronic **p<0.01, NS=not significant; control vs Brg1<sup>D1Cre</sup> ***p<0.001. (d) Time spent in each compartment of the conditioned place preference setup during the preconditioning phase. Control n=18, Brg1<sup>D1Cre</sup> n=17. (e) Additional time spent in the saline- and cocaine-paired compartment after conditioning in controls and Brg1<sup>D1Cre</sup> mice. Saline- and cocaine-conditioned controls n=9 for each, saline- and cocaine-conditioned Brg1<sup>D1Cre</sup> mice n=8 and n=9 respectively. Main drug effect F(1,31)=68.8; p<0.001. Genotype x drug F(1,31)=8.683; p<0.01. Two-way ANOVA followed by Bonferroni correction saline vs cocaine ***p<0.001, **p<0.01; control vs Brg1<sup>D1Cre</sup> *p<0.05. Data are represented as mean ± s.e.m.
Figure 4: Consequences of repeated social defeat on ventral tegmental area dopamine neurons spontaneous activity in control and $Brgr1^{D1Cre}$ mice. (a) Representative traces of dopamine neurons activity in undefeated (upper trace) and defeated (lower trace) control mice. Firing frequency (left chart) and percentage of spike in burst (right chart) in undefeated (n=51 neurons) and defeated (n=51 neurons) control mice. (b) Representative traces of dopamine neurons activity in undefeated (upper trace) and defeated (lower trace) $Brgr1^{D1Cre}$ mice. Firing frequency (left chart) and percentage of spike in burst (right chart) in undefeated (n=81 neurons) and defeated (n=45 neurons) $Brgr1^{D1Cre}$ mice. T-test (for frequency) and Wilcoxon test (spike in burst) *p<0.05, ***p<0.001. Data are represented as mean ± s.e.m.

Figure 5: Changes of nuclear and chromatin conformation in absence of BRG1. (a) Nuclear abnormalities (pointed by yellow bars) in the NAc of control and $Brgr1^{D1Cre}$ mice using Lamin B staining with percentage of grooved, irregular and multilobed nuclei (n=3/3 mice, and from 595 to 710 nuclei analyzed per mouse). T-test *p<0.05, p<0.01. (b) Representative DAPI staining in nuclei from control and BRG1$^{D1Cre}$ undefeated mice with heterochromatin domains circle in dashed white lines. Total surface of heterochromatin and number of heterochromatin areas in control and $Brgr1^{D1Cre}$ mice undefeated, 1h after a single defeat or 24h after repeated social defeat. Scale bar = 2 µm. n=11 to 12 nuclei in 3 to 4 animals in each condition. Main genotype effect $F(1,65)=62.47; p<0.001$. Main stress effect $F(2,65)=3.91; p<0.05$. Interaction genotype x stress $F(2,65)=3.61; p<0.05$. Two-way ANOVA followed by Bonferroni correction control vs $Brgr1^{D1Cre}$ ①p<0.1, ***p<0.001; acute vs chronic §§p<0.01. Data are represented as mean ± s.e.m.

Figure 6: Immediate early genes induction and ERK pathway activation in response to a single social defeat in control and $Brgr1^{D1Cre}$ mice. (a) First row of charts, c-fos gene induction by an acute social defeat in control and $Brgr1^{D1Cre}$ mice in the dorsomedial striatum (left chart), NAc core (middle chart) and NAc shell (right chart). Locations of the corresponding structures are indicated in orange on the schemes above. Undefeated control n=6, defeated control n=6, undefeated $Brgr1^{D1Cre}$ n=6, defeated $Brgr1^{D1Cre}$ n=6. Second line of charts, egr1 gene induction by social defeat in controls and $Brgr1^{D1Cre}$ mice in the dorsomedial striatum (CPu, left chart), nucleus accumbens core (middle chart) and shell (right chart). Undefeated control n=5,
defeated control n=6, undefeated \textit{Brg1}^{D1Cre} n=6, defeated \textit{Brg1}^{D1Cre} n=6. Two-way ANOVA followed by Bonferroni correction *p<0.1, **p<0.05, ***p<0.01, ****p<0.001. (b) ERK phosphorylation after an acute social defeat in controls and \textit{BRG1}^{D1Cre} mice in the NAc core (left chart) and NAc shell (right chart). Undefeated control n=5, defeated control n=6, undefeated \textit{Brg1}^{D1Cre} n=5, defeated \textit{Brg1}^{D1Cre} n=6. Two-way ANOVA followed by Bonferroni correction *p<0.05, **p<0.01. (c) Representative H3S10P stainings (red) in the NAc of control mice undefeated and defeated (left panel). Scale bar = 15 \mu m. Induction of H3S10P by an acute social defeat in control and \textit{Brg1}^{D1Cre} mice (right panel). Undefeated control nuclei n=28 (5 mice), undefeated \textit{Brg1}^{D1Cre} nuclei = 22 (4 mice), defeated control nuclei = 28 (5 mice), defeated \textit{Brg1}^{D1Cre} nuclei = 28 (5 mice). Main genotype effect F(1,102)=4.42; p<0.05. Maine stress effect (F1,102)=8.83; p<0.01. Two-way ANOVA followed by Bonferroni correction undefeated vs defeated ****p>0.001, NS: not significant; control vs \textit{Brg1}^{D1Cre} **p<0.01. Data are represented as mean ± s.e.m.
Figure 1

a) Lysate control, Anti-GR, Anti-BRG1, Anti-BRM precipitation Ab

b) GR, BRG1, Overlay

c) Basal condition, Acute social defeat, Repeated social defeat

d) Nb of GR foci, Nb of BRG1 foci, Nb of GR and BRG1 foci

- Basal
- Acute defeat
- Repeated defeat
a) Repeated social defeat

New resident mouse x 10 days

Exp mouse | Social defeat | Sensory contact

~5min | 24h

b) Undefeated control | Defeated control | Defeated Brγ1\textsuperscript{D1Cre}

No interacting mouse

Interacting mouse

c) Time interacting (s)

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**NS**

Resilience

Control | Brγ1\textsuperscript{D1Cre} | Brm\textsuperscript{-/-}

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d) Elevated O maze

Time in open portion (s)

*** NS

Time in open portion (s)

*
Figure 3

a) Acute treatment

Locomotor activity (1/4turn) vs Time (min)

- Control saline-cocaine
- Brgr1D1Cre saline-cocaine
- Control cocaine-cocaine
- Brgr1D1Cre cocaine-cocaine

b) Chronic treatment

Locomotor activity (1/4turn) vs Time (min)

- Control saline-cocaine
- Brgr1D1Cre saline-cocaine
- Control cocaine-cocaine
- Brgr1D1Cre cocaine-cocaine

c) 1/4 turn in 15 min

- Control
- Brgr1D1Cre

Pre-conditioning

- Time in arms (s)
- Additional time in paired arm (s)

Post-conditioning

- Saline
- Cocaine

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Figure 4

(a) Control

(b) Brg1<sup>D1Cre</sup>

Spike in burst (%)

Frequency (Hz)

Undefeated control

Defeated control
Figure 5

a) Grooved, Irregular, Multi-lobed

b) Control, Brg1\textsuperscript{Cre}

Heterochromatin surface (μm²)

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<td><strong>4.6 ± 0.7</strong></td>
<td><strong>4.8 ± 0.6</strong></td>
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Figure 6

a) Dorsomedial striatum  Nucleus accumbens Core  Nucleus accumbens Shell

![Graphs showing c-FOS positive cells](image)

b) Nucleus accumbens Core  Nucleus accumbens Shell

![Graphs showing EGR1 positive cells](image)

c) DAPI  H3S10P  Overlay

![Images of un打败ed and defeated groups](image)

Legend:
- Undefeated control
- Defeated control
- Undefeated Brg1D1Cre
- Defeated Brg1D1Cre

H3S10P (foci per nucleus)
Figure S1

(a) 

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</tr>
<tr>
<td>Brm-/-</td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
<td><img src="image9.png" alt="Image" /></td>
</tr>
</tbody>
</table>

(b) 

<table>
<thead>
<tr>
<th>Region</th>
<th>Control</th>
<th>Brg1D1Cre</th>
<th>Brm-/-</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dorsomedial striatum</strong></td>
<td><img src="image10.png" alt="Image" /></td>
<td><img src="image11.png" alt="Image" /></td>
<td><img src="image12.png" alt="Image" /></td>
</tr>
<tr>
<td>Neuronal density (mm²)</td>
<td>2000 ± 500</td>
<td>1500 ± 400</td>
<td>2000 ± 300</td>
</tr>
<tr>
<td><strong>Nucleus accumbens</strong></td>
<td><img src="image13.png" alt="Image" /></td>
<td><img src="image14.png" alt="Image" /></td>
<td><img src="image15.png" alt="Image" /></td>
</tr>
<tr>
<td>Neuronal density (mm²)</td>
<td>2000 ± 500</td>
<td>1500 ± 400</td>
<td>2000 ± 300</td>
</tr>
</tbody>
</table>

Overlay: BRMBRG1

Nissl staining
Figure S2

a) Three chambers test

- Mouse in box
- Interaction time (s)
- Undefeated control
- Undefeated Brg1D1Cre
- Defeated control
- Defeated Brg1D1Cre

b) Dark/Light box

- Time in lit compartment (s)
- Undefeated control
- Undefeated Brg1D1Cre
- Defeated control
- Defeated Brg1D1Cre

NS, ** indicates statistical significance.
Figure S3

Adrenal weight

Body weight

Weight gain (g)

Weight (mg)

-1 0 1 2 3 4 5

Days

3 5 7 9 Post

** ***

Undefeated control
Defeated control
Undefeated \textit{Brg}^{\textit{D1Cre}}
Defeated \textit{Brg}^{\textit{D1Cre}}
a) Contextual and cued fear memory in Brg1\textsuperscript{D1Cre} mice

- Conditioning
- Contextual memory
- Cued memory

![Graphs showing freezing percentage over conditioning and memory tests](image)

b) Motor learning in Brg1\textsuperscript{D1Cre} mice

- Latency to fall (s)

![Graph showing latency to fall over different days](image)

c) Working memory in Brg1\textsuperscript{D1Cre} mice

- Correct choices (%)

![Graph showing correct choices over different days](image)
Figure S5

(a) Acute treatment  (b) Chronic treatment

Locomotor activity (1/4 turn)

- □ Control saline-cocaine
- ▲ Brm$^{+/}$ saline-cocaine
- ● Brg1$^{D1Cre};Brm^{-/-}$ saline-cocaine
- △ Control cocaine-cocaine
- ⬤ Brm$^{+/}$ cocaine-cocaine
- ♦ Brg1$^{D1Cre};Brm^{-/-}$ cocaine-cocaine

Cocaine

Time (min)

-80 -40 0 40 80 120

1/4 turn in 15 min

-80 -40 0 40 80 120 150 200 250 300 350 400 450 500

Acute  Chronic

Control
Brm$^{+/-}$
Brg1$^{D1Cre};Brm^{-/-}$

NS

***

NS
Figure S6

(a) 

(b) 

(c) 

<table>
<thead>
<tr>
<th>Control</th>
<th>Brm&lt;sup&gt;-/-&lt;/sup&gt;</th>
<th>Brg1&lt;sup&gt;Δ1Cre&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frequency (Hz)</td>
<td>Spike in burst (%)</td>
<td>Frequency (Hz)</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

** Statistical significance:**

- **P < 0.01**
- **P < 0.001**
Figure S7

- **Dorsomedial striatum**
  - Control saline
  - Control cocaine
  - Brg1D1Cre saline
  - Brg1D1Cre cocaine

- **Nucleus Accumbens core**
  - Control saline
  - Control cocaine
  - Brg1D1Cre saline
  - Brg1D1Cre cocaine

- **Nucleus Accumbens shell**
  - Control saline
  - Control cocaine
  - Brg1D1Cre saline
  - Brg1D1Cre cocaine
Figure S8

(a) Basal and glutamate (glu) stimulation at different concentrations (0.05, 0.1, 0.3, 0.6 µM) in control and BRG1^D1Cre conditions. Images show changes in fluorescence intensity over time.

(b-g) Graphs showing time course of \\( \Delta F/F \) for control and BRG1^D1Cre conditions with different concentrations of glutamate. A.U.C. (Area Under the Curve) and statistical analysis (NS for non-significant) are shown for each condition.