Histone post-translational modifications in the nuclei of striatal D1 and D2 neurons : development of a novel method of study and effects of cocaine

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Histone post-translational modifications in the nuclei of striatal D1 and D2 neurons: development of a novel method of study and effects of cocaine

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LIST OF ABBREVIATIONS

5hmC	5-hydroxymethylcytosines
A2AR	Adenosine 2A receptor
AAV	Adeno-associated virus
AC	Adenylyl-cyclase
AchE	Achetylcholine Esterase
AKAP	A-kinase anchoring proteins
AMPAR	α-amino-3-hydroxy-5-methyl-4 isoxazolepropionic acid receptor
AMPK	AMP-activated protein kinase
AP-1	Activating protein 1
ARPP-16	cAMP-regulated phosphoprotein, Mr ~ 16,000
ATP	Adenosine triphosphate
BAC-	Bacterial artificial chromosome
BacTRAP	BAC-Translating Ribosome Affinity Purification
BG	Basal ganglia
CaMKII/IV	Ca^{2+}/calmodulin-dependent protein kinase
cAMP	Cyclic adenosine monophosphate
ChIP	Chromatin immunoprecipitation
CB1	Cannabinoid receptor 1
CBP	CREB binding protein
Cdk5	Cyclin-dependent kinase 5
CoREST	Corepressor of REST (RE1-silencing transcription factor)
CPP	Conditioned place preference
CPu	Caudate-putamen
CRE	cAMP response-element
CREB	cAMP response-element-binding protein
D1R	Dopamine D1 receptor
DA	Dopamine
DAG	Diacylglycerol
DARPP-32	Dopamine- and cAMP-regulated phospho-protein of 32 KD
DAT	Dopamine transporter
DNA	Deoxyribonucleic acid
EGFP	Enhanced green fluorescent protein
EGR	Early growth response factors
Elk-1	Ets-like gene 1
ERK	Extracellullar signal-regulated kinase
FACS	Fuorescence Activated Cell Sorting
FRA	Fos-related antigen
FS	Fast-spiking
FSI	Fast-spiking interneuron
GABA	γ-aminobutyric acid
Gαi	Inhibitory α subunitof G protein
Gαs	Stimulatory α subunitof G protein
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GluR1	Glutamate receptor type
GPCR	G protein-coupled receptor
GPi	Internal globus pallidus
GPe	External globus pallidus
GTP	Guanosine triphosphate
H3	Histone H3
HAT	Histone acetyltransferase
HDAC	Histone deacetylase

HP-1 Heterochromatin protein-1

IEG Immediate-early genes

KO Knock-out

LGP Lateral globus pallidus

LTD Long-term depression

LTP Long-term potentiation

LTS Low-threshold spiking interneurons

mIPSCs miniature inhibitory post synaptic currents

MAPK Mitogen-activated protein kinase

MBD methyl-binding domain protein

MEK MAPK/ERK-kinase

MEEK MEK kinase

MEF2 Myocyte enhancer factor 2

MGP Medial globus pallidus/ entopeduncular nucleus

mRNA Messenger ribonucleic acid

MSK1 Mitogen- and stress-activated kinase 1

MSN Medium-sized spiny neuron

MYPT1 Myosin phosphatase-targeting subunit-1

NAc Nucleus accumbens

NES Nuclear export signal

NLS Nuclear localization signal

NET Norepinephrine transporters

NuRD Nucleosome remodeling deacetylase

NMDAR N-methyl-D-aspartic acid receptor

NOS Nitric oxide synthase

PFC Prefrontal cortex

PD Parkinson's disease

PDE Phosphodiesterase

PKA/C/D cAMP-dependent protein kinase A/C/D

PP1 Protein phosphatase 1

PP2A/2B Protein phosphatase 2A/2B

Ras-GRF1 Ras-guanyl nucleotide releasing factor 1

RCS Regulator of calcium signaling

RGS Regulator of G protein signaling

RL Reinforcement Learning

RRA Retrorubral area

RRF Retrorubral field

RSK Ribosomal subunit protein S6 kinase

SIK Salt inducible kinase

SERT Serotonin transporter

SNc Substantia nigra pars compacta

SNr Subtantia nigra pars reticulata

SRE Serum response element

SRF Serum response factors

STAT Signal transducers and activators of transcription

STEP Striatal-enriched phosphatase

STN Subthalamic nucleus

TCF Ternary complex factor

VGCC Voltage-gated calcium channels

VP Ventral pallidum

VTA Ventral tegmental area

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INTRODUCTION

GENERAL INTRODUCTION

The present thesis investigates the cell-specific mechanisms of dopamine action within the striatum. Dopaminergic signaling in the striatum plays a crucial role in mediating the effects of drugs of abuse. It is known that drugs of abuse, such as cocaine, induce long-lasting structural, electrophysiological and transcriptional changes in the nucleus accumbens (NAc) that can account for the persistent behavioral alterations of the addictive phenotype. The basis of some of these long-lasting changes could be epigenetic, providing a key mechanism by which the environment can influence gene expression and, hence, phenotype. In particular, increasing evidence support an important role for histone post-translational modifications (PTMs) such as phosphorylation, acetylation, or methylation, in promoting the aberrant transcriptional changes caused by chronic cocaine exposure. Recent publications have demonstrated that cocaine regulates histone acetylation and methylation in the NAc through the perturbation of histone deacetylases and methyltransferases.

Yet, the striatum is composed of multiple cell-types that exhibit contrasting transcriptional responses to drugs of abuse and mediate distinct aspects of drug reward and addiction. 95% of striatal neurons are GABAergic medium-sized spiny neurons (MSNs), which can be further differentiated based on the subtype of dopamine receptor they express, namely dopamine receptor D1 or D2. Both subpopulations express their own subsets of markers, project to different areas of the basal ganglia and more notably, activate opposing signaling cascades upon cocaine exposure. So far, epigenetic research is lacking single cell resolution and no studies have investigated the effect of drugs on histone modifications in isolated neurons.

We provide an innovative approach to separate the nuclei of D1 and D2 MSNs and to investigate in each of both nucleus types the alterations of histone PTMs and the enzymes controlling them, upon cocaine exposure. We demonstrate that our approach, based on flow cytometric amalysis, is suitable for the screen of PTMs of nuclear proteins, even the most labile. Using this technique, we found that different cocaine exposure protocols dynamically modify the histone acetylation and methylation profiles of D1 and D2 MSNs nuclei. In essence, we pinpoint their similarities and specific differences as well the distinct temporal regulation of histone marks induced by the drug in the two cell types.

As an introduction, we will first present the anatomy and physiology of the basal banglia highlighting the segregation of the striatonigral and stritaopallidal pathways. We will then present what is known about dopamine signaling in D1 and D2 MSNs, and how cocaine alters the function of these neurons. After having presented the main epigenetic mechanisms

for gene regulation, in particular those modified upon cocaine exposure, we will review the available techniques in order to study signaling pathways in a cell-specific manner. We will show that the study of epigenetic regulation in the striatum in a cell-specific manner is still a very new field, and that the technique that we propose here has the potential to bring new and crucial knowledge on long-term modifications caused by cocaine in the striatum.

CHAPTER I

THE BASAL GANGLIA

1. THE BASAL GANGLIA CIRCUITRY

The basal ganglia (BG) are a large collection of subcortical nuclei interconnected with the cerebral cortex, thalamus, and brainstem. Physiological function of the basal ganglia has been implicated in movement control, cognitive function, emotion, and learning. The main components of the BG are the striatum, the lateral or external globus pallidus (refered to as GPe in primates and LGP in rodent), the medial or internal globus pallidus (refered to as GPi in primates and MGP or entopeduncular nucleus in rodent), the subthalamic nucleus (STN) and the substantia nigra composed of substantia nigra pars compacta (SNc) and substantia nigra pars reticulata (SNr). Together, these nuclei form a highly organized and complex circuitry.

The basal ganglia connect the cerebral cortex with other neural systems that control behavior. The major input comes from glutamatergic neurons from nearly all areas of the cortex. The points of entry of this cortical information is mainly the striatum, and the subthalamic nucleus. It is now considered that the cortical information is processed within the striatum, integrated with the many others inputs to the basal ganglia (e.g. intralaminar thalamic nuclei, amygdala, hippocampus, dorsal raphe) which primarily innervate the striatum, and then the processed information is transmitted to the output nuclei of the system, the GPi and the SNr (Albin *et al.*, 1989; DeLong, 1990; Smith *et al.*, 1998). Neurons from GPi and SNr target then the thalamic nuclei, which project to several cortical premotor regions, and influence motricity.

The most widely accepted model of basal ganglia circuit function is based on the segregation of information processing into direct and indirect pathways. The direct pathway is composed of striatal GABAergic neurons, also referred as striatonigral neurons, projecting directly to the GPi/SNr, making synpatic contact with the GABAergic output neurons of this structure. The indirect pathway is also composed of GABAergic neurons, referred as striatopallidal neurons. However, the corticostriatal information is transmitted indirectly to the output nuclei via the GPe and the STN. GPe neurons innervate the glutamatergic neurons of the STN first, which then innervate the GABAergic output neurons in the GPi/SNr. This flow of transmission is described as two parallel cortex-basal ganglia-thalamus-cortex loops that diverge within the striatum and are differentially modulated by dopamine (Alexander *et al.*, 1986; Joel and Weiner, 1994; Haber *et al.*, 2000) as schematized in **Fig. 1**. The progressive

understanding of this organization enabled a major conceptual advance in the comprehension of basal ganglia function as we will see in the last part of this chapter.

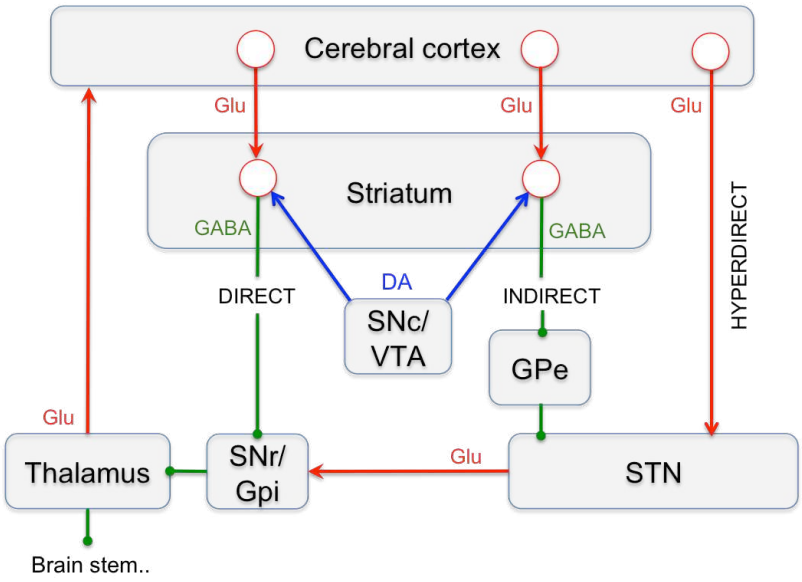

FIGURE 1: SCHEMATIC WIRING DIAGRAM OF THE BASAL GANGLIA.

Medium size spiny neurons receive excitatory glutamatergic inputs (red) from cerebral cortex and thalamus (not shown in diagram) and a modulatory dopaminergic input (blue) from the SNc and VTA. Dopamine activates the striatonigral pathway and inhibits the striatopallidal pathway. These opposite regulations control the disinhibition of thalamo-cortical glutamatergic neurons and promote motor activity. Abbreviations: DA: dopamine; Glu: glutamate; SNT: subthalamic nucleus; GPe: external globus pallidus; Gpi: internal globul pallidus; SNr: subtantia nigra reticulata; SNc: subtantia nigra pars compacta; VTA: ventral tegmantal area.

2. BASAL GANGLIA NUCLEI

2.1. THE STRIATUM

The striatum, the major input structure of the BG and its largest component, is comprised of a set of interconnected gray matter nuclei located deep in the forebrain. The term striatum refers to its layered appearance produced by the abundant fiber bundles passing trough it, forming white striae. In rodents, the striatum appears as a single nucleus, often referred to as the caudate-putamen.

2.1.1. THE DIVISIONS OF THE STRIATUM

Although the different classes of striatal projection neurons are homogeneously expressed throughout the striatum, anatomical divisions can be made within the striatum based on differences in neurochemistry, efferent and afferent connections, physiology, and contributions to BG-mediated behavior. The three major divisional categories are dorsal versus ventral striatum, patch versus matrix compartments, and direct versus indirect projection pathways.

2.1.1.1. DORSAL AND VENTRAL STRIATUM

The dorsal-ventral division of the striatum was originally based on differences in afferent connections, whereby the dorsal region receives inputs from associative cortex, sensorimotor-cortex and dopaminergic innervation primarily from the SNc (Kitai *et al.*, 1976; Donoghue and Herkenham, 1986; Nakano, 2000; Herrero *et al.*, 2002). The ventral striatum, on the other hand, receives information from orbitofrontal cortex and other limbic related afferents with a higher density of inputs from the amygdala, hippocampus, and VTA (Nakano, 2000; Herrero *et al.*, 2002; Voorn *et al.*, 2004). There is an intermediate zone, receiving polymodal information from the thalamus as well as input from higher associational cortical centers (Voorn *et al.*, 2004). These divisions are loosely based as there are no strict anatomical boundaries between them and innervation by all afferent brain regions is found throughout the striatum. The ventral striatum or nucleus accumbens is comprised of two regions: the core and the shell. The core is closely related to the dorsal striatum in terms of connections and function, whereas the shell has been proposed to be related to the extended amygdala. This suggests that the shell area of the accumbens might be conceived as a transitional zone between the striatum and the extended amygdala, or that elements of the extended amygdala intermingle to some extent with typical striatal components in this part of the accumbens

(Heimer *et al.*, 1991).

Distinctions between the dorsal and ventral striatum are also made regarding their function and relative contribution to behavioral processes. The dorsolateral striatum has been associated with procedural, stimulus-response, and spatial learning, whereas the ventromedial striatum is involved in motivation and reward learning and is the primary site of action for drugs of abuse. There has been significant research in the field of drug abuse to examine the contributions of the core versus shell of the ventral striatum in different biochemical and behavioral correlates of drug addiction. One conclusion from this work is that the core controls responses to conditioned reinforcers whereas enhancement of this reinforcement by drugs of abuse may depend on the shell (Parkinson *et al.*, 1999).

2.1.1.2. DIRECT AND INDIRECT PROJECTIONS

A major characteristic of the striatum is the division of its efferents into the direct striatonigral and indirect striatopallidal pathways. The direct pathway is comprised of projections from a subclass of GABAergic medium-sized spiny neurons which express the dopamine the dopamine D1 receptors and directly innervate the SNr. Indirect pathway neurons preferentially express D2 receptors and project to the GPi/SNr via two intermediate structures, the GPe and STN (**Fig. 1**). These anatomically defined cell populations are receiving more attention as it becomes increasingly clear that they have distinct physiological and signaling properties and that they can be modulated independently to result in different behavioral outcomes. In the normal state, these output pathways are highly regulated and work synergistically to produce coordinated effects on behavior. In the case of disruption or imbalance in signaling, such as occurs in Parkinson's disease, there are unique maladaptations which occur in each pathway that may have important consequences for targeting therapies to this brain area (Albin *et al.*, 1989; Gerfen, 2000). Studies dissecting the relative contributions of these pathways to behavioral or biochemical processes have been limited by the fact that these two types of projection neurons are morphologically indistinguishable and anatomically intermixed. Only recently have novel techniques emerged, largely based on genetically modified mouse technology (**Fig. 2**), which allow selective identification, purification, and/or targeting of these cells (Heintz, 2001; Gong *et al.*, 2003; Gong *et al.*, 2010). Studies utilizing these mice have revealed important differences between striatonigral and striatopallidal neurons which were not appreciated with conventional techniques. Chapters II and V of this thesis will present further data revealing important differences in intracellular signaling processes between these two cell populations.

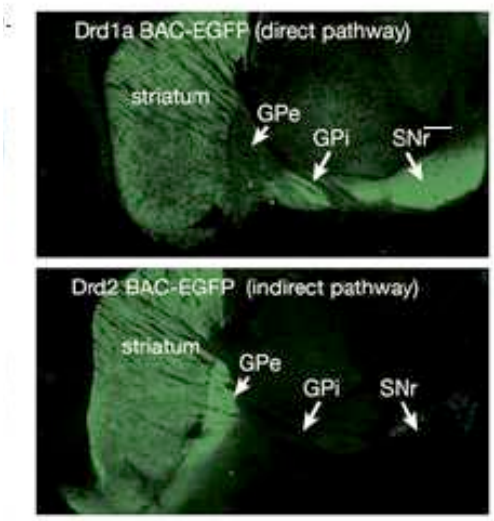

Figure 2: The direct and indirect pathway.

Sagittal sections from *drd1a*- and *drd2*- BAC (Bacterial artificial chromosome) transgenic mice showing labeling of the neuronal bodies and their axonal projections arising from the striatum (scale bar= 500 µm), adapted from Kreitzer and Malenka, 2008 . The study of BAC transgenic mice revealed that EGFP-labeled projection matched with the simplest model of the basal ganglia. Abbreviations: GPe: external globus pallidus; Gpi: internal globul pallidus; SNr: subtantia nigra reticulata.

2.1.1.3. PATCH AND MATRIX COMPARTMENTS

The striatum can also be divided into patch and matrix compartments which were regions originally identified histologically as rich or poor in acetycholinsterase staining (AchE) (Graybiel and Ragsdale, 1978). The AchE poor striosomes or patches were later shown to correspond to small striatal regions rich in µ-opiod receptor mRNA and protein (Herkenham and Pert, 1981). The patch and matrix areas differ in their connectivity whereby the larger matrix compartment (representing 80-90% of striatal volume) receives afferents from layer V cortical neurons and sends efferents to the GP and SNr (Donoghue and Herkenham, 1986). The striatal matrix is also associated with largely sensorimotor information. The patch compartment receives afferents from limbic areas as well as cortical layers Vb and VI and sends efferents to the SNc which can modulate dopaminergic input into the striatum (Graybiel, 1990). This suggests that there are unique biochemically and anatomically defined sub-regions within the striatum which likely have selective roles in the functions of this brain region.

2.1.2. CELL COMPOSITION

2.1.2.1. THE MEDIUM SPINY NEURONS

In rodent, the striatum is mainly comprised of medium-sized spiny neurons (MSNs), which represent about 95% of striatal cells; the remainning 5% are mostly cholinergic and GABAergic interneurons (Kreitzer, 2009; Tepper *et al.*, 2010). Morphologically, MSNs are medium-sized neurons with a soma of about 10 to 20 µm of diameter (Kawaguchi, 1997) with large and extensive dendritic trees (**Fig. 3**). The spiny neurons receive cortical and thalamic

inputs onto the tips of the numerous dentritic spines. There is also a diffuse dopaminergic input from the SNc that often contacts at the base of the dendritic spines, in a strong position to modulate the action of the cortical and thalamic inputs at the tips (Freund *et al.*, 1984; Smith *et al.*, 1994; Hanley and Bolam, 1997). In addition, they receive inhibitory inputs from striatal GABAergic interneurons, strategically located on their perikarya, and from cholinergic interneurons (Tepper *et al.*, 2010). Each striatal MSN receives 5-10,000 excitatory inputs. The input from each synapse is weak and many inputs are needed to trigger the discharge of the neuron. Therefore, MSNs are wired to integrate many convergent excitatory inputs, while they are highly sensitive to inhibition by interneurons. As a result and due to their conductance properties they have low firing rates in vivo (Kreitzer and Malenka, 2008).

For such a large nucleus, the striatum is unique in its complete lack of glutamatergic neurons. Indeed, all striatal cells including the MSNs are GABAergic with a few cholinergic interneurons. MSNs send axons collaterally within the striatum and project via the direct and indirect pathways to the output structures of the BG as described above. The division of MSNs into direct and indirect sub-populations is also accompanied by differences in the expression of neuropeptides and neurotransmitter receptors in each cell type. Direct pathway neurons express substance P and dynorphin and preferentially express type 1 dopamine receptors (D1R). Indirect pathway MSNs express enkephalin, type 2 dopamine receptors, and adenosine type 2 receptors (D2R) (Gerfen *et al.*, 1990).

Recently, the use of enhanced green fluorescent protein (EGFP)-tagged promoter elements of D1R and D2R in bacterial artificial chromosome (BAC) transgenic mice has allowed the fine quantification of the proportion of striatal neurons expressing the receptors within the striatonigral and striatopallidal pathways (Valjent *et al.*, 2009) (**Fig. 3B**). It was estimated that about 50% of MSNs express exclusively D1Rs, 35-45% exclusively D2Rs in the dorsal striatum. The population of MSNs co-expressing both D1R and D2R was about 5% in the dorsal striatum and core of the nucleus accumbens and 17% in the shell (Bertran-Gonzalez *et al.*, 2008; Matamales *et al.*, 2009).

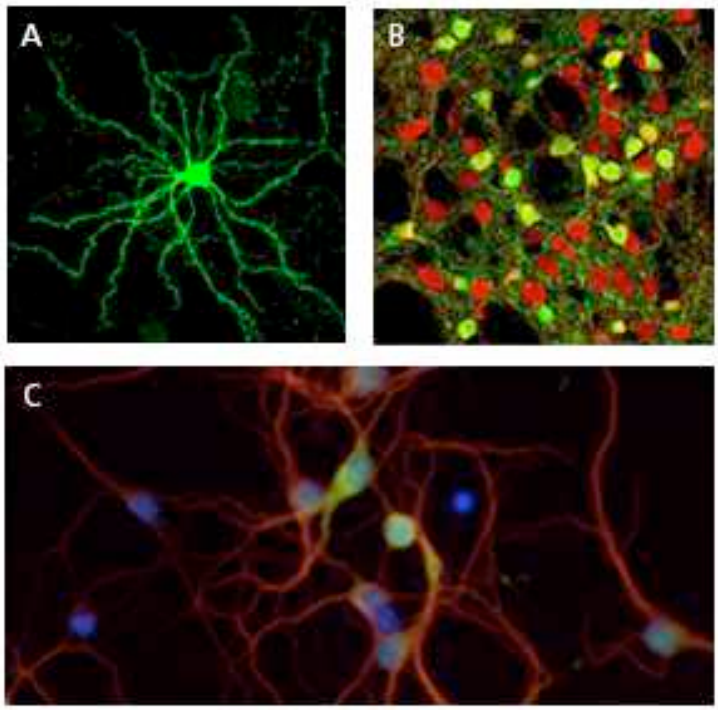

Figure 3: Medium-Sized Spiny Neurons (MSN).

A. A MSN in culture from a BAC *drd2* mouse (Day *et al.*, 2008). Note the high number of spines on the distal part of each dendrite. **B**. Confocal section of striatum showing the segregation between D1R and D2R-expressing neurons (Bertran-Gonzalez *et al.*, 2008). DARPP-32 immunofluresence identifies D1 and D2 MSNs (red) and EGFP labels D2 neurons (green) in the BAC *drd2* mouse. **C.** Mouse embryonic striatal neurons in coculture with cortical neurons (not seen on picture) immunostained with pCamKIIα (green), MAP2 (red) and DAPI (blue) DIV10. (Jordi, unpublished data).

Electrophysiological analyses have shown that D1 MSNs are less excitable than D2 MSNs possibly due to the different number of primary dendrites and/or differences in presynaptic cortico-thalamic input (Kreitzer and Malenka, 2007; Cepeda *et al.*, 2008; Gertler *et al.*, 2008).

There is now some evidences in the literature supporting the existence of a heteromer D1-D2 complex within a subpopulation of MSNs *in vitro* and *in vivo.* (Lee *et al.*, 2004; Dziedzicka-Wasylewska *et al.*, 2006; Hasbi *et al.*, 2009; Perreault *et al.*, 2010). The localization and function of these complexes *in vivo* remain to be precisely determined.

2.1.2.2. THE INTERNEURONS

The remaining striatal neurons are interneurons, in that they do not provide projection axons within the striatum but rather distribute axons within the striatum, most of which make synaptic contacts with spiny projection neurons. Striatal interneurons fall into four main categories and can be defined by their physiology, morphology, and expression of specific markers. One class of large cholinergic neurons which represent the main source of acetycholine (Ach) in the striatum and three types of GABA-ergic interneurons which express either parvalbumin, calretinin, or nitric oxide synthase (NOS, previously known as NADPH-diaphorase) (Kawaguchi *et al.*, 1995). These interneurons receive input from the cortex, SNc, GPe as well as from other interneurons and make synapses on MSNs where they can strongly regulate action potential firing (Tepper and Bolam, 2004; Kreitzer, 2009). At the

physiological level, the striatal interneurons can also be differentiated. Parvalbumin-positive neurons exhibit rapid and sustained firing rates and are alternatively known as fast-spiking (FS) interneurons. Somatostatin-positive interneurons have lower firing rates and are known as low-threshold spiking (LTS) interneurons. Although calretinin-positive interneurons have not been well characterized electrophysiologically, they may also exhibit some characteristics of LTS interneurons (Tepper and Bolam, 2004). Finally, cholinergic interneurons or tonically active neurons (TAN) fire tonically in basal conditions but irregularly in response to excitatory synaptic inputs (Graybiel, 1990; Wilson *et al.*, 1990; Mesulam *et al.*, 1992). These neurons may provide a neural substrate for attentional shifts and cessation of ongoing motor activity with the appearance of salient environmental stimuli (Ding *et al.*, 2010).

An elegant study performed by our group demonstrated that the striatal cell types, including interneurons, could be identified by staining their nuclei with a DNA intercalating fluorescent molecule (TO-PRO-3) (Matamales *et al.*, 2009) (**Fig. 4**). The criteria used to categorize them were nuclear diameter, nuclear shape, and heterochromatin. As opposed to traditional antibodies specific to one type of molecule (such as DARPP-32 in the following figure), the nuclear DNA morphology-based system is useful for classifying cells using just one type of marker.

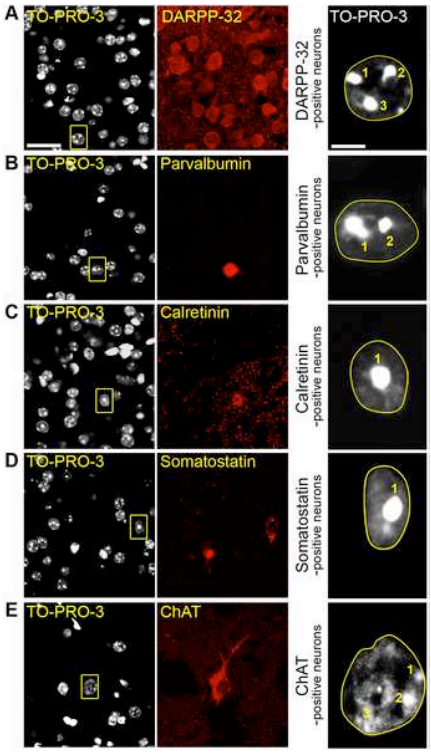

Figure 4 : The various striatal neuronal populations have distinct nuclear morphology.

Confocal images from mouse striatal slices labeled with various markers specific for each type of striatal neuron (Matamales *et al.*, 2009). **A**. DARPP-32 antibody identifies all MSNs, and the specific markers for interneurons (**B**, **C**, **D** and **E**).

As a conclusion, the striatum responds to and integrates signals from a variety of brain areas and neurotransmitter systems. It is therefore an excellent model system for studying neuronal signal transduction and integration.

2.2 THE GLOBUS PALLIDUS

The globus pallidus (GP) is separated in two nuclei by small fibers of passage and pallidal border cells : the globus pallidus external division (GPe) and the globus pallidus internal segment (GPi). These two divisions of the pallidal complex differ by their inputs and outputs and are functionally distinct. GPi and GPe both receive GABAergic input from the striatal medium spiny neurons, but their afferences originates from differerent classes of MSNs (Difiglia *et al.*, 1982). The GPe also receives glutamatergic projections from the subthalamic nucleus (STN) and, to a lesser extent, from the cerebral cortex and others structures (Kita, 2007). It projects mainly to the STN but also to the GPi/SNr and the striatum (Smith and Bolam, 1989). The GPi however, as part of the output BG structure, targets the motor thalamus, the ventral medial and parafascicular thalamic nuclei (Deniau and Chevalier, 1984; Deniau *et al.*, 2007).

2.3 THE SUBTHALAMIC NUCLEUS

After the striatum, the second major port of entry of neocortical and thalamic information into the BG is the subthalamic nucleus. The STN is the only excitatory structure of the BG, providing glutamatergic projections to the GPe (Bevan *et al.*, 1994) and to the output structures GPi/SNr (Nakanishi *et al.*, 1987; Smith and Parent, 1988). It receives glutamatergic and GABAergic inputs from the prefrontal cortex and the intralaminar thalamic nuclei, and the GPe respectively. The corticosubthalamic pathway is considered the fastest route by which cortical and thalamic information can influence activity in the output nuclei, since it bypasses the striatum. It has been referred to as the hyperdirect pathways (**Fig. 1**) (Kitai and Deniau, 1981; Nambu *et al.*, 1996; Bevan *et al.*, 2007) and could be involved in the inhibition of initiated movements (Aron and Poldrack, 2006).

2.4 THE SUBSTANTIA NIGRA

The substantia nigra (SN) is comprised of a compact and a diffuse clustering of neurons, the pars compacta (SNc) and the pars reticulata (SNr) respectively. Both clusters lie dorsal to the cerebral peduncle in the ventral midbrain. The SNc, together with the adjacent regions, ventral tegmental area (VTA) and the retrorubral area field (RRA or RRF), constitutes the set

of midbrain dopaminergic nuclei that project to the striatum, designated as A9, A8, and A10 respectively, according to the nomenclature of Dahlström and Fuxe (1964). This classification is based on their localization and efferent projections. A8 neurons project predominantly to the dorsolateral part of the striatum. The nigral A9 neurons present in SNc project exclusively to the dorsal striatum creating the nigrostriatal pathway. The A10 neurons from the VTA project to limbic and cortical areas by the mesolimbic and mesocortical pathway. More precisely, neurons located in the lateral part of the VTA project to the nucleus accumbens and the ventromedial part of the dorsal striatum. A different division, adding a level of compexity to the system, was advocated by Fallon and Moore based on the finding that neurons of A10 and A9 are continuous in providing input to the forebrain (Fallon and Moore, 1978). They defined two sets of DA neurons located either on the dorsal or lateral parts of the VTA and SNc. The dorsal tier group innervates the ventral basal forebrain structures (e.g. olfactory tubercle, amygdala), the limbic striatum and the cortex. The ventral tier group projects to more dorsal structures of the basal forebrain (e.g. the septum) and the entire striatum.

3. FUNCTION AND DYSFUNCTION OF THE BASAL GANGLIA

The basal ganglia only make up about 6 or 7% of the entire human brain, yet, damage to this system can have devastating results in exerting even the simplest of motor tasks. Most of our knowledge about its functions has arisen from studying the clinical phenomenology of human basal ganglia disorders. For example, Parkinson's disease and Huntington's disease are neurodegenerative diseases with BG damages which result in debilitating disruption of coordinated movement and cognition. Psychiatric disorders such as schizophrenia, obsessive-compulsive disorder, and attention deficit hyperactivity disorder have also been connected in part to the BG, possibly involving imbalances in neurotransmitter systems within this brain area. Also, the maladaptive reward learning associated with drug addiction has been linked to alterations in cellular processes in particular areas of the BG. It is clear that the BG are implicated in a wide range of functions, from motor coordination to cognition. However, accumulating evidence points to a fundamental role in basic action selection processes that we review below (Mink, 1996; Redgrave *et al.*, 1999; Grillner *et al.*, 2005; Humphries *et al.*, 2006).

3.1. ACTION SELECTION & THE CONTROL OF MOVEMENT

Action selection is the process of choosing an action among many possible alternatives. As an animal evolves in its environment, it is essential that he select the right sequence of movements over the ones that would interfere with the desired activity. The BG is believed to

resolve conflicts between different behavioral alternatives, by simultaneously removing inhibition from the desired motor mechanism to allow that movement to happen, and inhibiting the competiting ones that are not desired. It allows the brain to focus on a single aim at a time to drive the most efficient behavior possible. This mechanism is based on the segregation and the fine balance of the direct and indirect pathway activation (Gerfen and Surmeier, 2011). Normal basal ganglia functions are achieved when there is a equilibrium between the activities of the two pathways.

In the case of the D1 receptor, the classical model of BG circuitry predicts that dopamine stimulation of D1Rs on direct pathway neurons will increase locomotion. This is due to the excitatory action of dopamine acting via D1Rs in direct pathway neurons which results in increased GABAergic input to the GPi/SNr. This inhibition of the GPi/SNr in turn relieves inhibition of thalamic neurons thereby facilitating excitation of cortical motor neurons (**Fig. 1**). In agreement with this, application of selective D1R agonists increases locomotor behavior (Desai *et al.*, 2005).

Dopamine acting via D2 receptors is also expected to increase locomotor activity. Stimulation of D2Rs is inhibitory on indirect pathway cells, resulting in decreased GABAergic input to the GPe. The GPe can then exert a strong inhibitory influence on the STN resulting in less excitation of the GPi/SNr. This has the effect of reducing the inhibitory influence of the GPi/SNr on excitatory thalamocortical neurons thereby facilitating locomotion. As a result, agents which block D2Rs such as antipsychotics, decrease locomotion and in some cases result in catalepsy, characterized by loss of voluntary movement and muscular rigidity (Hauber, 1996; Manzanedo *et al.*, 1999).

Movement disorders of basal ganglia origin, such as Parkinson's disease (PD) are thought to result from imbalanced activities between the two pathways; with the polarity of the imbalance determining the kinetics of the disorder (Albin *et al.*, 1989). A recent series of experiments using optogenetics in mice nicely illustrates this dual function of the two pathways (Kravitz *et al.*, 2010). In PD, the progressive loss of midbrain dopamine neurons leads to reduced dopamine levels in the striatum and the severe hypokinetic motor deficits characteristic of PD. Studies in both animal models (Filion and Tremblay, 1991; Mallet *et al.*, 2006) and in human PD patients (Obeso *et al.*, 2000) have indicated that striatal dopamine depletion leads to enhanced indirect-pathway MSN output and decreased direct-pathway MSN output, and a consequent decrease in activity in GPe and increase in GPi. Because the indirect pathway normally inhibits unwanted movements, its overactivity may lead to the inhibition of wanted movementss and the disruption of learned motor routines. Conversely, underactivity of the direct pathway may contribute to difficulties in initiating and performing movements in PD.

In summary, the BG circuitry is central to release inhibition so that appropriate movements are facilitated whereas others are supressed. Importantly, the outputs are not believed to generate movements but only to influence the motor system towards certains patterns of movement that are likely to be encoutered as the animal moves in its environment.

3.2. REINFORCEMENT LEARNING & THE REWARD PREDICTION ERROR

One way by which animals acquire complex behavior is by interacting with their environment and learning to obtain reward (and to avoid punishments). This reward-driven (or punishment-driven) learning is called reinforcement learning (RL) and is the basis of motivational and goal-directed behaviors. It allows the striatum to learn pattern of outputs that, depending on the context, bias movement selection toward actions that lead to reward.

Each striatal MSN receives inputs from several thousand different cortical neurons on its spines. This convergent input from cortex is normally complemented by a tonic dopamine signal from SNc. When an animal receives a reward, the SNc produce short bursts of dopamine at higher frequency, that could modulate the efficiency of cortical inputs, and reinforce immediatly preceding inputs that might have led to the reward (Ljungberg *et al.*, 1992). The cortex is therefore using the striatum to select the appropriate action which reinforces behaviors that are likely to lead to a reward. More precisely and according to one prominent theory, dopamine can signal a "reward prediciton error" (Schultz *et al.*, 1997; Montague *et al.*, 2004). Shultz *et al*. (1997), recorded neurons from the VTA and SNc in behaving monkeys and found that DA fired when animals received an unexpected reward or, following associative learning, in response to the conditioned stimulus but not anymore to the reward itself. Though, in the absence of predicted reward, the dopaminergic activity is reduced. Hence, the reward needs to occur unexpectedly to induce learning, and learning slows as rewards become increasingly predicted. Dopaminergic neurons are thus said to encode a reward prediction error signal facilitating their synaptic plasticity, to ensure that actions maximising the future acquisition of reward are selected more often.

However, an alternative model of RL proposes a different perspective in DA function (Redgrave and Gurney, 2006). The availability of limited afferent sensory processing and the precise timing of dopaminergic signals support that they might have more to do with the discovery of new actions rather than adjusting the relative probabilities of selecting pre-existing actions to maximize anticipated reward. The authors suggest that the reinforcing function of the phasic DA signal acts to reinforce the reselection of actions that immediately precede an unpredicted biologically salient event.

ADDICTION: A MALADPATIVE REWARD LEARNING?

Drug abuse, and its transition to addiction and relapse, can be understood as forms of aberrant learning in which drugs have subverted the natural conditioning mechanisms we employ to anticipate important events and make plans. This aberrant engagement of learning processes is thought to originate from the usurpation of the neuronal circuitry involved in motivation and reward (see also Chapter III section 2.2) As a result, drug-associated cues can trigger craving and compulsive drug-seeking behavior in some drug users. Voluntary control over drug use is lost, even decades after the last drug intake.

Blockade of the dopamine reuptake transporter by drugs like cocaine enhances and prolongs phasic increases in dopamine concentrations (Church *et al.*, 1987; Suaud-Chagny *et al.*, 1995; Giros *et al.*, 1996). This mechanism would lead to a massively enhanced dopamine signal after primary rewards and reward-predicting stimuli. The enhancement by drugs of abuse would let these nonrewarding stimuli appear as strong or even stronger than natural rewards. Postsynaptic neurons could misinterpret such signals as a exceptionally powerful reward-related event and undergo long-term changes in synaptic transmission (Schultz *et al.*, 1997; Wolf, 2002; Mameli and Luscher, 2011).

CHAPTER II

DOPAMINE SIGNALING IN STRIATAL NEURONS

The striatum contains an abundance of neurotransmitters and neuromodulators and striatal neurons express a wide variety of receptors and ion channels. These include ionotropic and metabotropic glutamate receptors, GABA receptors, several classes of serotonin receptors, adenosine receptors, opiate receptors, and several types of dopamine receptors. A common theme in the signaling pathways activated by these receptors is that they are all somehow connected to dopamine signaling, which appears to be either modulating or modulated by the other neurotransmitters.

In this section we discuss what is known about dopamine in the striatum and the signaling properties of dopamine receptors, which are key regulators of basal ganglia function.

1 FUNDAMENTALS OF DOPAMINE SIGNALING

1.1. DOPAMINERGIC PATHWAYS

Dopaminergic neurons form three major circuits, the nigrostriatal, mesocorticolimbic and tuberohypophysial pathways (Dahlstroem and Fuxe, 1964). The tuberohypophysial pathway is outside the scope of this thesis and will not be discussed. The nigrostriatal and mesocorticolimbic dopamine systems are both anatomically and functionally interwined (Haber *et al.*, 2000) (**Fig. 5**). A central target of these two pathways is the striatum.

Cell bodies of the mesocorticolimbic dopamine pathway are located in the ventral tegmental area (VTA) and project to limbic regions including the NAc shell, limbic cortex (prefrontal- cingulate- and entorhinal cortices), amygdala terminalis, ventral pallidum (VP, ventral analogue of the globus pallidus) and the olfactory tubercle (**Fig. 5**). The mesocorticolimbic dopaminergic pathway plays an essential role in regulation of reward, motivation and goal-directed behaviors (Wise and Bozarth, 1987). The nigrostriatal system consists of dopaminergic neurons that originate in the substantia nigra (SN) and project to the dorsal striatum and the NAc core subregion. The nigrostriatal dopaminergic pathway has

traditionally been implicated in motor control, e.g. regulation of voluntary movement and stereotyped behaviors (Graybiel *et al.*, 1994).

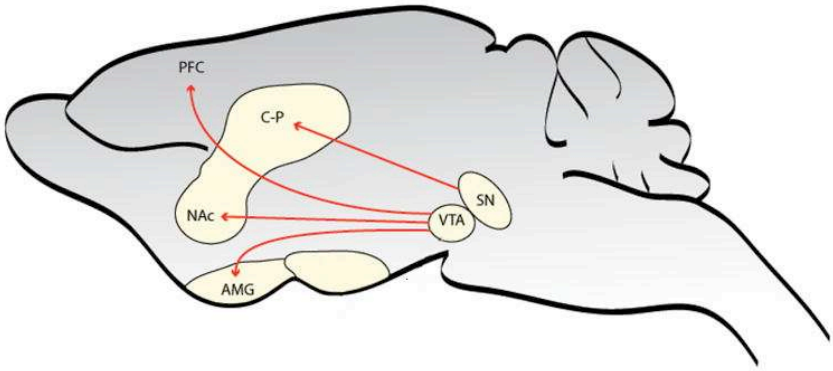

Figure 5: Schematic representation of the mesocorticolimbic and nigrostriatal dopamine projections in the mouse brain. The major dopamine projections are shown in red. The substantia nigra (SN) neurons project primarily to the caudate putamen (C-P), while the ventral tegmental area (VTA) neurons project primarily to the nucleus accumbens (NAc), the prefrontal cortex (PFC) and the amygdala (AMG). Modified from Narayanan et *al.,* 2010.

1.2. DOPAMINE RECEPTORS

1.2.1. RECEPTOR SUBTYPES AND COUPLING

Dopamine's actions are carried out by five subtypes of DA receptors selectively expressed in discrete brain regions: D1, D2, D3, D4 and D5. They all belong to the superfamily of G protein-coupled receptors (GPCRs) with 7 transmembrane domains. G proteins are composed of 3 different subunits (α, β, and γ) forming a heterotrimeric complex attached to the membrane. By stimulating the receptor, DA induces its conformational change leading to the activation of Gα subunit that detaches from Gβγ. Depending on their action on adenylyl cyclase (AC) activity, the enzyme that converts adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP) (Stoof and Kebabian, 1981), Gα subunits can be of the stimulatory or inhibitory type. Positive coupling of GPCRs to AC is mediated by the stimulatory Gα, $G\alpha_s$ subunit, whereas inhibitory Gα protein, $G\alpha_i$, inhibits AC and abolishes cAMP production (**Fig. 6**) (Albert *et al.*, 1990). DA receptors can be further differentiated in two distinct classes with respect to their coupling to AC.

The D1-like family includes the D1 receptor (D1R) and D5 receptor subtypes which couple to stimulatory $G\alpha_s$ proteins and activate AC (Stoof and Kebabian, 1981), thereby increasing the intracellular concentration of the second messenger cAMP (**Fig. 6**). $G\alpha_{olf}$, an isoform of $G\alpha_s$ coded by a different gene, is highly expressed in the striatum, as opposed to $G\alpha_s$ and mediates D1R signaling to AC in this brain region (Herve *et al.*, 1993; Zhuang *et al.*, 2000; Herve, 2011). Likewise the subunit gamma 7 is abundantly expressed in medium spiny neurons (Watson *et al.*, 1994), particularly in neurons that also express D1 receptor, suggesting its interaction with $G\alpha_{olf}$ in a common signaling pathway (Wang *et al.*, 2001b). Even though less is known about the β subunits that combine with $G\alpha_{olf}$, some data converge to a role for β1 (Wang *et al.*, 2001) and β2 (Schwindinger *et al.*, 2010).

The D2-like family of receptors includes D2, D3, and D4 receptors subtypes (D2R, D3R, D4R) which are coupled to the inhibitory $G\alpha_{i/o}$ proteins and inhibit AC, thereby decreasing cAMP and protein kinase A (PKA) activity (Stoof and Kebabian, 1981) (**Fig. 6**). D2R signaling via inhibition of AC acts in opposition to agents that stimulate AC, decreasing the phosphorylation of PKA substrates

Adenylyl cyclase catalyzes the conversion of ATP to cyclic AMP, which binds to the regulatory subunits of the PKA holoenzyme to disinhibit the catalytic subunits. Four isoforms of AC are expressed in the striatum (ACII, V, VII and IX) at significant levels (Mons and Cooper, 1994). ACV mRNA levels are highly expressed in MSNs suggesting that this isoform is particularly involved in D1R signaling in the striatum (Mons and Cooper, 1994; Iwamoto *et al.*, 2003). Finally, cAMP is hydrolyzed to its inactive derivate AMP by phosphodiesterases (PDE) including PDE1B (Polli and Kincaid, 1994), PDE4B (Siuciak *et al.*, 2008), and PDE10A (Fujishige *et al.*, 1999).

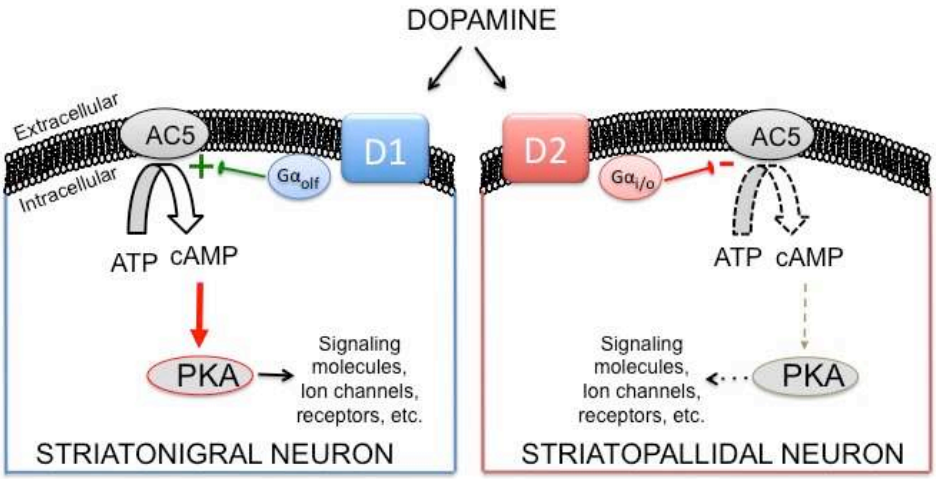

Figure 6: Differential coupling of striatal dopamine (DA) receptors.

DA acts in a bidirectional manner whereby it exerts stimulatory biochemical effects via D1 receptors and inhibitory biochemical effects via D2 receptors. D1 receptors activate adenylyl cyclase AC5 through $G\alpha_{i/o}$, consequently activating PKA. D2 receptors inhibit AC5 through $G\alpha_{i/o}$ thereby inhibiting PKA.

1.2.2. DISTRIBUTION OF D1RS AND D2RS

D1R mRNA has been found in the dorsal and ventral striatum, mainly in striatonigral MSNs and the olfactory tubercle. In addition, D1Rs have been detected in the limbic system, hypothalamus, and thalamus (Dearry *et al.*, 1990; Fremeau *et al.*, 1991; Weiner *et al.*, 1991). D5Rs are concentrated in the hippocampus and entorhinal cortex with very low expression in the striatum.

D2Rs are highly expressed in the striatum where they are mostly expressed in striatopallidal MSNs, and in the olfactory tubercle. It is also found in the SNc and in the VTA, where it is expressed by dopaminergic neurons (Meador-Woodruff *et al.*, 1989; Weiner *et al.*, 1991). D2Rs are thus located both post-synaptically on MSNs and pre-synaptically as autoreceptors on dopaminergic nerve terminals where they participate in the autoregulation of the dopamine release (Jaber *et al.*, 1996). D2Rs exist as two different splice variants termed D2short and D2long which are differentially expressed pre-synaptically (D2short) or post-synaptically (D2long) (Centonze *et al.*, 2004). D3Rs are expressed in the striatum where they are concentrated in the ventral striatum/nucleus accumbens. D3 and D4 receptors are also expressed in limbic structures including the amygdala, hippocampus and PFC (Sealfon and Olanow, 2000; Guillin *et al.*, 2007).

Within the MSNs, D1 and D2 receptors are present on both dendrites and spines (Huang *et al.*, 1992; Levey *et al.*, 1993)**.** D2Rs are also found in axons terminals forming symmetrical, rather than asymmetrical synapses (Hersch *et al.*, 1995). D2Rs have a high affinity for DA and are tonically activated by low basal concentrations of DA in the extracellular space, whereas D1Rs are thought to be stimulated following burst firing of DA neurons which is more efficient to release DA (Grace, 1991).

1.3. CAMP/ PKA CASCADE

cAMP activates a major effector enzyme, the PKA (Miyamoto *et al.*, 1968; Beavo *et al.*, 1974). PKA phosphorylates a variety of intracellular targets such as voltage-gated and ligand-gated ion channels (Surmeier *et al.*, 2007), transcription factors (Konradi *et al.*, 1994), neurotransmitter receptors (Cepeda and Levine, 1998), and other proteins which change their signaling properties and can influence cellular excitability (see Missale *et al.*, 1998 for review).

A well studied target for the cAMP/PKA signaling cascade in striatal neurons is the dopamine- and cyclic AMP-regulated phosphoprotein of 32kDa (DARPP-32) (Greengard *et al.*, 1999; Svenningsson *et al.*, 2004) which is an inhibitor of protein phosphatase 1 (PP1) (Hemmings *et al.*, 1984). We will detail DARPP-32 implication in D1 signaling in the next section. Others include cAMP-regulated phosphoprotein, Mr ~ 21,000 (ARPP-21)

(Hemmings and Greengard, 1989; Ouimet *et al.*, 1989), or regulator of calcium signaling (RCS), which is an inhibitor of Ca^{2+} /calmodulin targets when phosphorylated by PKA (Rakhilin *et al.*, 2004), and cAMP-regulated phosphoprotein, Mr ~ 16,000 (ARPP-16) , recently shown to be an inhibitor of PP2A-55d when phosphorylated by a Greatwall family kinase (Gharbi-Ayachi *et al.*, 2010; Mochida *et al.*, 2010).

The action of PKA is counterbalanced by specific protein phosphatases. In some cases, it has been demonstrated that phosphatases belonging to the PP1 and PP2 families are responsible for the dephosphorylation of PKA substrates. Important proteins that modulate PKA activity are the A-kinase anchoring proteins (AKAPs). AKAPs bind to the regulatory subunit dimer of the tetrameric PKA protein and thereby target PKA to defined cellular compartments in the vicinity of its substrates. Although PKA is preferentially located in the cytoplasm in resting conditions, a significant fraction of the dissociated C subunit of PKA localizes to the nucleus upon increase of intracellular cAMP (Meinkoth *et al.*, 1990). The functional importance of nuclear PKA was demonstrated using a specific inhibitor of this enzyme in the nucleus, which prevented the induction of long-lasting long-term potentiation in hippocampal slices (Matsushita *et al.*, 2001).

2. D1R-MEDIATED SIGNALING PATHWAY

2.1. DARPP-32 /PP1 CASCADE

Thirty years ago, a phospho-protein named DARPP-32 was discovered to be enriched in the dopaminoceptive neurons of the striatum and to be phosphorylated in response to dopamine and cAMP (Walaas *et al.*, 1983). Since then, a variety of molecular, cellular and functional approaches have shown that DARPP-32 is an essential mediator of the effects of dopamine and other neurotransmitters in the striatum (Fienberg and Greengard, 2000; Svenningsson *et al.*, 2004; Le Novere *et al.*, 2008).

DARPP-32 is a major target for the cAMP/PKA signaling cascade (Greengard *et al.*, 1999; Svenningsson *et al.*, 2004) and is expressed in both the D1R-enriched striatonigral and D2R-enriched striatopallidal neurons (Ouimet *et al.*, 1998; Bateup *et al.*, 2008). In D1R, DA stimulates the cAMP/PKA cascade and increases phosphorylation of DARPP-32 at threonine 34 (T34) (**Fig. 7**), which converts DARPP-32 into a potent high-affinity inhibitor of the multi-functional serine/threonine protein phosphatase, PP1 (Hemmings *et al.*, 1984). DARPP-32 is expressed at very high concentrations (~50 μM) and therefore a substantial portion of PP1 activity is inhibited in vivo in response to dopamine stimulation (Ouimet *et al.*, 1998; Bateup *et al.*, 2008).

PP1 is a major, highly conserved serine/threonine phosphatase that regulates an enormous variety of cellular functions including cell cycle, muscle contraction, carbohydrate metabolism, neuronal signaling, and transcription (see Bollen, 2001 for review). The PP1 catalytic subunit (PP1c) is a ~36 kDa globular protein highly conserved in eukaryotes. Among the four PP1c isoforms (α, β/δ, γ1, and γ2) expressed in the mammalian brain, PP1α and PP1γ1 are highly expressed in the striatum, whereas PP1β/δ is less abundant (da Cruz e Silva *et al.*, 1995). PP1c can form complexes with over 50 regulatory subunits, most of which target it to specific subcelullar locations and bring it into proximity to its substrates. Most of these proteins interact with a small hydrophobic groove on the surface of PP1c through a short conserved bonding motif- the RVxF motif- which is often preceded by further basic residues. Some of the PP1-interacting partners are potent inhibitors of PP1c, such as DARPP-32, inhibitor-1 (Aitken *et al.*, 1982) and the much less characterized inhibitor of protein phosphatase 5 (IPP-5) (Wang *et al.*, 2008a). Regulation of PP1c in response to extracellular and intracellular signals occurs through changes in the levels, conformation or phosphorylation status of targeting subunits. The activation of DARPP-32/PP1 cascade amplifies PKA signaling by preserving the phosphorylated state of its targets, such as several ion channels in striatal neurons, including AMPA and NMDA glutamate receptor (AMPAR, NMDAR) and voltage-gated Na^+ and Ca^{2+} channels (see Svenningsson *et al.*, 2004 for review). Therefore, by regulating PP1 function, DARPP-32 can regulate the physiological properties of neurons.

In contrast to T34 phosphorylation, phosphorylation of DARPP-32 at T75 has been shown to inhibit PKA activity both towards DARPP-32 and towards other PKA substrates (Bibb *et al.*, 1999). T75 is phosphorylated by cyclin-dependent kinase 5 (Cdk5) which is highly expressed in the striatum. T75 is dephosphorylated by PP2A (**Fig. 7**) and distinct isoforms of this enzyme are activated in response to both calcium signaling via AMPAR and NMDAR, and PKA signaling stimulated by D1R activation (Ahn *et al.*, 2007a; Ahn *et al.*, 2007b). Specifically, the B56δ subunit can be phosphorylated by PKA, increasing PP2A activity (Ahn *et al.*, 2007a). D1R activation triggers the PKA dependent phosphorylation of PP2A containing the B56δ subunit. This activates the catalytic activity of PP2A that dephosphorylates T75 phosphorylation, and thereby removes the inhibition on PKA (Nishi *et al.*, 2000; Ahn *et al.*, 2007a). Hence, DARPP-32 has the unique ability to act as an inhibitor of phosphatase (PP1) or as inhibitor of kinase (PKA) depending on its state of phosphorylation.

In addition, DARPP-32 is phosphorylated at 3 others sites: serine 45 and serine 97 (serine 102 in the rat) by casein kinase 2 (CK2) and at serine 130 (serine 137 in the rat) by casein kinase 1 (CK1) under resting conditions (**Fig. 7**) (Girault *et al.*, 1989; Girault *et al.*, 1990; Desdouits *et al.*, 1995a). These phosphorylations modulate the likelihood of phosphorylation at T34 by two different mechanisms. First, *in vitro* phosphorylation by CK2 increases the efficiency of phosphorylation of T34 by PKA (Girault *et al.*, 1989). Moreover,

in vitro S130 phosphorylation decreases the rate of dephosphorylation of T34 triggered by protein phosphatase 2B (PP2B or calcineurin) (Desdouits *et al.*, 1995b). Hence, the overall result is the potentiation of D1R signaling through amplification of the PKA/DARPP-32 pathway.

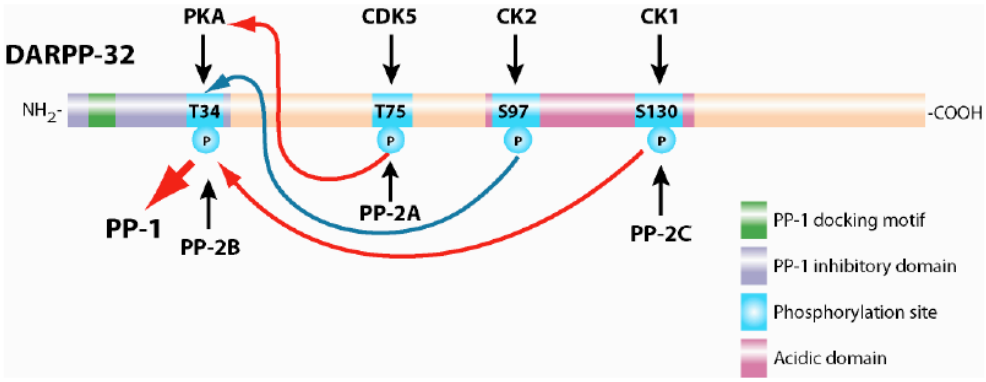

Figure 7: DARPP-32 phosphorylation sites.

Schematic representation of DARPP-32 protein showing the location of four known phosphorylation sites. The kinases (upper row) and phosphatases (lower row) regulating these sites are shown. Green arrows represent facilitatory interactions while red arrows indicate inhibitory interactions (Svenningsson *et al*., 2004).

Taken together, these findings suggest that Cdk5 and PKA signaling are mutually antagonistic. In resting condition or period of low cAMP/PKA activity, Cdk5 phosphorylates DARPP-32 at T75, thereby reducing the efficacy of dopaminergic signaling. During periods of high cAMP/PKA activity, dopamine decreases the state of phosphorylation of DARPP-32 at T75 and increase phosphorylation at T34 thereby removes this inhibitory constraint. As a consequence, DARPP-32 inhibits PP1 allowing heightened phosphorylation of PKA targets (see **Figure 8** for a summary).

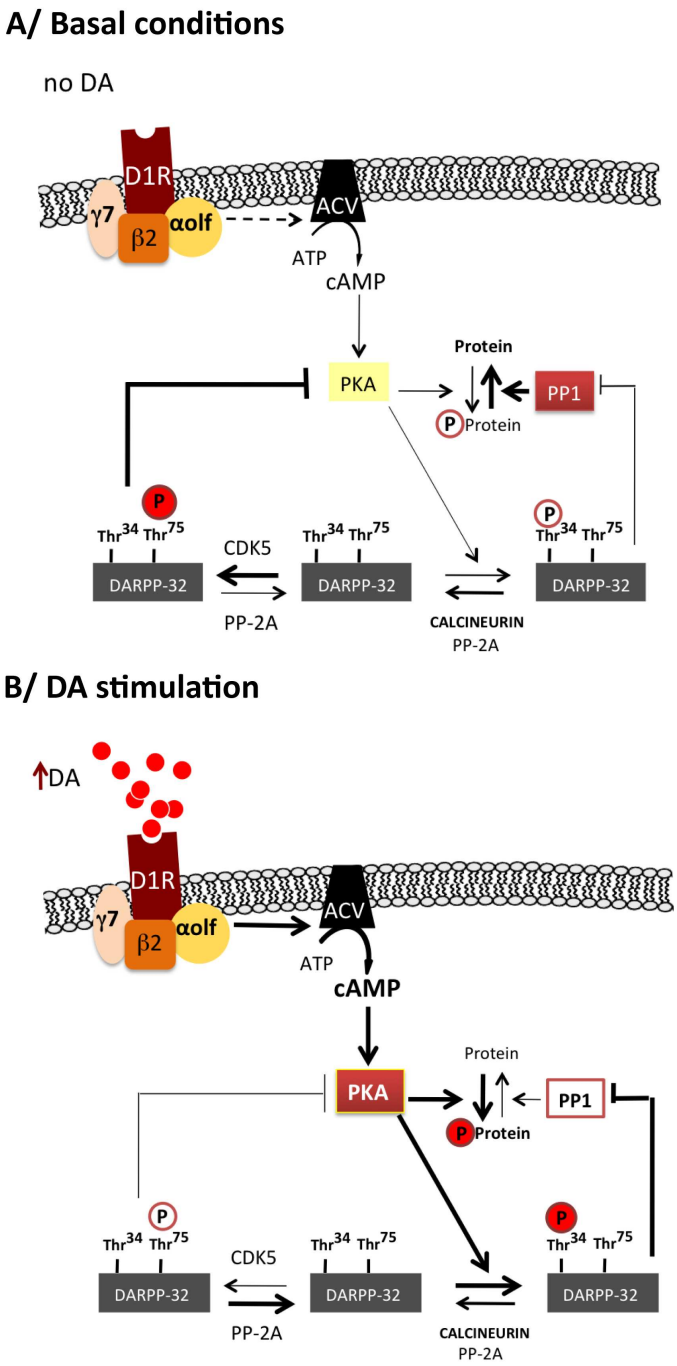

Figure 8: Key role of DARPP-32 specific phosphorylation sites on the regulation of PP1.

A. cAMP/PKA/DARPP-32 signaling pathway under basal conditions in MSNs. Cdk5 increases phosphoT75-DARPP-32, thus inhibiting PKA activity. In parallel, the small amount of DA is not able to increase cAMP in the cytoplasm. PKA is not activated and cannot phosphorylate T34-DARPP-32. The inhibition of PP1 is then removed allowing the dephosphorylation of multiple substrates. Inhibition of PKA and activation of PP1 leads to reduction in phosphorylation of wide range of proteins.

B. cAMP/PKA/DARPP-32 signaling pathway under stimulating conditions in MSNs. When DA stimulates D1R, the up-regulation of cAMP triggers the activation of PKA that phosphorylate T34-DARPP-32. In this state of phosphorylation, DARPP-32 inhibits PP1 activity, amplifying the effect of PKA in phosphorylating protein substrates. Concomitantly, PP2A is activated and decreases the phosphorylation of DARPP-32 on T75. This reduces PKA inhibition. Both, PKA activation and PP1 inhibition, results in a higher increase in the phosphorylation of multiple substrates in the MSNs. Courtesy of Dr. Alcacer.

2.2. ROLE OF DARPP-32

A hypothesis concerning the overall function of DARPP-32 in striatal signaling was elucidated by studies of the DARPP-32 knock-out mouse. These studies showed that basal electrophysiology and locomotor behaviors were unaffected in the knock-outs, but the biochemical, electrophysiological, gene transcriptional, and behavioral responses to administration of a variety of agents were absent or significantly reduced in the DARPP-32

knock-out mice (Fienberg *et al.*, 1998). Interestingly, many of these responses could be re-instated in the knock-out mice by increasing the doses of the drugs. These observations suggest that DARPP-32 functions as an amplifier of signaling. In response to stimulation and activation of second messenger cascades, protein kinases directly phosphorylate target proteins. When this activity is combined with DARPP-32-mediated inhibition of the phosphatase PP1, high levels of phosphorylation and activation are sustained. When DARPP-32 is absent, inhibition of PP1 is relieved allowing it to dephosphorylate substrates, thereby shutting off or reducing the effects of the drug.

2.3. ERK1/2 CASCADE

ERK (extracellular signal-regulated protein kinase) is a serine/threonine kinase that belongs to the mitogen-activated protein kinase (MAPK) family. MAPK modules comprise three classes of enzymes that act in a cascade of activatory phosphorylation: the MAP kinase or ERK is activated by a MAP/ERK kinase (MEK), which is itself activated by a MEK kinase (MEKK) (**Fig. 9**). MEKs are dual specificity kinases that trigger the activation of MAPKs by phosphorylating a threonine and a tyrosine in their activation loop. ERK1/2 module phosphorylation occurs through tyrosine kinases and G protein-coupled receptors activation that induce a small G protein, Ras, to move from an inactive GDP-bound state to an active GTP-bound state. The GTP-bound form of Ras binds to Raf bringing it to the plasma membrane where its protein kinase activity is increased and the kinase cascade is activated (Vojtek *et al.*, 1993; Morrison and Cutler, 1997). Once activated, Raf phosphorylates and activates the dual specificity protein kinases MEK1/2, which in turn phosphorylate and activate ERK1/2 (**Fig. 9**).

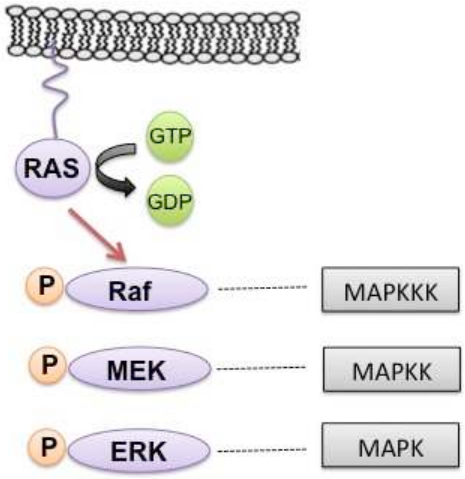

Figure 9: MAP kinases cascade of activatory phosphorylation.

Activation of Ras allows the exchange of GDP for GTP on Ras protein. Ras-GTP activates Raf (MAPKKK) that in turn phosphorylates MEK (MAPKK) and ultimately activates ERK (MAPK) through a double phosphorylation.

ERK1/2 phosphorylate numerous substrates in all cell compartments such as cytoskeletal proteins, regulatory enzymes, and transcription factors (see Krishna and Narang, 2008 for review). ERK activity is controlled through dephosphorylation by MAP kinase phosphatases (Sun *et al.*, 1993) that are dual specificity phosphatases and also by classical phosphatases including STEP and PP2A (Camps *et al.*, 2000; Raman *et al.*, 2007).

The "classical" route to Ras–ERK activation through receptor tyrosine kinases, adaptor proteins (such as Grb2), and GEFs (such as Sos) can operate in neurons in response to neurotrophins. However, ERK can be activated through another route, in response to direct membrane depolarization (Rosen *et al.*, 1994) or glutamatergic signaling (Yun *et al.*, 1999). ERK activation that is induced by these stimuli is Ras-dependent, but activation of Ras in response to these signals requires calcium influx (Rosen *et al.*, 1994). This calcium influx can be facilitated by NMDA (N-methyl-D-aspartate)-type glutamate receptors or voltage-gated calcium channels (**Fig. 10**).

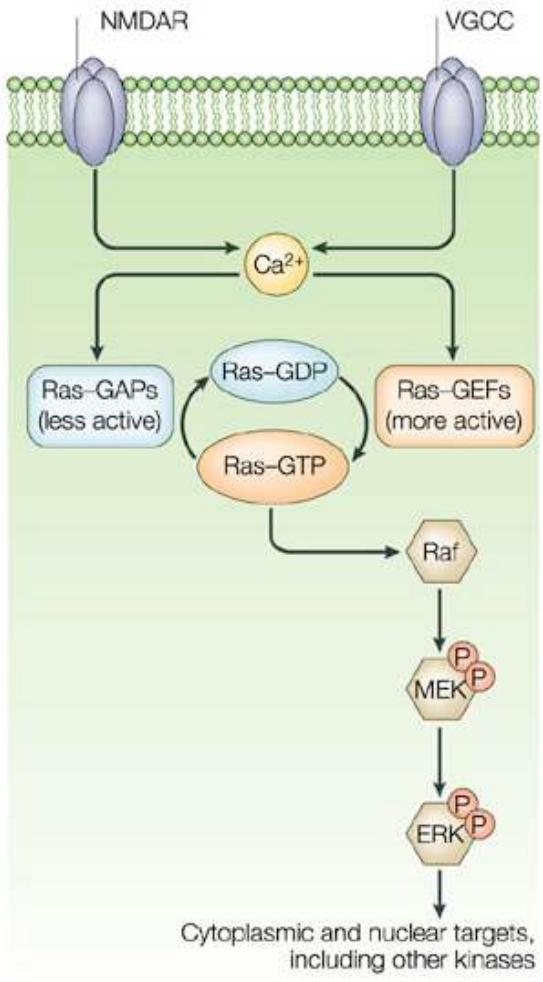

Figure 10: Activation of ERK by synaptic signaling.

Calcium influx, either through NMDA (N-methyl-D-aspartate)-type glutamate receptors (NMDARs) or voltage-gated calcium channels (VGCCs) triggers an increase in the levels of Ras–GTP. This leads to the activation of Raf, mitogen-activated protein kinase (MAPK)/ERK kinase (MEK) and ERK, allowing phosphorylation of both nuclear and cytoplasmic ERK substrates. Calcium can activate Ras signaling through activation of Ras–guanyl nucleotide exchange factors (GEFs), inhibition of Ras–GTPase-activating proteins (GAPs), a change in the localization of these enzymes (which would alter their likelihood of "seeing" Ras), or a combination of all of these factors. The precise route to Ras activation might differ depending on the neuronal cell type and/or the extracellular stimulus. Adapted from Thomas and Huganir, 2004.

ERK activation forms an essential pathway for cells to generate adaptive responses to changing environments. These cellular responses include alteration in cellular structure or metabolism, cellular growth, differentiation, and apoptosis (Lewis *et al.*, 1998; Chen *et al.*, 2001; Johnson and Lapadat, 2002; Yoon and Seger, 2006). In addition, pharmacological inhibition of MEK has been used to investigate the role of ERK in short and long-term neuronal responses in vivo (Miller and Marshall, 2005; Valjent *et al.*, 2006b; Languille *et al.*, 2009). In that context, ERK activation has been shown to mediate the regulation of gene expression and synthesis of new proteins that end up in long-term neuronal events such as the modulation of synaptic plasticity and/or dendritic spine morphology that can be critical for memory processes (Sweatt, 2004). In particular, the activation of ERK is a major trigger for the immediate early genes induction including *c-fos*, *jun-B*, *zif268* and *fosB* (Sgambato *et al.*, 1998; Vanhoutte *et al.*, 1999; Radwanska *et al.*, 2006).

2.4. ERK: COINCIDENCE GATE BETWEEN D1R AND NMDAR

Multiple evidences implicate ERK in all stage of drug addiction (Lu *et al.*, 2006; Brami-Cherrier *et al.*, 2009). Drugs of abuse induce specifically a robust increase of ERK phosphorylation in the mesocorticolimbic system (Valjent *et al.*, 2004; Corbille *et al.*, 2007). Non addictive substances such as caffeine or fluoxetine do not have such a strong effect. In particular both acute and repeated cocaine administration cause consistent up-regulation of ERK phosphorylation in the striatum, nucleus accumbens (NAc), as well as in other brain areas (Zhai *et al.*, 2008). Cocaine-induced ERK activation occurs selectively in striatonigral D1R-containing MSNs and requires not only D1R activation but also the concomitant stimulation of NMDAR (Valjent *et al.*, 2000; Valjent *et al.*, 2005; Girault *et al.*, 2007). ERK activation can be abolished by the administration of D1R or NMDAR antagonists (Valjent *et al.*, 2001). These findings show that ERK pathway activation is the result of simultaneous release of DA and glutamate (Girault *et al*., 2007).

The precise mechanism by which the glutamate leads to MAPK activation in the MSNs remains to be fully characterized. Several possible pathways coupling NMDAR activation to ERK1/2 phosphorylation through Ca^{2+} influx have been proposed in other systems (Sweatt, 2004; Thomas and Huganir, 2004). In the striatum, dopamine regulation of ERK1/2 involves several levels of interaction with NMDAR signaling. Activation of the Ca^{2+}-activated Ras guanine-nucleotide exchange factor Ras-GRF1 is an important contributor (Fasano *et al.*, 2009). D1R appear to potentiate NMDAR thorugh tyrosine phosphorylation of NR2B subunit (Pascoli *et al.*, 2011). An additional interaction involves DARPP-32 (Valjent *et al.*, 2005) (**Fig. 11**). The activation of PKA/DARPP-32 module upon D1R stimulation is required for activation of the ERK signaling pathway in adult mice (Valjent *et al*., 2005). When phosphorylated at T34 residue, DARPP-32 inhibits PP1, an effect which appears to be

important at several levels in ERK cascade. On the one hand, PP1 inhibition prevents ERK dephosphorylation by STEP (striatal-enriched phosphatase), by maintaining this tyrosine phosphatase in a phosphorylated inactive state. On the other hand, it is also critical upstream of ERK, because MEK phosphorylation in response to psychostimulants was dramatically reduced in DARPP-32 knockout mice (Valjent *et al.*, 2005) (**Fig. 11**). This may in part result from the role of DARPP-32/PP1 inhibition in the regulation of NMDAR (Snyder *et al.*, 1998) . Although other links with D1R could exist, the regulation of protein phosphatases through DARPP-32 appears critical for the activation of ERK1/2 in the striatum.

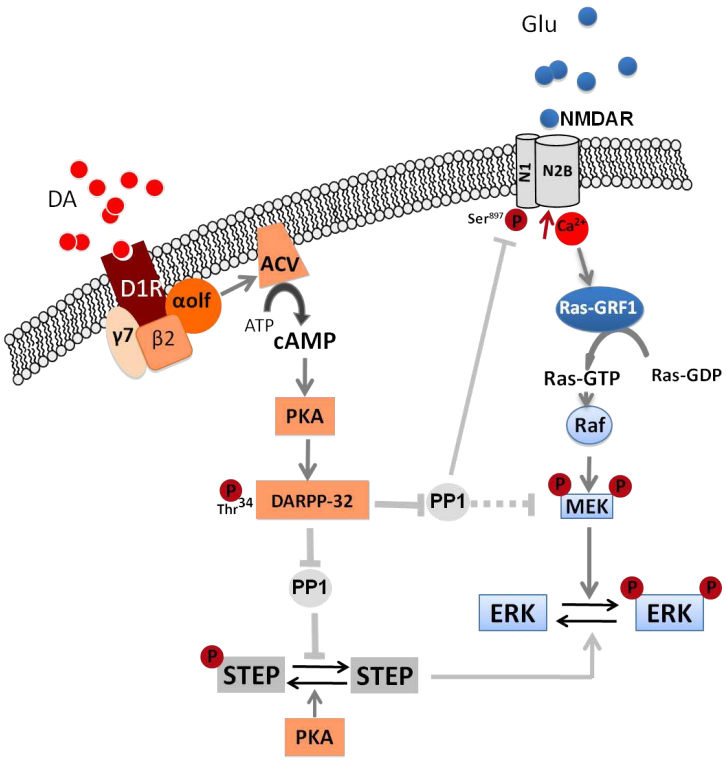

Figure 11: Convergence of D1R and NMDAR signaling onto ERK cascade: role of PP1 inhibition.

D1R-dependent PKA activation and NMDAR-dependent calcium signaling converge to activate MAPK in the striatal MSNs. The phosphorylation of Thr-34-DARPP-32 by PKA activation amplifies glutamate-induced ERK signal transduction by inhibiting PP1, thus desinhibiting the MAPK cascade at different levels. Abbreviations: ACV: adenylyl cyclase V; ATP: adenosine triphosphate; cAMP: cyclic adenosine monophosphate; D1R: D1-receptor; DA: dopamine; DARPP-32: DA- and cAMP regulated phosphoprotein of 32kDa; ERK: extracellular signal regulated kinase; Glu: glutamate; MEK: MAPK ERK kinase; NMDAR: N-methyl, D-aspartate receptor; PKA: protein kinase A; PP1: protein phosphatase 1; Raf: MAPK ERK kinase-kinase; Ras-GRF1: ras guanosine releasing factor 1; STEP: striatal enriched phosphatase. Courtesy of Dr. Alcacer.

3. D2R-MEDIATED SIGNALING PATHWAY

Unlike in D1R, D2R activation mediates the inhibition of cAMP accumulation (De Camilli *et al.*, 1979; Stoof and Kebabian, 1981) conferring to dopamine the ability to exert inhibitory biochemical effects in neurons which express these receptors (Gerfen *et al.*, 1990; West and Grace, 2002).

Here, we first review the differential regulation of DARPP-32 phosphorylation occurring in the striatopallidal neurons. Then, we describe the role of a second type of receptor enriched in these neurons, adenosine A2A receptor, whose function is primordial in regulating D2R neurons. It is important to note, that, as is typical of $G\alpha_{i/o}$-coupled receptors, D2-like receptors modulate other signaling pathways in addition to adenylyl cyclase, including phospholipases, ion channels, MAP kinases, and the Na^+ /H^+ exchanger (Huff *et al.*, 1998). Many of these pathways are regulated by $G\beta\gamma$ subunits that are released by receptor activation of $G\alpha_{i/o}$ proteins. It is beyond the scope of this thesis to describe these pathways in detail.

3.1. REGULATION OF DARPP-32 PHOSPHORYLATION IN STRIATOPALLIDAL NEURONS

The opposing action of D1 and D2 receptors stimulation is also true for the regulation of DARPP-32 phosphorylation. D2Rs activation, unlike D1Rs, results in a decrease in T34 phosphorylation and an increase in T75 phosphorylation which are mediated by two signaling pathways. One pathway involves the $G\alpha_{i/o}$ mediated inhibition of AC/cAMP signaling which decreases PKA and PP2A activity. The other pathway is calcium-dependent and involves PP2B dephosphorylation of T34 (Nishi *et al.*, 1997). This opposing regulation of T34 and T75 phosphorylation results in feedback loops in each cell type whereby DARPP-32 amplifies both the D1R-mediated potentiation of PKA activity and the D2R-mediated inhibition of PKA signaling. Interestingly, *in vivo* studies have confirmed the specific regulation of D2R on the state of phosphorylation of DARPP-32 (Bateup *et al.*, 2008). This study used BAC transgenic mice that express differentially tagged DARPP-32 protein selectively in D1R-enriched striatonigral and D2R-enriched striatopallidal neurons allowing cell-type specific analysis of DARPP-32 phosphorylation. The authors confirmed that D2Rs activation by the agonist quinpirole decreases T34-DARPP-32 phosphorylation and increased T75 phosphorylation in striatopallidal neurons. Consistently, the blockade of D2R with the anti-psychotic haloperidol - a strong D2R antagonist- increases T34-DARPP-32 phosphorylation and, this effect was restricted to D2 neurons (Bateup *et al.*, 2008).

Adenosine A2A receptors (A2AR) stimulate the activity of adenylyl cyclase (see below), an effect which leads to PKA-dependent phosphorylation of DARPP-32 at T34 (Svenningsson *et al.*, 1998), and decreased phosphorylation at T75 (Lindskog *et al.*, 2002).

3.2 A2AR-D2R INTERPLAY IN STRIATOPALLIDAL NEURONS

Adenosine has been shown to be an important modulator of dopamine signaling. A2ARs are GPCRs highly expressed in the striatum where they are selectively localized to indirect striatopallidal MSNs (Schiffmann *et al.*, 1991). A2ARs are coupled to $G\alpha_{olf}$ proteins which stimulate the AC/cAMP/PKA cascade (Kull *et al.*, 2000; Corvol *et al.*, 2001) and therefore antagonize D2R activity. This regulation is well illustrated by a study pointing out the involvement of the cAMP/PKA/T34-DARPP-32 module in the A2AR-triggered signaling events (Svenningsson *et al.*, 2000). Administration of D2R antagonist induces an increase in phospho-T34. In A2AR KO mice, this effect is abolished. Similarly, a reduction in phospho-T34 is observed in mice that had been pretreated with a selective A2AR antagonist (Svenningsson *et al.*, 2000). This suggests that blockade of tonic dopamine D2R increases DARPP-32 phosphorylation and that this effect is counteracted by blockade of A2AR-mediated transmission.

Moreover, the antagonistic interaction between A2AR and D2R receptors is reinforced by their ability to form heteromeric complexes in striatopallidal neurons (Hillion *et al.*, 2002; Canals *et al.*, 2003). Indeed, A2AR activation decreases the affinity of DA for their D2R (Ferre *et al.*, 1991; Dasgupta *et al.*, 1996) resulting in an amplified effect of A2ARs activity in rat striatal neurons. The exact physiological significance of this interaction still needs to be defined (Ferre *et al.*, 2007).

CHAPTER III

COCAINE-INDUCED NEUROCHANGES

1 NEUROCHEMICAL ACTION OF COCAINE

Cocaine acts as an indirect agonist of the monoaminergic systems, including the dopaminergic system. Cocaine blocks the dopamine-, norepinephrine- and serotonin re-uptake transporters (DAT, NET, and SERT, respectively) thereby prolonging the availability of the monoamines in the extracellular space (Ritz *et al.*, 1990) (**Fig. 12**). Although psychostimulants have the ability to produce feelings of pleasure and relieve negative emotional states (Nesse and Berridge, 1997), they also have highly diverse behavioral and neuropharmacological properties. Via its actions on the NET, cocaine has profound effects on the autonomic sympathetic nervous system responsible for increases in heart rate, blood pressure, respiration and body temperature, vasoconstriction and pupil dilation (Pitts and Marwah, 1989; Das, 1990). Cocaine use is therefore associated with a high risk of death due to cardiovascular collapse, respiratory failure, stroke, and cerebral hemorrhage.

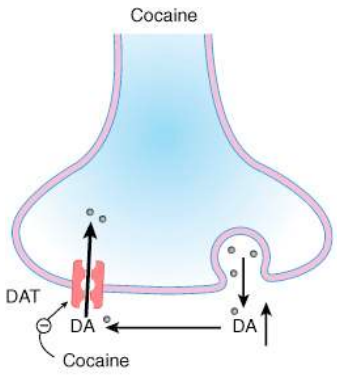

Figure 12: Mechanism of action of cocaine on synaptic terminal of dopamine (DA) neurons.

Cocaine inhibits the dopamine transporter (DAT), decreasing DA clearance from the synaptic cleft and causing an increase in extracellular DA concentration. (Katzung, 2012).

2. THE BRAIN REWARD PATHWAYS

2.1. CENTRAL ROLE OF DOPAMINE

All drugs of abuse including cocaine converge to the mesolimbic pathway where they, in a way or another, increase extracellular dopamine within the nucleus accumbens (Wise and

Rompre, 1989), especially in the shell subregion (Di Chiara and Imperato, 1988). As a central component in the brain reward pathways, the mesolimbic pathway is implicated in the rewarding properties of both natural stimuli (food, drink, mating opportunities) and addictive drugs.

Rewards are pursued with the anticipation that their consumption will produce desired outcomes, i.e. will "make things better" (Berridge and Robinson, 2003). As with natural rewards, drugs are reinforcing i.e. behaviors with rewarding effects tend to be repeated over time (White, 1989). Although natural rewards also produce an increase in DA release, their effects are not nearly as robust as those of addictive drugs. The ability of drugs of abuse to stimulate brain reward circuitry with a strength, time course, and reliability exceeding almost any natural stimulus, powerfully consolidate responses to drug-associated stimuli (Berke and Hyman, 2000). This suggests that drugs of abuse "hijack" the system normally implicated in the rewarding and reinforcing effects of stimuli involved in survival functions (Robbins and Everitt, 1996) but unlike natural stimuli do not serve any biological need. In addition, the effects are persistent, adding to the consolidation of responses to drug-associated stimuli (Berke and Hyman, 2000) and further promoting the repeated use of the addictive substance.

A large body of work, including pharmacological lesion, transgenic and microdiaysis studies, has established that DA release is necessary for reward (Koob and Bloom, 1988; Wise and Rompre, 1989; Di Chiara, 1998) an effect hypothesized to provide positive reinforcement for drug self-administration and, as such, the initiation of the addiction cycle. The "dopamine hypothesis" based on the fact that cocaine blocks DAT (Kuhar *et al.*, 1991), has been however challenged by the observation that mice lacking the DAT still experience the reinforcing effects of cocaine and/or display conditioned place preference (CPP) to cocaine (Rocha *et al.*, 1998; Sora *et al.*, 1998). Yet, the complete DAT knockout may have resulted in compensatory adaptations that alter normal functioning of the reward pathways. In a subsequent report, Chen *et al*. (2006) provided compelling evidence for the role of the DAT in cocaine reward. These authors showed that transgenic mice expressing a functional DAT that is insensitive to cocaine, do not display drug-induced increases in locomotion and nucleus accumbens (NAc) dopamine release or drug reinforcement.

2.2 GLUTAMATE AND REWARD

It has become increasingly evident that in addition to dopamine, glutamate plays an essential role in drug reward and reinforcement. Dopaminergic neurons in the VTA receive extensive glutamatergic input from the PFC, amygdala and hippocampus. Cocaine stimulate glutamate

release in the PFC and NAc (Reid *et al.*, 1997) which is potentiated with repeated exposure (Reid and Berger, 1996). Glutamate enhances dopaminergic transmission by increasing activity of the dopaminergic neurons in the VTA, and by facilitating dopamine release from the presynaptic terminals in the NAc (Taber *et al.*, 1995; Floresco *et al.*, 1998). Many of the actions of this excitatory transmitter result from its stimulatory interaction with the dopamine system. For example, basal and psychostimulant-induced locomotion, which are critically dependent on dopamine, are stimulated and inhibited by glutamatergic agonists and antagonists, respectively (Pulvirenti *et al.*, 1989; Swanson and Kalivas, 2000). Perhaps the most striking evidence for the importance of glutamate in addiction processes, comes from the observation that many of the enduring neuroplastic changes associated with repeated psychostimulant administration involve glutamatergic transmission (Carlezon and Nestler, 2002; Boudreau and Wolf, 2005; Hemby *et al.*, 2005; Lu *et al.*, 2005). Of particular interest is the synaptic plasticity that occurs in reward-related brain regions (see next section for details). It was demonstrated that a single *in vivo* cocaine exposure increases AMPA-mediated currents at excitatory synapses onto dopamine cells in the VTA (Ungless *et al.*, 2001). Furthermore, the structural plasticity in the NAc core and mPFC associated with cocaine-induced behavioral sensitization is localized to portions of the dendritic tree that might contain dopamine/glutamate synapses (Li *et al.*, 2003).

In addition to dopamine and glutamate, other neurotransmitters including gammaaminobutyric acid (GABA) (Brebner *et al.*, 2002), norepinephrine (Davis *et al.*, 1975) serotonin (Filip *et al.*, 2005), acetylcholine (Smith *et al.*, 2004), endogenous opioids (Van Ree *et al.*, 2000), and endocannabinoids (Maldonado *et al.*, 2006) are involved in reward processes. It is beyond the scope of this thesis to describe their involvement in detail.

3. CHRONIC CELLULAR & MOLECULAR ADAPTATIONS TO COCAINE

Drug addiction is defined as a compulsive drug use despite negative consequences such as medical illness, failures in significant life roles or the need to engage criminal activity to obtain drugs. An important characteristic of addiction is its stubborn persistence in spite of treatment and others attempts to control drug taking (McLellan *et al.*, 2000; Hser *et al.*, 2001). Although some individuals can stop compulsive use of drugs on their own, for 15 to 20% of individuals (Deroche-Gamonet *et al.*, 2004) addiction proves to be a chronic and recalcitrant condition with relapses occurring decades after abstinence (McLellan *et al*., 2000).

There is considerable evidence that both environment and genetics influence the progression from substance use to dependence and ultimately addiction. Estimates from twin and adoption studies give ranges of 40% to 60% heritability. Genetic contributions to addiction result from complex genetic differences, ranging from alleles that control drug metabolism to hypothesized genetic control over drug sensitivity and environmental influences (Crabbe, 2002; Uhl and Grow, 2004). Studies in human addicts have identified several genes that are associated with cocaine dependence. These include certain alleles of the D2 receptor gene (Noble *et al.*, 1993), DAT (Gelernter *et al.*, 1994; Guindalini *et al.*, 2006), the serotonin transporter (Mash *et al.*, 2000), the cannabinoid receptor CB1(Comings *et al.*, 1997), prodynorphin (Chen *et al.*, 2002), the mu-opioid receptor (Zubieta *et al.*, 1996) and myelin-related genes (Albertson *et al.*, 2006), However, the difficulty with association studies is that they cannot distinguish whether genes play a role in establishment of addiction, underlie aspects of withdrawal, or rather represent a compensatory homeostatic mechanism.

The development of addiction involves a transition from casual to compulsive patterns of drug use. It is hypothesized that cellular and molecular changes occur during initial drug exposure, which then accumulate over time and, in vulnerable individuals, drive compulsive drug use. Here, we review the principal drug-induced neuro-adaptations in NAc-related circuitry found at synaptic, cellular, and gene expression levels.

3.1. SYNAPTIC PLASTICITY

The word plasticity is often used in neuroscience to refer to the ability of experiences to modify the organization and behavior of neural circuits in the brain. There are several forms of plasticity. One is the activity-dependent alteration of the connections among neurons, such as the creation of new synapses and the pruning of existing ones. Another is the long-lasting activity-dependent modification of the strength of synapses. The third mechanism concerns changes in the intrinsic excitability of neurons (Malenka and Bear, 2004; Malenka, 2011). Since synaptic plasticity can convert drug-induced signal, such as dopamine release, into long-term alterations in neural function, it is considered a good candidate mechanism for the persistence of addiction-related behaviors.

The best characterized cellular mechanisms of plasticity are long-term potentiation (LTP) and long-term depression (LTD). LTP and LTD describe long-lasting changes in the efficiency of synaptic transmission that occur in response to repeated stimulation. LTP and LTD involve changes in signaling pathway as well as in the synthesis and localization of proteins affecting spine maturation and stability. Both LTP and LTD have been linked to

many forms of experience-dependent plasticity such as learning and memory (Martin and Shapiro, 2000; Malenka and Bear, 2004). There is now good evidence that LTP and LTD occur in DA neurons and the target of their projections, suggesting their implication in drug-induced alteration of neural circuits (Hyman and Malenka, 2001).

Administration of NMDAR antagonists into the VTA prevents the development of sensitization to psychostimulants (Vanderschuren and Kalivas, 2000). Because NMDARs are critically involved in triggering major forms of LTP and LTD (Malenka and Bear, 2004), these findings suggested that addicted drugs might trigger synaptic plasticity in the VTA. Subsequently, it was shown that a single *in vivo* cocaine exposure was enough to cause synaptic adaptations at excitatory synapses on VTA neurons (Ungless *et al.*, 2001) (**Fig. 13**). Twenty four hours after cocaine injection the AMPA/NMDA ratio was significantly increased in VTA slices suggesting a greater synaptic strength in the cocaine-treated animals. This effect, persisted for 5 to 10 days, and was dependent of NMDAR activation and appeared to be selective to DA neurons (Ungless *et al.*, 2001). This robust yet transient cocaine-evoked plasticity could initiate the neural processes leading to addiction (Vanderschuren and Kalivas, 2000; Everitt and Wolf, 2002).

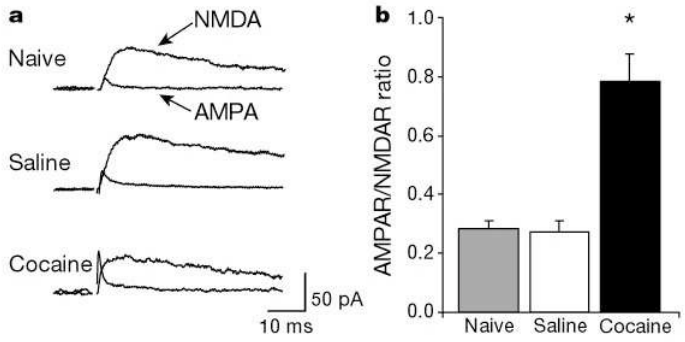

Figure 13: A single exposure to cocaine induced an increase in the AMPAR/NMDAR ratio of glutamatergic synaptic currents in VTA dopamine cells.

a, Sample EPSCs in neurons from naive animals or animals pretreated with cocaine or saline. **b**, Peak AMPAR- and NMDAR-mediated EPSCs expressed as a ratio. Single cocaine injection ($n = 6$) results in a significantly increased AMPAR/NMDAR ratio compared with saline-injected ($n = 6$; $P < 0.05$) or naive animals ($n = 5$; $P < 0.05$) (Ungless *et al.*, 2001).

A possible mechanism underlying the drug-induced formation of LTP is the phosphorylation of transcription factor CREB (cAMP response element binding protein), which is induced by numerous drugs of abuse in the VTA (Shaw-Lutchman *et al.*, 2002; Walters *et al.*, 2003). The activation of CREB leads to increased expression of the GluR1 AMPAR subunit in the VTA (Olson *et al.*, 2005), which may contribute to the observed LTP. The functional output of the LTP induced in DA neurons is still elusive but tends toward a role in enhancing the rewarding properties of drugs of abuse (Malinow and Malenka, 2002; Harris and Aston-Jones, 2003; Dong *et al.*, 2004).

In the NAc, plasticity has also been demonstrated even though much less work has been performed on drug effects in this area. The response to cocaine (chronic) in MSNs can induced a decrease of synaptic strength (Thomas *et al.*, 2001; Kourrich *et al.*, 2007) or an increased, depending on the exact paradigm (Kourrich *et al.*, 2007). More recently, a functional consequence of the cocaine-induced potentiation at glutamatergic synapses in NAc has been proposed (Pascoli *et al.*, 2012). The authors established a causal link between cocaine-evoked plasticity and locomotor sensitization. They showed that cocaine-induced ERK dependent plasticity, selectively in the NAc D1 MSNs, was required for locomotor sensitization to cocaine and that depotentiation of cortical Nac inputs by optogenetic stimulation *in vivo* abolished the cocaine-induced locomotor sensitization. Hence, the D1 MSNs synaptic potentiation may be a mechanism underlying a core component of addiction (Pascoli *et al.*, 2012).

3.2. STRUCTURAL PLASTICITY

The synaptic plasticity mechanisms are accompanied by structural changes in striatal neurons. Two general types of structural plasticity have been observed: changes in the size of cell bodies (Sklair-Tavron *et al.*, 1996) and changes in dendritic arborization or spine morphology (Robinson and Kolb, 2004).

First, numerous studies reported that cocaine and other drugs of abuse produce persistent changes in the structure of dendrites and dendritic spines on medium spiny neurons in the NAc and in the PFC (Norrholm *et al.*, 2003; Robinson and Kolb, 2004; Lee *et al.*, 2006; Li *et al.*, 2012). Dendritic spines in the NAc are the primary sites of excitatory synapses from glutamatergic inputs. These spines also receive dopaminergic inputs from the VTA (Hyman *et al.*, 2006). Therefore, altering the density of NAc dendritic spines could have important effects on the modulation of information processed from upstream limbic structures and is very likely to play a crucial role in addiction. However, the correlation between these neuroadaptations and addiction-related behavior such as sensitization to cocaine is still a matter of debate.

Cocaine regulates many cytoskeleton regulatory genes. For example, cocaine decreases Homer1 and PSD95, scaffolding proteins associated with the postsynaptic cytoskeleton (Yao *et al.*, 2004; Szumlinski *et al.*, 2006) and reduces the activity of RhoA (Kim *et al.*, 2009b) and Rac1 (Dietz *et al.*, 2012), two small GTPases regulating actin filaments. Repression of Rac1 has been shown to be responsible for the expansion of dendritic spines in NAc neurons and for enhanced cocaine reward (Dietz *et al.*, 2012). This highlights the importance of actin dynamics in cocaine-induced structural plasticity.

A key transcriptional regulator of enhanced spinogenesis is ΔFosB. ΔFoB mediates the structural plasticity that is induced in NAc by cocaine through the regulation of genes related to dendritic spine architecture such as synaptogamin6, microtubule-associated protein 2 (McClung and Nestler, 2003), activity-regulated cytoskeleton-associated protein (ARC, (Renthal *et al.*, 2009); and Cdk5 (Bibb *et al.*, 2001) It is both necessary and sufficient for cocaine-induced changes in dendritic spine density (Maze *et al.*, 2010) . Local inhibition of Cdk5 prevents cocaine-induced spine proliferation in NAc (Norrholm *et al.*, 2003) (**Fig. 14**). This could be mediated through Cdk5-dependent inhibition of MEF2, which in turn increases dendritic spines in NAc MSNs (Pulipparacharuvil *et al.*, 2008). Additionally, increased MEF2 activity in the NAc enhances sensitized behavioral responses to cocaine and implicate MEF2 as a key regulator of structural synapse plasticity and sensitized responses to cocaine (Pulipparacharuvil *et al.*, 2008). There is also evidence that WAVE1, a cytoskeleton-associated protein, regulates spine morphogenesis in a Cdk5-dependent manner (Kim *et al.*, 2006; Sung *et al.*, 2008). Interestingly, WAVE1 has a putative MEF binding site in its proximal promoter regions. Thus, induction of Cdk5 by chronic cocaine via ΔFosB could result in regulation of WAVE1 activity, whereas MEF2 can regulated its expression level to mediate longer-term changes involved in addiction.

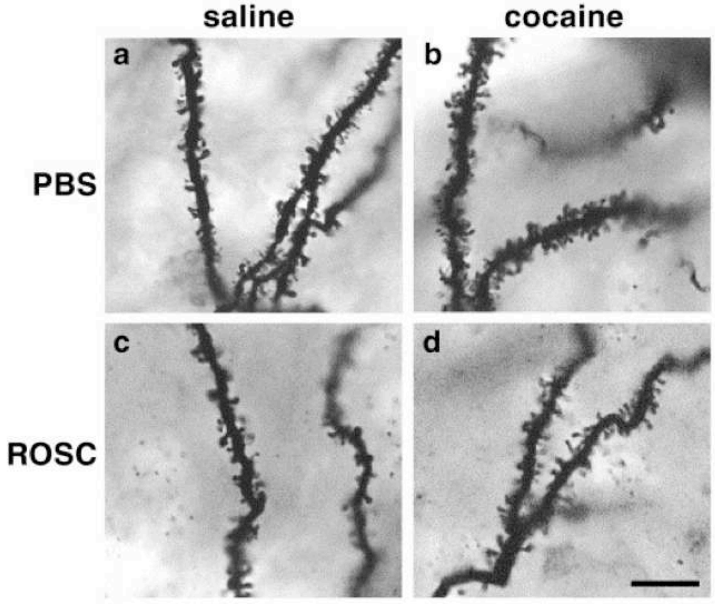

Figure 14: Effect of cocaine and Cdk5 inhibitor on spines formation.

Representative laser confocal photomicrographs of dendritic processes from NAc shell of rats administered a 4-week regimen of daily cocaine or saline injections while receiving intra-accumbens infusion of Cdk5 inhibitor roscovitine (ROSC) or PBS. Panels a–d illustrate dendritic segments from NAc shell after treatment with (a) saline + PBS, (b) cocaine + PBS, (c) saline + ROSC, or (d) cocaine + ROSC. Scale bar=10 μm. This shows that repeated cocaine administration increase dendritic spine density on medium spiny neurons (panel a-b) and that this increased is blocked by Cdk5 inhibitor (panel c-d), suggesting that it is a Cdk5-dependent mechansim (Norrholm *et al.*, 2003).

Finally, specific cell types in which these molecular signaling pathways occur may add another level of regulation of structural plasticity. Although repeated treatments with cocaine induce dendritic spines in both D1 and D2 MSNs, the long-term stability of new spines differs between the two cell types. It appears that newly formed spines are more persistent in D1 (Lee *et al.*, 2006) supporting the concept that intracellular signaling pathways downstream of D1R can mediate longer-term stabilization of spines than in D2. Indeed, cocaine-induction of ΔFosB occurs in D1 neurons (Hope *et al.*, 1994; Nestler, 2008) correlating with the persistent increased of spines in this cell type.

It will be particularly interesting to determine how the specific molecular changes occurring in the two cell types lead to different changes in neuronal structure in response to cocaine and how these changes affect addictive behaviors.

3.3. MOLECULAR PLASTICITY

The persistence of addiction-related behaviors (e.g. tendency to relapse) suggests that they may be mediated, in part, through changes in gene expression (Nestler *et al.*, 1993). Repeated exposures to drug of abuse perturb transmission at particular synapses in the brain possibly via intracellular cascades to the nucleus, where they first initiate and then maintain changes in the expression of specific genes. Induction and regulation of transcription factors as well as chromatin remodeling represent important mechanisms through which signal transduction pathways influence gene expression. In this section, we summarize some of the major transcriptional mechanisms that are thought to underlie neuronal and behavioral plasticity.

3.3.1. TRANSCRIPTION FACTORS ACTIVATED BY COCAINE

3.3.1.1. CREB

CREB is a member of the leucine-zipper transcription factors family, constitutively bound to a specific sequence of DNA called CRE (cAMP-response-element) that is present in the promoter region of many genes including *c-fos*, *dynorphin*, and *enkephalin* (Shaywitz and Greenberg, 1999). CREB is activated by phosphorylation of its Ser133 residue and this event is necessary to induce CREB interaction with its co-activators CREB binding protein (CBP) and p300 (Lundblad *et al.*, 1995). Both co-activators have an intrinsic histone acetyl-transferase activity. The acetylation of histones allows the decompaction of chromatin promoting the transcriptional activity of CREB and the recruitment of RNA polymerase II that triggers mRNA synthesis (Kwok *et al.*, 1994; Bannister and Kouzarides, 1996). CREB is phosphorylated by PKA, CaMK II/IV, p90 ribosomal S6 kinases 1/2 (RSK1/2), mitogen- and stress-activated kinases 1/2 (MSK1/2), and PKC (Johannessen and Moens, 2007).

CREB is phosphorylated and activated in several reward-related regions by acute and chronic cocaine (Walters *et al.*, 2003; Carlezon *et al.*, 2005). The induction of CREB activity appears to become greater and more persistent with repeated drug exposures. Virally mediated overexpression of CREB in the NAc decreases sensitivity to the rewarding effects of cocaine, whereas reduction in CREB activity - via overexpression of a negative-mutant form of CREB in the NAc - has the opposite effects (Carlezon *et al.*, 1998; Barrot *et al.*, 2002). The ability of CREB to decrease reward is mediated by the induction of the expression of dynorphin peptide (**Fig. 15**) (Cole *et al.*, 1995). Dynorphin acts on k opiod receptors in VTA neurons to decrease dopamine release (Spanagel *et al.*, 1992) and is associated with

dysphoria (Hyman and Malenka, 2001). Thus, persistent activation of CREB and the resulting induction of dynorphin, may therefore serve as a negative feedback signal to regulate excessive DA release resulting from frequent cocaine consumption. In addition, a recent study showed that mice with a genetic deletion of CREB in the medium-spiny neurons have an increase sensibility to psychostimulant (Madsen *et al.*, 2012). These results suggest that CREB has a negative effect on cocaine addiction-related behaviors, although the role of CREB is certainly more complex since a recent study demonstrated that CREB activity increases the motivation for cocaine during active self-administration (Larson *et al.*, 2011).

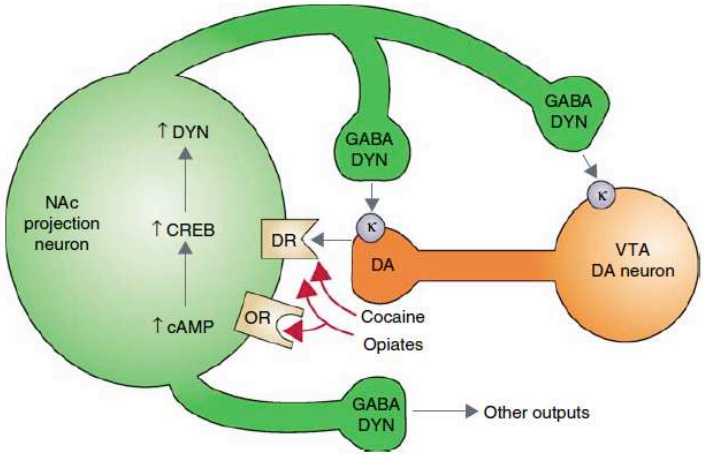

Figure 15 : Feedback between the NAc and VTA via CREB activation.

Cocaine has been shown to activate prodynorphin gene expression in the NAc and dorsal striatum via D1R stimulation, the cyclic AMP pathway, and the phosphorylation of CREB. The resulting dynorphin peptides are transported to presynaptic terminals including terminals found on recurrent collateral axons that feedback on dopaminergic neurons. Dynorphin peptides are agonists at inhibitory k opiate receptors, resulting in decreased dopamine release. This mechanism may contribute to emotional and motivational aspects of drug withdrawal. (McClung and Nestler, 2008).

3.3.1.2. ELK-1

Elk-1 is a transcription factor belonging to the TCFs (ternary complex factors) family, proposed to be important for the long-term effects exerted by ERK activation in striatal neurons (Vanhoutte *et al.*, 1999; Valjent *et al.*, 2000; Valjent *et al.*, 2001). Elk-1 is phosphorylated by ERK at Ser383/389, two residues crucial for the transcriptional activity of Elk-1 (Janknecht *et al.*, 1993). This phosphorylation occurs in the cytoplasm and upon ERK activation Elk-1 translocates to the nucleus (Lavaur *et al.*, 2007). This translocation has been observed as well upon cocaine *in vivo* or glutamate in cell culture (Lavaur *et al.*, 2007). Once phosphorylated, Elk-1 can interact with two SRF proteins (serum response factors) forming a TCF with intrinsic binding activity towards SRE (serum response element) sites (Yang *et al.*, 1999). These SRE sites are located in the promoters of many

genes including *zif268*, *c-fos*, *junB*, and *nur77* (Treisman, 1990). Specific inhibition of Elk-1 phosphorylation, by a cell-penetrating peptide, altered the cocaine-induced regulation of these genes (Besnard *et al.*, 2011). In a chronic cocaine administration paradigm, inhibition of Elk-1 phosphorylation reversed cocaine-induced increase in dendritic spine density and delayed the establishment of cocaine-induced psychomotor sensitization and conditioned-place preference (Besnard *et al.*, 2011). Hence, Elk-1 phosphorylation downstream from ERK may be a key molecular event involved in long-term neuronal and behavioral adaptations to cocaine.

3.3.2. TRANSCRIPTION FACTORS INDUCED BY COCAINE

Drug-induced gene expression has been shown to occur in successive "waves". Many activated genes that are rapidly and transiently induced are the so-called immediate-early genes (IEGs). The transcription of these particular genes is induced within minutes and does not require protein synthesis. IEGs usually, but not always, encode transcription factors such as *c-fos*, *junB*, *c-jun*, *Fra-1*, *Fra-2* and *zif268*. They reach a peak of expression within 1 hour and return to basal level by 2 hours after cocaine injection. In the striatum, induction of IEGs occurs preferentially in D1 MSNs (Bertran-Gonzalez *et al.*, 2008) although depending on environment and time frame factors they have been detected in the D2 MSNs as well (Lee *et al.*, 2006). We review in this section the implication of ΔFosB and Zif268 in the cocaine-induced plasticity.

3.3.2.1. ΔFOSB

Addictive drugs are known to induce members of the Fos family of transcription factors *c-fos*, *fosB*, *Fra1* and *Fra2* (Graybiel *et al.*, 1990; Hope *et al.*, 1992) in the dorsal and ventral striatum mainly. Fos family proteins form heterodimers with Jun family proteins (c-Jun, JunB, JunD) that bind to activator protein-1 (AP-1) sites present within the promoters of certain genes to regulate their transcription. Fos proteins are encoded by immediate early genes, which show very rapid, yet transient, induction in response to diverse type of stimuli (cf next section). Maximal induction of Fos proteins occurs within 1-2 h after drug administration and returns to normal levels within 8-12 h. Although all members of the Fos family proteins are induced by acute cocaine exposure, their expression is attenuated upon repeated drug treatment (tolerance), except for ΔFosB, a truncated form of FosB. Biochemically modified isoforms of ΔFosB (molecular mass 35-37 kDa) accumulates for at least several weeks after the drug treatment ceases (**Fig. 16**) (Hope *et al.*, 1994; Nye *et al.*,

1995) because of its extraordinary long half-life (Chen *et al.*, 1997; Carle *et al.*, 2007) and which is even further enhanced when it is phosphorylated on Ser27 (Ulery-Reynolds *et al.*, 2009). ΔFosB accumulation seems to occur preferentially in the D1 MSN subtype (Nye *et al.*, 1995; Muller and Unterwald, 2005; Lee *et al.*, 2006). ΔFosB stability provides a molecular mechanism by which drug-induced changes in gene expression can persist despite long periods of drug withdrawal (Nestler, 2001).

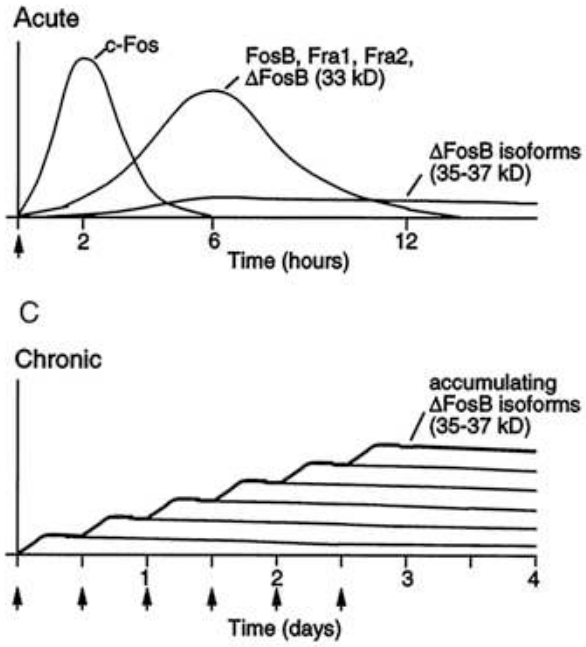

Figure 16: The gradual accumulation of ΔFosB in response to drugs of abuse.

Top panel: Several waves of Fos-like proteins [comprised of c-Fos, FosB, ΔFosB, and Fra1 or Fra2] are induced in the Nac and dorsal striatal neurons by acute administration of a drug of abuse. Also induced are biochemically modified isoforms of ΔFosB; they, too, are induced (although at low levels) after acute drug administration, but persist in brain for long periods because of their stability. Bottom panel: With repeated drug administration, each acute stimulus induces a low level of the stable ΔFosB isoforms, which is indicated by the lower set of overlapping lines that indicate ΔFosB induced by each acute stimulus. The result is a gradual increase in the total levels of ΔFosB with repeated stimuli during a course of chronic treatment, which is indicated by the increasing stepped line in the graph.

ΔFosB plays a substantial role in regulating drug-related phenotypes including locomotor sensitivity, reward, and cocaine self-administration. ΔFosB overexpression specifically in D1 MSNs of the striatum increased locomotor sensitivity to cocaine and the rewarding properties of cocaine (Kelz *et al.*, 1999) or self-administration at low doses of the drug (Colby *et al.*, 2003).

ΔFosB controls the expression of several genes involved in addiction-like behaviors in animal models. For instance, ΔFosB regulates proteins that are important for glutamatergic transmission, synaptic function, and plasticity, including AMPA receptor subunits GluR2 (Kelz *et al.*, 1999) and Ca^{2+} calmodulin-dependent kinase II (CamKII) (McClung and Nestler, 2003).

Although ΔFosB presence in the brain is long-lasting, it is not permanent and cannot explain entirely why drug relapse occurs years after abstinence. One possibility is that ΔFosB may function as a "molecular switch" that helps initiate and maintain over mid-term periods certain aspect of the addicted state. ΔFosB effect could then prolonged by more persistent changes in neuronal functions such as, for example, an increased in spines density, elevation of the GluR1 glutamate receptor subunit, or long-lasting chromatin alterations of the chromatin.

3.3.2.2. OTHERS

Increasing evidence links several other transcription factors to drug exposure. For example, Zif268 is another transcription factor induced by cocaine under the control of ERK and has been implicated in the lon-lasting effect of the drug (Valjent *et al.*, 2006a). The nuclear factor-NF-kB (NFKB) is induced in the NAc by chronic cocaine exposure (Russo *et al.*, 2009) and has been linked to synaptic plasticity and memory (Meffert *et al.*, 2003). Moreover, the brain glucocorticoid receptor plays an important role in mediating the reinforcing effects of cocaine (Deroche-Gamonet *et al.*, 2003) and is required in dopamine receptor D1-expressing neurons to facilitate cocaine seeking (Ambroggi *et al.*, 2009). Others include early growth response factors (EGRs; (Freeman *et al.*, 2008) and signal transducers and activators of transcription (STATs) (Berhow *et al.*, 1996).

In conclusion, since the acute actions of drugs of abuse dissipate as the drug leaves the brain, it cannot explain alone the development of addictive behaviors. Addiction and vulnerability to relapse are likely to result from both synaptic and molecular alterations the repeated drug experience leaves in the brain. We have seen that a large body of literature indicates that modification of the pattern of neural activity in the brain may underlie behavioral adaptations in addiction. Alteration in gene expression may also play a complementary role by modifying the responses to drug exposure. It is interesting to note that, like the locomotor sensitization and drug-seeking behaviors associated with addiction (Neisewander *et al.*, 1996; Grimm *et al.*, 2001), changes in protein content often become

greater with increasing periods of withdrawal (Swanson *et al.*, 2001; Grimm *et al.*, 2003; Bowers *et al.*, 2004). However, the time points of these studies are relatively short-term as effects are analyzed within 3 weeks of withdrawal. Data are lacking to know precisely whether addiction-related genes are expressed months or years after drug exposure, and if any, what their contributions to relapse are. One model has been proposed by Kalivas and Volkow, 2005 (**Fig. 17**), suggesting that the cellular adaptations induced by drug exposure occur in a chronological sequence and match the development of addiction.

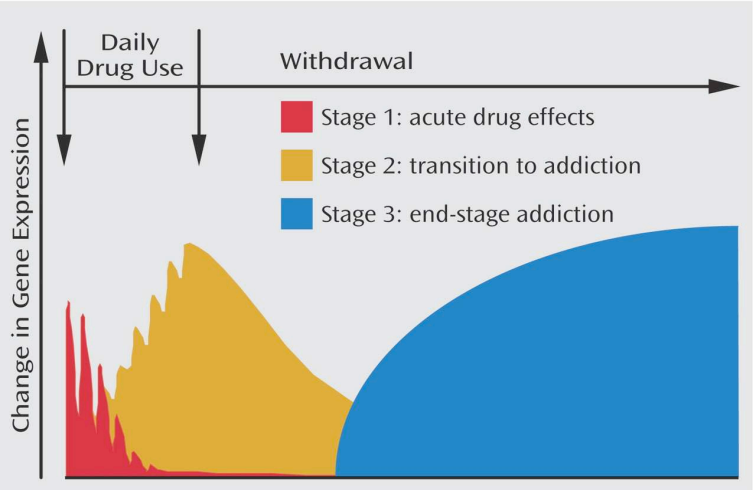

Figure 17 : The three stages of addiction by Kalivas and Volkow

Acute drug effects occur within minutes-hours after exposure. Neuroadaptations mediating the transition from recreational drug use to addiction endure for a finite period after discontinuation of repeated drug administration and initiate the changes in protein expression and function that emerge during withdrawal and underlie the behavioral characteristics of end-stage addiction, such as craving, relapse, and reduced ability to suppress drug seeking.

3.4. CHROMATIN REMODELING

Signaling in the nucleus not only controls the activity of transcription factors that mediate gene expression, but also changes chromatin structure thereby modifying the transcriptional rate of specific genes. Histone tail modification, DNA methylation among others mechanisms mediate the DA response to the nucleus. We will review these mechanisms in the chapter IV.

CHAPTER IV

EPIGENETICS

1 OVERVIEW

More than half a century ago, Conrad H. Waddington, a British developmental biologist, described the idea of an « epigenetic landscape » to represent the process of cellular decision-making and to explain how the same genotype could give rise to phenotypic variation during development (Waddington, 1957). Waddington's hypotheses were made before the discovery of the DNA code. Today an epigenetic trait refers to stable phenotype, heritable through generations or at least cell division, resulting from changes in a chromosome without alterations in the DNA sequence. For example, even though the vast majority of cells in a multicellular organism share an identical genotype, the development generates a diversity of cell types with diverse, yet stable, profiles of gene expression and distinct cellular functions. Thus, cellular differentiation may be considered as an epigenetic phenomenon. It is becoming clear that many of the same processes of gene regulation that are involved in the normal differentiation are also engaged in the adult organism to mediate cellular adaptation to environmental stimuli. Within the central nervous system, post-mitotic neurons are fully differentiated but nonetheless, deploy a surprising level of plasticity through epigenetic modifications to translate external stimuli into functional and morphological changes that produce behavioral responses.

After a presentation of the major molecular actors involved in epigenetic regulation, we review their implication in learning and memory and cocaine addiction mechanisms.

2. GENERAL ORGANIZATION OF THE CHROMATIN

2.1 NUCLEOSOMES AND THE 30-NM FIBER

Eukaryotic cells contain from 10^7-10^{11} DNA base pairs (bp) in a nucleus just a few μm in diameter. The DNA molecules comprising the human genome, span about 2 meters in length

and require a physical organization and compaction to fit in the nucleus. This vast quantity of DNA is packaged by the histone proteins into a hierarchical structure called chromatin and is compacted over 10,000-fold (reviewed in Kornberg and Lorch, 1999). The nucleosome is the fundamental repeating unit of chromatin. It consists of 146 bp of superhelical DNA wound around an histone octamer, formed by pairs of each of the core histone proteins H2A, H2B, H3 and H4 (Kornberg, 1974) **(Fig. 18A**). This level is also referred as to the "beads-on- a-string" because of its appearance under the electron microscope (Bram and Ris, 1971; Olins and Olins, 1974). The structure of the nucleosome core particle has been solved by X-ray crystallography at high resolution (Luger *et al.*, 1997; Harp *et al.*, 2000) (**Fig. 18B**). All four histone proteins share a highly similar structural motif, the histone fold, comprising three alpha helices connected by two loops. They are basic proteins and so provide a good platform to package negatively charged DNA. The packaging of DNA into nucleosomes shortens the fiber length about seven fold. Structurally, histones can be divided into the core domain, which makes up approximately 75% of the protein and is composed of histone fold motifs that physically interact with themselves to form the H2A/2B and H3/4 heterodimers, and the flexible tail domain, which makes up the remaining 25% of the protein.

This structure can be further wound into the “30-nm fiber”, also referred as to the higher-order structure, that consist of nucleosome arrays in their most compact form (Finch and Klug, 1976). Adjacent nucleosomes are joined by a stretch of free DNA termed "linker DNA" and stabilized by the histone linker H1 (Satchwell and Travers, 1989). The spacing between two nucleosomes varies during differentiation along with development, and is associated with changes in gene transcription system (Sperling and Weiss, 1980).

The hierarchy continues with increasing DNA packing density until the metaphase chromosome is ultimately attained (Belmont *et al.*, 1999; Strukov *et al.*, 2003).

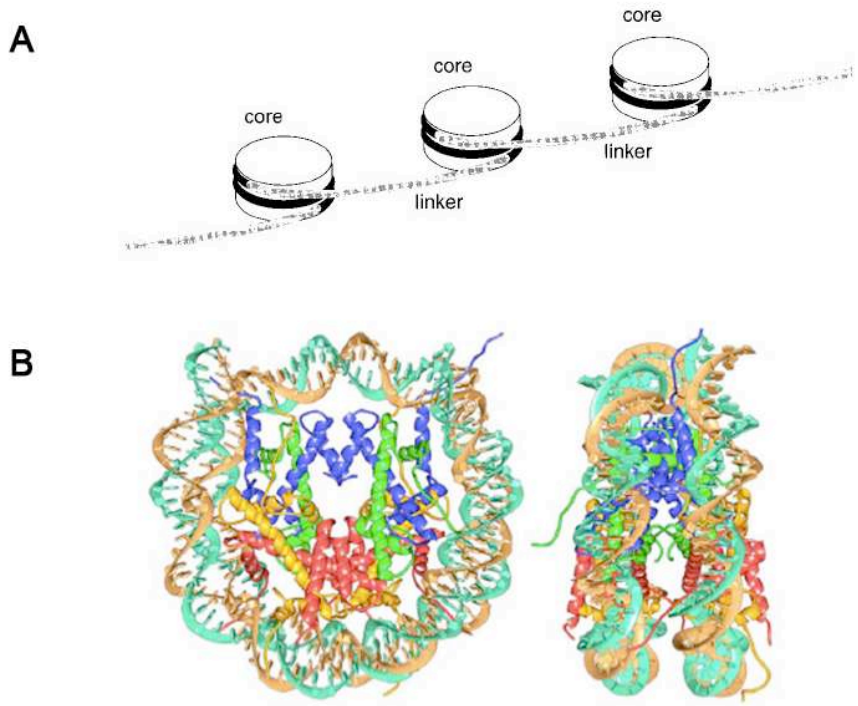

Figure 18 : The nucleosome core particule

A. Schematic of nucleosome core particule and linker. The histone octamer is represented as a disk, and the DNA as a ribbon, with shading to distinguish core DNA (dark) from linker DNA(light) (Kornberg and Lorch, 1999).**B.** Crystal structure of nucleosome core particule, front and side views. Histone are represented as ribbons (H3 blue. H4 green, H2A yellow, H2B red). The DNA superhelix is shown in brown and turquoise. Adapted from Luger *et al*., 1997.

2.2 EUCHROMATIN AND HETEROCHROMATIN

In a simplistic view, the higher order of chromatin structure has two structurally and functionally distinguishable domains: euchromatin and heterochromatin (**Fig. 19**). Euchromatin consists largely of coding sequences which only account for a small fraction (less than 4%) of the genome in mammals. It refers to regions of decondensed chromatin, making it "poised" for gene expression, although not necessarily transcriptionally active. Conversely, heterochromatin refers to highly condensed, gene-poor, and transcriptionally silent regions such as centromeric and telomeric regions. Both chromatin regions contain particular histones modifications and binding proteins, though there is a high degre of overlap between them.

Euchromatin is typically enriched in acetylated histones H3 and H4 and methylated H3K4 (Noma *et al.*, 2001). Hallmark features are hypoacetylation of histones, enrichment in

meK_9H3, $meK_{27}H3$ and $me_3K_{20}H4$ (Rea *et al.*, 2000; Jenuwein and Allis, 2001; Schotta *et al.*, 2004), association of heterochromatin protein-1 (HP1) (Bannister *et al.*, 2001), and DNA cytosine methylation (5mC) in organisms showing this modification (Suzuki and Bird, 2008). Heterochromatin can be further differentiated between constitutive heterochromatin, that contains permanently repressed genes located in centromeres and telomeres, from facultative heterochromatin that comprises genes that are differentially expressed through development and /or differentiation and can be silenced (Trojer and Reinberg, 2007).

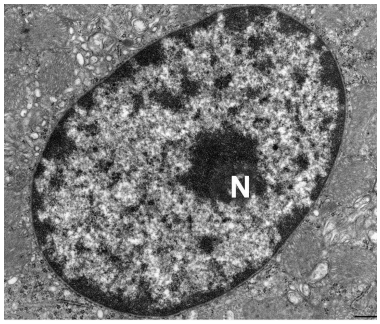

Figure 19: Heterochromatin in a nucleus of parietal cell (stomach, rat).

Obtained by conventional transmission electron microscopy. Heterochromatin appears dark (N: nucleolus) (Fedorova and Zink, 2008).

Local chromatin architecture is now generally recognized as an important factor in the regulation of gene expression. However, such condensation of DNA provides a considerable obstacle to the proteins driving transcription, DNA replication and repair. Hence, the dynamics of chromatin structure is critical to allow remodeling and localized decondensation that facilitates the progress of the nuclear machinery. During activation of gene transcription, this inaccessible DNA is made available to DNA binding proteins via modification of the nucleosome. As we will detail in the next chapter, the architecture of chromatin is strongly influenced by post-translational modifications of the histones, histone variants or chromatin remodeling complexes that work together to alter the chromatin fibers and to regulate gene transcription (Cheung *et al.*, 2000a; Strahl and Allis, 2000).

3. CHROMATIN MODIFICATIONS

In eukaryotic cells, changes in chromatin structure are achieved by distinct mechanisms including histone modifications, DNA methylation, non-coding RNAs, ATP-dependent chromatin remodeling, and histone variants incorporation.

3.1. MODIFICATIONS OF HISTONES

The unstructured histone N-terminal tails that protrude from the nucleosome core particle are accessible to various enzymes and are subject to an array of post-translational modifications, although modifications in the histone-fold domain have also been found (van Leeuwen and Gottschling, 2002; Fuchs *et al.*, 2006). More than 70 different sites for histone PTMs and eight types of histone PTMs have been reported, largely from the extensive application of mass spectrometry and antibody-based detection techniques, as well as from metabolic-labeling studies (Berger, 2007; Kouzarides, 2007; Ren *et al.*, 2007). They include so far, phosphorylation, acetylation, methylation, that we will review here and ubiquitination, sumoylation, ADP ribosylation, glycosylation and S-nitrosylation **(Fig. 20)**. These covalent modifications are controlled by proteins termed "the tools" of the epigenome (Borrelli *et al.*, 2008). The "writers" modify specific histone loci by adding PTMs that are reversed by enzyme with opposing enzymatic activities "the erasers". Another class of proteins, "the readers", translate these modifications into the appropriate cellular responses, as we will see in the section 4.2.

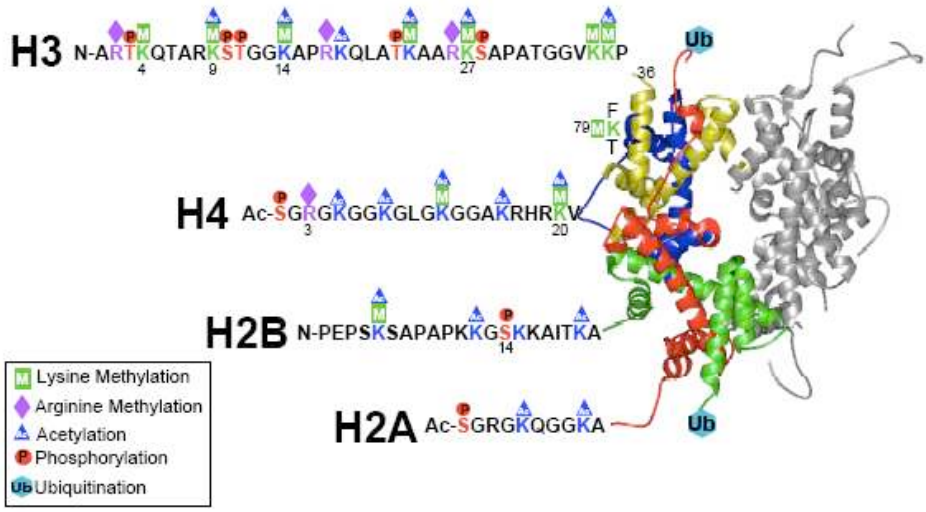

Figure 20: Histone post-translational modifications

The sequences of amino-terminal tails of human H3, H4, H2B and H2A are aligned with each modification coded as indicated. The label corresponds to the amino acid position. Note that lysine residues can be mono-, di-, tri-methylated and arginine residues can be mono- and di-methylated. For clarity, these different methylation events are collectively represented as a single methyl mark for each of the residues. Ac, acetylation; M, methylation; P, phosphorylation, Ub, ubiquitination (courtesy of Dr. Scott Briggs).

3.1.1. HISTONE ACETYLATION

Histone acetylation occurs at the ε-amino groups of evolutionarily conserved lysine residues located at the N-termini. All core histones are acetylated *in vivo*; modifications of histones H3 and H4 are, however, much more extensively characterized than those of H2A and H2B. The acetylation of core histones is probably the best understood type of modification. Important positions for acetylation are K9 and K14 on histone H3, and K5, K8, K12 and K16 on histone H4 (Kouzarides, 2007) (**Fig. 20**). Steady-state levels of acetylation of the core histones result from the balance between the opposing activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs) (Kouzarides, 2007) (**Fig. 21**).

HATs catalyze the addition of acetyl groups onto the ε-amino group of lysine residues, and HDACs catalyze their removal (Carmen *et al.*, 1996; Johnson and Turner, 1999; Sterner and Berger, 2000). The HATs are grouped into three main families; GNAT, MYST and CBP/p300 and their target lysine are generally well described (Roth *et al.*, 2001; Kouzarides, 2007). HDACs have been classified into four different classes based on the sequence homology to yeast histone deacetylases (de Ruijter *et al.*, 2003; Gallinari *et al.*, 2007). Phylogenetically, class I, II and IV HDACs are closely related and share Zn-catalyzed hydrolysis of acetyl-lysine amide bond in common. Conversely, class III NAD^+-dependent HDACs, so called sirtuins, are not part of the classical HDAC family as they are similar to yeast SIR2 and mechanistically distinct. They have been implicated in the regulation of lifespan and metabolism (Michan and Sinclair, 2007) and more recently have been identified as targets for cocaine action in the NAc (Renthal *et al.*, 2009). There is currently one class IV HDAC, HDAC11, and it has characteristics of both Class I and Class II enzymes (Yang and Seto, 2008). In general, HDACs have relatively low substrate specificity by themselves, a single enzyme being capable of deacetylating multiples sites within histones. Their specificity depends on interaction with transcription factors that target them to specific chromosomal loci. They inhibit transcription through two distinct features, their intrinsic deacetylase activity and their interaction with co-repressors. For example, class II HDACs function by associating with the myocyte enhancer factor 2 (MEF2) family of transcription factors to repress MEF2 dependent transcription (Lu *et al.*, 2000).

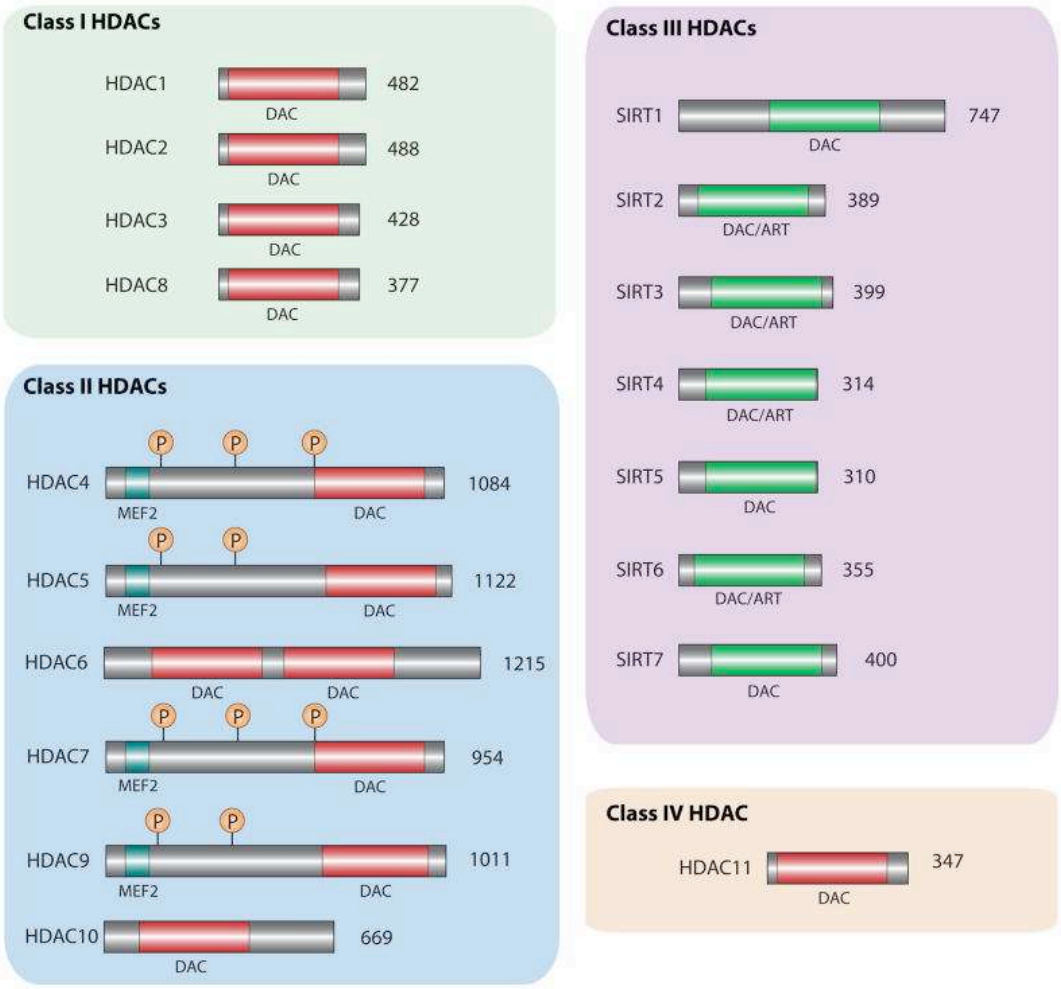

Figure 21 : Histone deacetylases (HDACs)

HDACs are divided into four major classes, Class I-IV, based on structural homology to yeast proteins. Class I and II use zinc as a cofactor, and have similar catalytic domains (DAC). Class II HDACs are distinguished from Class I by their large N-terminal regulatory region, which recognizes transcription factors (e.g. MEF2) and are phosphorylated to control subcellular localization. The Class IV HDAC, HDAC11, is structurally similar to both Class I and Class II but has not been well studied. The catalytic deacetylase domain on Class III HDACs requires NAD+ as a cofactor and, in addition to deacetylating proteins, this domain has also been reported to have ADP ribosyltransferase activity (ART) (Renthal and Nestler, 2009).

HDACs activity is tightly controlled through several mechanisms, most importantly by post-translational modifications, subcellular localization, and complex formation.

(i) Post-translational modifications

All mammalian HDACs are subjected to diverse post-translational modifications that regulate their activity. Among them, phosphorylation is the best studied. It has been reported that phosphorylation of class I HDACs promotes their deacetylase activity and their ability to form complexes with other partners. HDAC1, HDAC2 and HDAC3 are phosphorylated *in vivo* by casein kinase II (CKII) principally (Pflum *et al.*, 2001; Sun *et al.*, 2007) and dephosphorylated *in vitro* by protein phosphatase 1 (PP1) and λ-phosphatase (Galasinski *et al.*, 2002) or PP4c in case of HDAC3 (Zhang *et al.*, 2005). HDAC1, and to a lesser extent HDAC2, were found in complex with PP1 and the transcription factor CREB. HDAC1 and PP1 cooperate to deacetylate histones and to dephosphorylate CREB, thereby silencing CREB-driven transcription in cAMP unstimulated cells or in later times after stimulation, contributing to signal attenuation (Canettieri *et al.*, 2003). In brain cells, PP1-HDAC1 containing complexes are central actors in the modulation of the epigenetic histone code at the promoters of genes implied in memory formation (Koshibu *et al.*, 2009). Finally, the role of HDAC8 in the brain, if any, has not been documented. Concerning class II HDACs, their phosphorylation seem to regulate their function by controlling their subcellular localization as described below.

(ii) Subcellular localization

The nuclear localization of HDACs occurs via a nuclear localization signal or via co-localization together with other proteins/HDACs. Most HDACs are nuclear although some can be cytosolic, such as HDAC6, depending on the presence of other regulatory domains. HDAC1 and HDAC2 are exclusively found in the nucleus, due to the lack of a nuclear export signal (NES) (Johnstone, 2002). HDAC3, however, has both a nuclear import signal and a NES, suggesting that HDAC3 can also localize to the cytoplasm. Class II HDACs are much larger enzymes, containing an N-terminal regulatory domain that enables them to shuttle in and out of the nucleus in response to certain cellular signals. The shuttling of HDACs 4, 5 and 7 between the cytosol and the nucleus has been studied extensively in differentiating muscle cells (Fischle *et al.*, 2001; McKinsey *et al.*, 2001) but can be also controlled by synaptic activity in neurons (Chawla *et al.*, 2003)**.** Their nuclear localization is promoted by the binding of MEF2. By contrast, the export is initiated when the class IIa HDACs is phosphorylated on multiple serine residues, which serve as docking domains for the 14–3-3 family of chaperone proteins. Interaction with 14-3-3 induces a conformational change in HDAC, which masks the NLS from importin, which is adjacent to the 14-3-3 binding site, and exposes the NES to CRM1, leading to nuclear export and derepression of HDAC target genes (McKinsey *et al.*, 2000a; Fischle *et al.*, 2002; Verdin *et al.*, 2003). This mechanism is calcium-dependent, but is also sensitive to perturbed energy balance and oxidative stress. To

date, five families of serine/threonine kinases families have been implicated in this mechanism including the calcium-calmodulin-dependent protein kinases CaMKI and IV (McKinsey *et al.*, 2000b), the AMP-activated protein kinase (AMPK; McGee *et al.*, 2008), and the AMPK related kinases, salt inducible kinase (SIK1; Takemori *et al.*, 2009), Mark2 (Chang *et al.*, 2005) and the protein kinase D (PKD; Vega *et al.*, 2004). Conversely, two phosphatases have been identified, myosin phosphatase-targeting subunit-1 (MYPT1)/protein phosphatase (PP)1β (Parra *et al.*, 2007) and PP2A (Illi *et al.*, 2008).

(iii) Ability to form complexes with regulatory proteins

HDACs recruitment and specificity is further complicated by the fact the enzymes are typically present in multiple distinct complexes, often with other HDAC members. For instance, HDAC1 and HDAC2 are present in at least 3 multi-protein complexes of the mSin3, the nucleosome remodeling deacetylase (NuRD) and the corepressor of REST (repressor element 1 silencing transcription factor (CoREST) complexes (Zhang *et al.*, 1999). Furthermore, the class IIa HDACs themselves do not possess intrinsic HDAC activity, but instead recruit a larger repressive complex that contains HDAC3 for this purpose (Fischle *et al.*, 2002).

3.1.2. HISTONE METHYLATION

Histone methylation mainly occurs on the side chain of lysine and arginine residues. Unlike acetylation and phosphorylation, methylation does not alter the charge of the histone protein (see also section 4.1). The histone lysine methyl mark is deposited by the lysine methyltransferase enzymes (HKMT) from S-adenosylmethionine (SAM) to the specific lysine ε-amino group. They are highly specific when compared with the acetyl transferases (Zhang and Reinberg, 2001). In addition, methylation shows further complexity in that lysine molecules may be mono, di or tri methylated with the enzymes themselves showing specificity not only for the residue, but also for the level of methylation. For example, the methyltransferases, G9a/GLP bind me_1K_9H3 and me_2K_9H3 via ankyrin repeat domains (Collins and Cheng, 2010), which contain a hydrophobic cage present in methyl-lysine binding modules of diverse folds. This cage binds $me_{1/2}K_9H3$ with approximately equal affinity, but it is too narrow to accommodate me_3K9H3, which is independently regulated by SUV39H1.

Arginine methylation is performed by two classes of arginine methyltransferase, the type-I and type-II enzymes belonging the PRMTs family. Both types catalyze the formation of

monomethyl-arginine but differ in the dimethyl-arginine products: type I PRMTs form asymmetric dimethyl-arginine; and type II form symmetric dimethyl-arginin. They transfer a methyl group from SAM to the ω-guanino group of arginine within a variety of substrates. With respect to histone arginine methylation, the most relevant enzymes are PRMT1, 4,5 and 6 (Bedford and Clarke, 2009).

Unlike acetylation, early studies measuring the turnover of methylated histones suggested that histone methylation was stable and irreversible since the half-life of methylated histones was very similar to the half-life of the histones themselves (Byvoet *et al.*, 1972). The first breakthrough came with the identification of lysine-specific demethylase 1 (LSD1, Shci *et al.*, 2004). LSD1 in complex with the Co-REST repressor catalyzes the removal of methyl groups from nucleosomal $me_{2/3}K_4H3$. Furthermore, the precise complex association determines which lysine is to be demethylated by LSD1. When LSD1 is complexed with the androgen receptor, it changes its substrate to me_2K_9H3. Hence, it can either act as a repressor by mediating demethylation of meK_4H3, a specific tag for epigenetic transcriptional activation, or as a coactivator by mediating demethylation of me_3K_9H3, a specific mark for epigenetic transcriptional repression (Klose and Zhang, 2007). Unlike the lysine methyltransferases, the demethylation of lysines seems less specific and is subject to controversy (Tan *et al.*, 2008). The demethylation of arginine is still elusive. However, the conversion of arginine to citrulline via a deimination reaction was discovered as a way of reversing arginine methylation (Cuthbert *et al.*, 2004; Wang *et al.*, 2004). Although this pathway is not a direct reversal of methylation, this mechanism reversed the dogma that methylation on this amino acid was irreversible. More recently, the jumonji protein JMJD6 was shown to be capable of performing a demethylation reaction on histones H3R2 and H4R3 (Chang *et al.*, 2007). However, these findings have yet to be recapitulated by other independent researchers.

3.1.3. HISTONE PHOSPHORYLATION

Phosphorylation at serine and threonine residues of histones has been shown to occur on all the core histones as well as on linker histone H1 and H2A variant, H2A.X, *in vitro* and/or *in vivo*. Among histone phosphorylation, $pS_{10}H3$ has been extensively studied. Interestingly, this modification is involved in both transcription and cell division, two events requiring opposite alterations in the degree of chromatin compaction. $pS_{10}H3$ was first noted as a distinctive marker for dividing cells since the highly condensed, metaphase chromosomes are heavily phosphorylated at this site in mammalian cells and in all organisms analyzed thus far (Gurley *et al.*, 1973; Paulson and Taylor, 1982; Hsu *et al.*, 2000). However, other studies found that

phosphorylation of histone pS10H3 could also occur during interphase, affecting only a small fraction of nucleosome (Barratt *et al.*, 1994), and that pS10H3 was linked with the induction of immediate-early gene expression (Mahadevan *et al.*, 1991). These studies revealed a strong temporal match between $pS_{10}H3$ and IEG induction as the strength and duration of the phosphorylation correlated closely with IEG activation such as *c-fos*/*c-jun*. In addition, Chromatin immuneprecipitation experiments confirmed the enrichment of this histone mark in the promoters of *fos* and *jun* (Chadee *et al.*, 1999). These concomitant events are called the nucleosomal response and have been shown to be mediated by the MAPK signaling cascade (Mahadevan *et al.*, 1991).

In neurons, $pS_{10}H3$ is important for synaptic activity-induced chromatin remodeling (Brami-Cherrier *et al.*, 2007). It has been shown to be induced in various neurons type including hippocampal neurons in an activity-dependent manner (Chwang *et al.*, 2007); (Chandramohan *et al.*, 2008) and in the medium-spiny neurons after stimulation of dopamine receptor DrD1 and DrD2 (Bertran-Gonzalez *et al.*, 2008). Interestingly, histone H3 phosphorylation induced by synaptic activity is associated with a change in nuclear structure (Wittmann *et al.*, 2009), which might suggest that nuclear geometry and transcriptional response are functionally linked. In mammals, this phosphorylation event is mediated by the mitogen-activated stress-kinases 1 and 2 (MSK1/2) (Soloaga *et al.*, 2003), and inhibited by PP1. In striatal neurons, Stipanovich *et al*. (2008) identified DARPP-32, a PP1 inhibitor, as a key player in the nucleosomal response following drug-induced nuclear translocation (Stipanovich *et al.*, 2008). The ensuing nucleosome response and IEG induction are thought to be important in long-term effects of drugs of abuse and in physiological reward-controlled learning.

3.2. METHYLATION OF DNA

An additional important chromatin mark is DNA methylation, traditionally viewed as a highly stable epigenetic mark in post-mitotic cells. DNA methylation occurs at the 5 position of cytosine nucleotides primarily when a cytosine (C) occurs next to guanine (G) in the so called CpG islands. These CG-rich regions overlap with the promoter of 50-60% of human genes and are known as molecular switches that can turn-off or turn-on the expression of the downstream gene. Thus, DNA methylation play a critical role in regulating gene transcription and is involved embryogenesis and differentiation (Goll and Bestor, 2005), X-inactivation (Heard *et al.*, 1997) and imprinting (Li *et al.*, 1993). In mammalian cells, two classes of DNA methyltransferases catalyze DNA methylation: DNA methyltransferase-1 (DNMT1), essential for maintaining DNA methylation patterns in proliferating cells, and DNMT3A and

DNMT3B, required for de novo methylation (Newell-Price *et al.*, 2000; Kim *et al.*, 2009a). A variant of DNA methylation, 5-hydroxycytosine methylation, (5-hydroxymethylcytosines or 5hmC), also seems to be important in gene regulation but has not yet been investigated in addiction models (Kriaucionis and Heintz, 2009; Pastor *et al.*, 2011). Conversely, the process of demethylation of DNA is less well understood no DNA demethylase have been identified so far. A recent study though, identified an active demethylating machinery in mammalian cells targeting 5-hmC-containing DNA (Guo *et al.*, 2011). These authors propose the conversion of 5-methylcytosine to 5-hydroxycytosine methylation, by the hydroxylase TET1, as a key initiating step for DNA demethylation, through an oxidation-deamination mechanism.

The addition of a methyl group to CpG sites, in the promoter regulatory regions of many genes, displaces the binding of transcription factors and attracts methyl-binding proteins that instigate chromatin compaction of DNA and gene silencing. Such complexes involve several DNA methyl-binding domain protein (MBDs), which are required for normal cell growth and development (Kim *et al.*, 2009a). The prototypical example of an MBD is methyl cpG binding protein 2 (MeCP2), which is mutated in the neurodevelopmental disorder Rett syndrome (Kriaucionis and Bird, 2003; Chahrour and Zoghbi, 2007) and dramatically affects synaptic plasticity in the hippocampus and memory formation (Amir *et al.*, 1999; Moretti *et al.*, 2006; Chao *et al.*, 2007).

The combination of specific enzymes and epigenetic modifications influence chromatin state. In simplified terms, chromatin exist in two states: condensed and transcriptionally inert or open and transcriptionally active (**Fig. 22**).

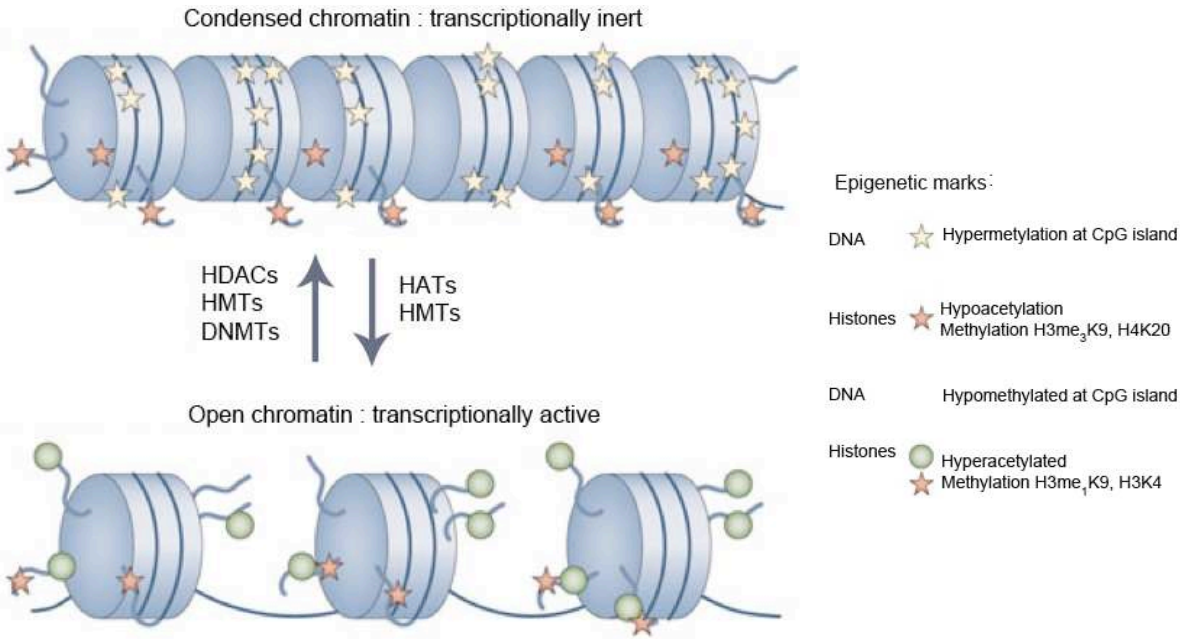

Figure 22: Specific enzymes and epigenetic modifications are characteristic of the chromatin states.

Transcriptionally inactive chromatin consists of a condensed, methylated DNA coiled around histone octamers that are deacetylated and methylated at particular lysine residues. Conversely, expanded chromatin is characterized by nonmethylated DNA loosely coiled around acetylated nucleosome complexes. This structure makes gene promoters accessible to the transcriptional apparatus. Abbreviations: DNMTs, DNA methyltransferases; HATs, histone acetyl transferases; HDACs, histone deacetylases; HMTs, histone methyltransferases. Adapted from Perry *et al*., 2010.

3.3. ADDITIONAL FACTORS MODIFYING THE CHROMATIN

They are additional processes involved in creating the highly diverse and specialized epigenome landscape such as microRNAs, the activation of ATP-dependent chromatin remodeling factors and the incorporation of histone variants.

Like histone modifications and DNA methylation, non-coding RNAs can alter the transcriptional potential of a gene without changing the DNA sequence. They include microRNAs (miRNAs), small RNA molecules, 22-nucleotide long, that control negatively their target gene expression posttranscriptionally. A single miRNA can target and bind hundreds of mRNAs, repressing their translation and sequestering them for storage or degradation. They are expressed in a cell type- and maturational stage-specific manner

(Schratt, 2009). In the central nervous system, miRNA pathways create a powerful mechanism to dynamically adjust the protein content of neuronal compartments without the need for new gene transcription.

Chromatin remodeling refers to dynamic structural changes that involve the disruption and reformation of DNA–histone complexes. As a result of this process, nucleosomes "slide" along the DNA molecule, facilitating the access of nuclear transcriptional complexes to previously inaccessible DNA. The activation of ATP-dependent chromatin remodeling factors is associated with both transcriptional activation and repression (Bowman, 2010).

As for histone variants, they have a potential to change the nature of the nucleosome through structural changes and charge related differences. They allow the cell to increase the post-translational modification profile. With the exception of H4, all core histones have variant counterparts such as H3.3, macroH2A, H2A.Z, H2A.X (Hake and Allis, 2006). Histone variants play important roles in controlling chromosomal architecture and their deregulation is expected to be linked to cancer (Kusch and Workman, 2007), although their physiological implication in the brain is poorly understood.

4. MODE OF ACTION OF CHROMATIN MODIFICATIONS

Histone modifications exert their effects via three main mechanisms. The first is a direct perturbation through the alteration of the overall charge and structure of chromatin. The second is an indirect effect and involves the positive or negative regulation of the binding of effector molecules. Finally, the chromatin can serve as signaling platform by integrating both phenomenons.

4.1. DIRECT STRUCTURAL PERTURBATION

The N-terminal histone tails appear to mediate chromatin condensation through multiple mechanisms, one of which involves simple DNA charge. Most of the information available comes from studies involving “tail-less” nucleosome arrays that have the major portions of the core histone tails removed by selective proteolysis with trypsin. These trypsinized nucleosomes are only able to form a moderate folded conformation and cannot oligomerize (Garcia-Ramirez *et al.*, 1992; Fletcher and Hansen, 1995; Schwarz *et al.*, 1996). It is widely assumed that histone acetylation and phosphorylation which negate the positive charge of histones decrease the attraction between the negatively charged DNA and the nucleosome. A possible explanation is that changes in charges in the histone tails alter protein-protein

interaction, for example internucleosomal tail-tail interactions and/or binding of the tails to exposed protein domains on the surface of other nearby nucleosomes. These events could play a role in inducing a different arrangement of the tails opening up the nucleosmes (Luger and Richmond, 1998; Hansen, 2002) thereby facilitating DNA access to protein machineries such as those involved in transcription. Evidence for such charge-specific effects has been displayed in artificially reconstituted nucleosome arrays (Tse *et al.*, 1998). The incorporation of acetylated histones prevented the formation of the 30-nm like fibres. In addition, recent evidence suggests that histone acetylation may alter the structure of histone tails by increasing their alpha-helical content (Wang *et al.*, 2000). However, the precise mechanism by which histone tails, protruding from the nucleosome, can modify chromatin compaction remains undefined. In contrast, methylation does not alter the overall charge of the histone tails, nonetheless, increasing methyl addition (mono, di or tri) does augment the Lewis basicity and hydrophobicity of lysine residues. Apart from the electrostatic component, protein protein interactions, for example internucleosomal tail-tail interactions and/or binding of the tails to exposed protein domains on the surface of other nearby nucleosomes, may also play a role in regulating chromatin structure.

4.2. REGULATION OF THE BINDING OF CHROMATIN MODULES

Histone modification can also indirectly affect chromatin structure by serving as marks for the recruitment and activity of protein complexes. These multivalent proteins have particular domains within them that can bind to the specifically modified histone, and engage gene repression or activation activities (Clapier and Cairns, 2009). For instance, bromodomains recognize acetylated motif and the 14-3-3 protein family binds to phosphohistone. Notably, there are numerous modules recognizing lysine methylation such as the chromodomain, tudor, MBT, part of the so-called Tudor family **(Fig. 23)**. An example of specific acetylated lysine binding is provided by bromodomains, which are often found in HATs and chromatin-remodeling complexes or in the general transcription factor TAF250 (Mujtaba *et al.*, 2007). The ATP dependent-remodeling complex SWI/SNF (SWItch/Sucrose NonFermentable) contains a bromodomain that targets it to acetylated histones and allows it to "open" the chromatin at specific locations and thus plays an important function in gene activation (Hassan *et al.*, 2002). On the other hand, histone PTMs can also disrupt the binding of some transcriptional modulators to their target histone motifs. For instance, $H3me_3K_4$, a permissive mark, prevents the transcriptional repressor NuRD complex from binding to the H3 N-terminal tail (Nishioka *et al.*, 2002; Zegerman *et al.*, 2002).

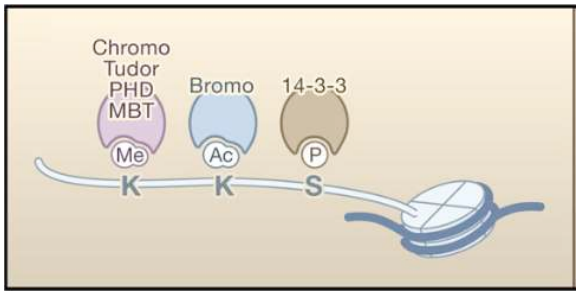

Figure 23: Recruitment of chromatin modules.

Domains used for the recognition of methylated lysines, acetylated lysines, or phosphorylated serines (Kouzarides, 2007).

4.3. HISTONE MODIFICATION CROSS-TALK

Mounting evidence suggests that different histone modifications can influence each other on several levels. This coordination, also referred as cross-talk, occurs either between histone modifications themselves or between histones and other epigenetic regulator.

(i) The first level of the complexity of histone modification cross-talk originates from the modular organization of chromatin itself (Fischle *et al.*, 2003). Distinct modifications of a particular site can have different readouts with various biological functions. Particularly, lysine and arginine residues can undergo several post-translational modifications. They can be acetylated, mono-ubiquitinated, or mono- or di-, and tri- methylated (**Fig. 24A**). Obviously, different marks on the same site cannot co-exist, and therefore, they exclude each other. For example, it was shown that di-methylation of H3-K4 occurs at both inactive and active euchromatic genes, whereas tri-methylation is present exclusively at active genes pericentric heterochromatin (Santos-Rosa *et al.*, 2002).

Therefore, the cross-talk choices of single histone residues and the enzyme system that modifies them provide one level of complexity that impact on gene activity and physiological processes.

(ii) The second level of complexity comes from the combination of marks on a single histone tail since the specificity of the enzymes targeting them can be influenced by preexisting modifications **(Fig. 24B).** For example, on histone H3, which has the highest density of post-translational modifications, methylation of H3-K9 inhibits *in vitro* acetylation of K14, K18 and K23 by HATs, such as p300, and methylation of H3 on K4 by HMTs (Wang *et al.*, 2001a). Interestingly, methylation of K9 and H3-S10 phosphorylation are reciprocally interfering between each other (Rea *et al.*, 2000). In mammalian cells, $pS_{10}H3$ inhibits methylation of K9 by the HMT SUV39H1, and conversely, meK_9H3 strongly reduces phosphorylation at Ser10 by the kinase Aurora. $pS_{10}H3$ and $acK_{14}H3$ seem to be

interconnected as well but the order in which they take place is not clear. Indeed, Real *et al*. (2000), showed that the Ipl1/aurora kinase phosphorylates H3-S10 more efficiently with a preexisting acK14 (Rea *et al.*, 2000) whereas, Cheung *et al*. (2000), published that $pS_{10}H3$ provides a docking site for the HAT Gcn5 promoting acetylation on K14 (Cheung *et al.*, 2000b). In neuronal cells, a strong acetylation on K14 and a low phosphorylation of S10 is observed in basal conditions (Brami-Cherrier *et al.*, 2005), which suggests that acK14H3 precedes H3-S10 phosphorylation induced by acute psychostimulant.

Furthermore, the communication between marks can occur between different histone tails and affect one or more nucleosomes **(Figure 24C).** A good illustration is provided by the ubiqitination of histone $H2BK_{123}$ that is required for methylation of $H3K_4$ and $H3K_{79}$, which are both involved in transcriptional silencing (Ng *et al.*, 2002; Sun and Allis, 2002; Shahbazian *et al.*, 2005).

(iii) Finally, there may be cooperation between modifications in order to efficiently recruit binding modules or as seen in the previous paragraph.

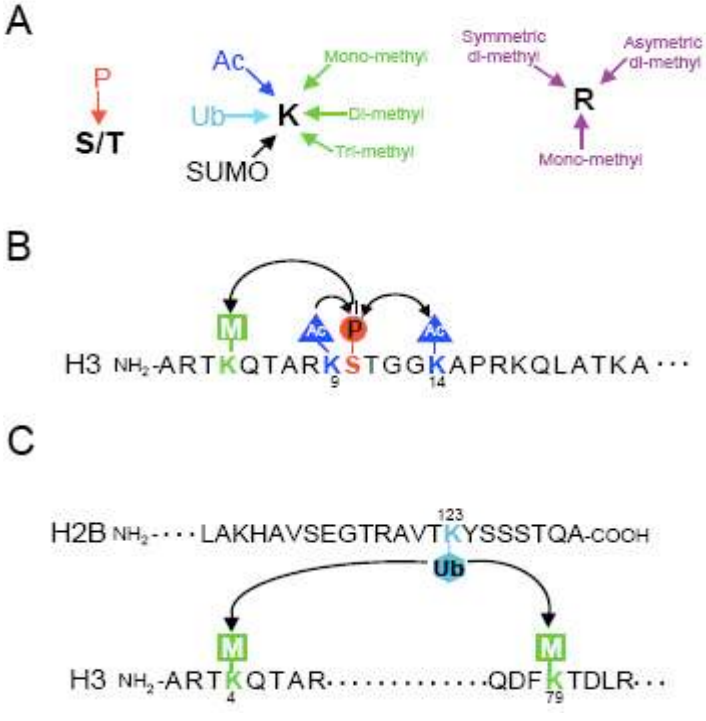

Figure 24 : Histone cross-talk

A. Potential "choices" of the modification status of different histone residues. Whereas serine (S) and threonine (T) residues are phospho-acceptor (P) sites, lysine (K) and arginine (R) residues have multiple choices of post-translational modification possibilities. Lysine residues can be acetylated (Ac), monoubquitinated (Ub), sumolated (Su), or (mono-, di-, tri-) methylated. Arginine might be mono- or di-methylated (symmetrical or asymmetrical). **B.** "Cross-talk" at the level of a single histone H3 amino-terminal tail. pS10H3 promotes acetylation on K14 following epidermal factor treatment. H3K9 and H3K14 acetylation stimulate pS10H3 during transcription. **C.** Interhistone "cross-talk" between H2B and H3. In yeast, only H2B is known to be ubiquilated. This modification is necessary for methylation of H3 on K4 and K79. Adapted from Fischler *et al.,* 2003.

Thus, in contrast to the straight flow of most signal transduction cascades, where the modification of one protein impacts directly on downstream effectors, signaling to and from chromatin appears to be far more complex.

Since histone proteins can be modified at a number of sites, specific modifications could work together as a code which would ultimately dictate whether a specific gene is transcribed. This idea was introduced as the "histone code hypothesis" by Allis and colleagues (Strahl and Allis, 2000; Jenuwein and Allis, 2001), where the code serves an epigenetic indexing system extending the capacity of the genome to store, inherit, and release information in normal and pathological development. More recently, Campos and Reinberg (2009) supported this hypothesis, suggesting that certain combinations of modifications will lead to transcriptional activation, whereas others would lead to transcriptional repression. Indeed, analysis of histone modifications across the human genome using ChIP-Seq (chromatin immunoprecipitation sequencing) has demonstrated that a specific combination of 17 modifications tended to co-occur at the level of the individual nucleosome and was associated with increased gene expression (Wang *et al.*, 2008b).

5 INTERPLAY OF SPATIAL GENOME ORGANIZATION AND GENE EXPRESSION

Together with the classical epigenetic mechanisms reviewed above, the spatial organization of the genome and its dynamics is implicated in the modulation of gene expression (see Schneider and Grosschedl, 2007 for review).

Chromosome territories are non-randomly arranged within the nucleus and are compartmentalized into functional nuclear domains, associated with regulation of transcriptional activity and posttranscriptional RNA processing. An interesting example is the concept of transcription factories defined by RNA polymerase II-enriched nuclear foci (Cook, 1995; Faro-Trindade and Cook, 2006) (**Fig. 25**). It has been shown that widely separated active genes, located on decondensed chromatin loops, are recruited and transcribed by the same RNA polymerase II present in the so-called transcription factories (Osborne *et al.*, 2004). Genes moving in these sites would be transcribed whereas transcription from gene moving out would stop (Osborne *et al.*, 2004). Also, transcriptional activation of highly expressed genes such as immediate early genes would relocalize to these preassembled transcription sites (Osborne *et al.*, 2007). Although many details of the transcription factory model are still unverified, it gives a compelling diverging view of the classical dogma of active genes recruiting a mobile transcription machinery.

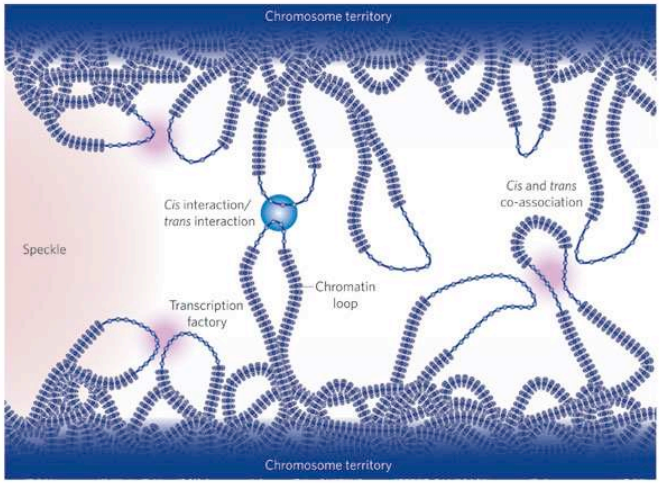

Figure 25: Transcription factories

Active genes on decondensed chromatin loops that extend outside chromosome territories can colocalize both *in cis* and *in trans* at sites in the nucleus with local concentrations of Pol II (namely transcription factories; dark pink) and adjacent to splicing-factor-enriched speckles (pale pink). Interactions can also occur between regulatory elements and/or gene loci and lead to coregulation in *trans* (blue circle) (Fraser and Bickmore, 2007).

An additional example of the interplay between gene spatial organization and transcription is provided by studies correlating the transcriptional state of some genes and their location near the nuclear periphery. In mammals, chromosomal regions with low gene-density are located close to the nuclear periphery (Croft *et al.*, 1999; Sadoni *et al.*, 1999), a region generally associated with gene silencing (Deniaud and Bickmore, 2009). Particularly, some studies show that the position within the nucleus influences the expression of some, but not all genes (Chuang *et al.*, 2006; Levsky *et al.*, 2007). This implies that the relocation of genes to the nuclear periphery could be a form a epigenetic mechanism and modulate the expression of subset of genes.

More specifically, lamins, components of the nuclear envelope, are also likely to play a role in the regulation gene expression. Direct evidences are provided by defects in the epigenetic regulation of chromatin and alterations in chromosome positioning found in various types of laminopathies (Parnaik, 2008).

6 PHARMACOLOGY OF CHROMATIN MODIFICATIONS

In the field of neuroepigenetics, much effort is in progress to develop selective, brain-penetrant, small-molecules probes of chromatin modifying enzymes (see Cole, 2008 for review). Here, we focus on the compounds identified from screens or by design that can modulate the activity of classical histone deacetylases as well as histone and DNA methyltransferases.

HDACs have become attractive therapeutic agents for the treatment of cancers and some disorders of the central nervous system (Kalin *et al.*, 2009). Using HDAC inhibitors

(HDACi), it is possible to manipulate the global acetylation state of the nucleosome core histones, thereby affecting transcriptional activation, even though it has been shown that this spread hyperacetylation induces transcription on a restricted subset of genes (Fass *et al.*, 2003; Vecsey *et al.*, 2007). As illustrated in figure 21, HDACs fall in 4 classes. The classical HDACi act exclusively on class I and class II HDACs by binding to their zinc-containing catalytic domain. Once HDACi are bound to an enzyme's catalytic core, deacetylase activity is blocked, histones become hyperacetylated, and target genes activated through chromatin remodeling They are four major types of HDACi: (1) carboxylic acids (e.g. butyrate, valproate), (2) hydroxamic acids (e.g. TSA (trichostatin A) and SAHA (suberoylanilide hydroxamic acid)), (3) ortho-aminoanilines (e.g., MS-275), and 4) natural products (e.g., trapoxin, FK228). Among these, the most widely studied are sodium butyrate, phenylbutyrate, trichostatin A, and SAHA. The butyrates are known to cross the blood–brain barrier (Carey and La Thangue, 2006).

These inhibitors have been explored in the context of animal models of neurodegenerative disorders such as Huntington's disease (HD) (Ferrante *et al.*, 2003), psychiatric disorder such as depression (Tsankova *et al.*, 2006), or more broadly on learning and memory (Levenson *et al.*, 2004; McQuown *et al.*, 2011)

There are also histone methyl transferase (HMT) inhibitors, expected to activate gene expression and to act synergistically with both HDAC and DNA methyltransferase inhibitors. The H3K9 methyltransferases, SU(VAR)-3– and G9a, are inhibited specifically by Chaetocin, a competitve inhibitor versus the co-substrate SAM (Greiner *et al.*, 2005), and BIX-01294 (diazepin-quinazolin-amine derivative) (Kubicek *et al.*, 2007) respectively.

In addition to HDAC and HMT inhibitors, DNA-demethylating agents, such as 5-aza-deoxycytidine (5-Aza) (Sorm *et al.*, 1964) could potentially be used to restore deficit in 5′-cytosine methylation observed on particular gene promoters in schizophrenia patients (Huang and Akbarian, 2007). 5-Aza demethylates DNA either passively, by incorporation into DNA as a modified nucleosides that subsequently interfere with DNA, or by inhibiting DNA methyltransferases (DNMTs). Besides, the inhibitor zebularine (Yoo *et al.*, 2004) is commonly used in animal model of learning and memory (Lubin *et al.*, 2008) and cocaine addiction (Anier *et al.*, 2010).

7. EPIGENETIC MECHANISMS IN ADDICTION

Epigenetic research indicates that regulation of gene activity is critically important for normal functioning of the genome. The epigenetic mechanisms described in the previous sections are

an integral part of a multitude of brain functions that range from the development of the nervous system over basic neuronal functions to higher order cognitive processes. They are essential for promoting neural cellular diversity and neural network connectivity and plasticity. In this context, numerous preclinical studies link perturbations in these processes to the molecular pathophysiology of CNS diseases (Tremolizzo *et al.*, 2002; Kumar *et al.*, 2005; Tsankova *et al.*, 2006; Pandey *et al.*, 2008).

Drug addiction has long been considered and interpreted as a disorder of learning and memory (Berke and Hyman, 2000; Kelley, 2004; Hyman, 2005). Hence, the neurobiological adaptations associated with these long-lasting behaviors are likely to be narrowly related.

In this section, we first review some evidences of epigenetic mechanisms mediating learning and memory before focusing on the one contributing to cocaine addiction.

7.1 EPIGENETIC MECHANISMS IN LEARNING AND MEMORY

Long-term memories require stable gene expression changes (Kandel, 2001). Hence, epigenetic mechanisms remodeling the chromatin structure at genes important for learning and memory are likely candidates to guarantee such enduring changes. Modifications of both DNA and histone proteins are associated with memory formation and consolidation.

Experiments testing spatial, emotional and contextual memory induce changes in histone acetylation, methylation, and phosphorylation in different brain regions. For instance, contextual fear conditioning a hippocampus-dependent form of memory, coincides with an hyperacetylation at multiple sites on H3 and H4 tails ($H3K_9$, $H3K_{14}$, $H4K_5$, $H4K_8$, and $H4K_{12}$) (Peleg *et al.*, 2010), as well as an increase in $H3K_9$ dimethylation, $H3K_4$ trimethylation, $H3S_{10}$ phosphorylation, and $H3S_{10}/H3K_{14}$ phosphoacetylation in the hippocampus (Chwang *et al.*, 2006; Gupta *et al.*, 2010). Interestingly, experimental manipulation of histone PTMs through the inhibition of their modifying- enzyme can alter memory formation. Examples are provided by the administration of HDACi in the hippocampus (Levenson *et al.*, 2004) or the deletion of the specific HDAC2 isoforms in neurons (Guan *et al.*, 2009) that enhanced memory formation and LTP. Likewise, selective inhibition of nuclear PP1 in forebrain neurons, resulted in improved long-term memory (Koshibu *et al.*, 2009).

Importantly, it appears that a specific combination of histone modifications is necessary to produces learning-related gene expression. Peleg *et al*. (2010) showed that the co-occurrence of acetylation at $H3K_9$, $H3K_{14}$, $H4K_5$, $H4K_8$, and $H4K_{12}$ in the hippocampus following fear conditioning is associated with changes of hundreds of genes in young mice,

and required for proper learning. Aged mice failed to upregulate $H4K_{12}$ acetylation, and alteration in this single residue correlated with impaired learning and memory.

DNA methylation represents another molecular mechanism underlying learning and memory. Indeed, contextual fear conditioning is associated with increased methylation and decreased transcription of the memory suppressor gene PP1 and at the same time demethylation and transcriptional activation of the memory promoting gene reelin (Miller and Sweatt, 2007). Consistent with the idea that methylation changes are necessary for memory formation, inhibition of DNMTs within the hippocampus, produces a hypomethylated state in naive animals, resulting in impaired expression of contextual fear (Lubin *et al.*, 2008).

7.2 EPIGENETIC MECHANISMS IN COCAINE ADDICTION

In recent year, research mainly from Nestler and colleagues have described how the epigenome contributes to neural and behavioral adaptation (Renthal and Nestler, 2008) demonstrating how drug exposure leads to histone modification and expression of genes relevant for the development of drug-seeking behavior.

7.2.1. COCAINE AND HISTONE MODIFICATIONS

Repeated exposure to the psychostimulant cocaine is thought to promote alterations in histone acetylation, phosphorylation and methylation levels (summarized in **Table 1**), as well as DNA methylation levels, in the NAc. It is thus hypothesized that these modifications may be involved in mediating drug-induced behaviors (Renthal and Nestler, 2008).

7.2.1.1. COCAINE AND HISTONE ACETYLATION

Histone acetylation is strongly modified by cocaine (Borrelli *et al.*, 2008; Renthal and Nestler, 2008). First, the total cellular level of H3 and H4 acetylation is increased in the NAc after acute and chronic exposure to cocaine (Kumar *et al.*, 2005; Wood *et al.*, 2006; McQuown and Wood, 2010). Acute cocaine exposure induces an increase in H4 acetylation and H3 phosphoacetylation around the promoter of the IEG *c-fos* and *fosB* in the NAc. This modification occurs within 30 min and disappears in 3 h consistent with the induction of IEG (Kumar *et al.*, 2005; Renthal *et al.*, 2007). This effect occurs after both passive cocaine administration and under conditions of chronic self-administration. Conversely, after repeated drug exposure, *c-fos* is not induced anymore, and H4 acetylation on *c-fos* promoter remains

unchanged. However, the gene *fosB* shows only a partial desensitization after chronic cocaine (Hope *et al.*, 1992) and its promoter shows an increase in H3 acetylation but not H4 acetylation (Kumar *et al.*, 2005). In addition, chronic cocaine induces specific set of genes in the NAc such as Cdk5 and BDNF whose expression, in the case of BDNF, can last for weeks, and that may be involved in the desensitization of IEGs (Grimm *et al.*, 2003; McClung and Nestler, 2003). Consistent with such stable changes, histone H3 acetylation ($H3K_9$ and $H3K_{14}$) was observed on BDNF promoter up to 1 week after the last injection of cocaine (Kumar *et al.*, 2005). This suggests that distinct patterns of chromatin remodeling are associated with specific genes that exhibit either acute or long-lasting expression profile.

Renthal *et al*. (2009) mapped thousands of gene promoters which show a cocaine-induced increase of acetylation correlating with an increase of transcription of genes such as *cart* (cocaine- and amphetamine-regulated transcript), *per1* (Period 1), *arc* (activity regulated cytoskeletal-associated protein) etc. In addition, they observed that chronic cocaine tend to induce hyperacetylation of the vast majority of the promoters versus hypoacetylation, and only a small subset of gene promoters showed an increased acetylation of both H3 and H4 (Renthal *et al.*, 2009) (**Fig. 26**). This suggests that changes of histone acetylation are enough to induce transcription and that H3 and H4 changes are mechanistically distinct.

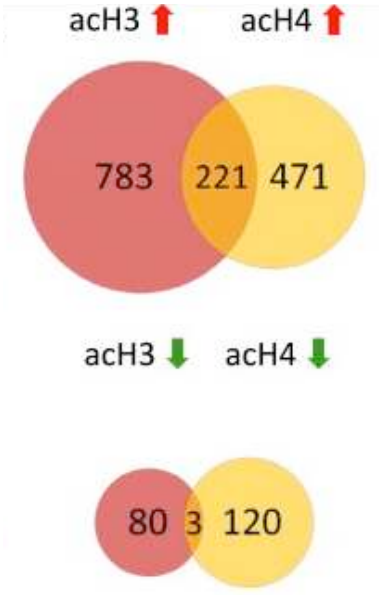

Figure 26: Regulation of H3 and H4 acetylation at gene promoters after chronic cocaine.

Venn diagrams of gene showing that cocaine induced hyperacetylation of histone H3 (K9 and K14) and histone H4 (K5, K8, K12 and K16) for the majority of the gene promoters studied in Renthal *et al*., 2009. 1254 promoters showed an hyperacetylation of H3 and H4 versus 200 that were hypoacetylated. Note that only a small fraction of regulated genes displayed increased acetylation at both H3 and H4.

In this perspective, looking at enzymes controlling acetylation could help decipher the precise signaling pathway responsible for acetylating specific region of the genome. As reviewed above, HATs catalyze the addition of acetyl groups onto histones. It has been shown

that CREB-binding protein (CBP), one of the most studied HATs, is required for the cocaine-induced H4 hyperacetylation on *fosb* promoter. Additionally, CBP-deficient mice are less sensitive to the locomotor-activating effects of cocaine. These findings suggest that CBP is important in mediating some of the cocaine-induced increases in histone acetylation and ultimately the downstream behavioral responses (Levine *et al.*, 2005).

Moreover, HDACs have also been shown to play a role in addiction-related behavior. Recent studies demonstrated that HDAC inhibitors such sodium butyrate and trichostatin A (TSA), both non-specific HDAC inhibitors, alter the behavioral responses to cocaine even though the outcome is unclear. Romieu *et al*. (2008) showed that TSA injection in the NAc preceding cocaine injection decreases the locomotor-activating effect of pyschostimulants, whereas Kumar *et al*. (2005) and Kalda *et al*. (2007) showed the opposite. Endogenous HDAC5 plays a role in regulating gene expression in response to chronic but not acute cocaine exposure. Renthal *et al*. (2007) showed that repeated injection induced phosphorylation and nuclear export of HDAC5 thus reducing histone deacetylation and increasing gene expression of the putative HDAC5 target genes such as *Nrk1/Subtance P* (Renthal *et al.*, 2007). Furthermore, after chronic cocaine, HDAC5 or HDAC4 (but not HDAC9) overexpression in the NAc reduces the rewarding effects of cocaine (Kumar *et al.*, 2005) whereas HDAC5 KO mice exhibit a hypersensitivity to reward (Renthal *et al*., 2007). Likewise, the findings of Taniguchi *et al*. (2012) support the role for HDAC5 in limiting cocaine reward behavior. However, this group showed that cocaine stimulated the nuclear import of HDAC5 in the NAc, and not its export, contradicting the previous findings on cocaine regulation of HDAC5 function in vivo (Rentlhal *et al.,* 2007). Interestingly, the authors demonstrated that the nuclear import of HDAC5 was mediated through cAMP-activated dephosphorylation by PP2A as with the cocaine-induced nuclear accumulation of DARPP-32 (Stipanovich *et al.*, 2008). These findings indicate that in the striatum negative feedback processes, such as enhanced HDAC5 nuclear levels, could attenuate the reward impact of future cocaine exposures.

Moreover, class I HDAC1 has been shown to be a relevant player in the cellular responses to psychostimulants, more precisely in the desensitization of IEGs following chronic drug exposure. After repeated amphetamine treatment, HDAC1 is recruited by the transcription factor ΔFosB to the *c-fos* gene promoter where it deacetylates surrounding histones and attenuates gene activity. Accordingly, targeted knock-out of HDAC1 in striatum abolishes amphetamine-induced desensitization of the *c-fos* gene (Renthal *et al.*, 2008).

7.2.1.2. COCAINE AND HISTONE PHOSPHORYLATION

Histone H3 phosphorylation and phospho-acetylation are considered to be marks of transcription and have also been shown to be important in the development of cocaine-induced behaviors. For example, cocaine administration rapidly induces H3 phosphorylation at serine 10 and phospho-acetylation at $pS_{10}acK_{14}H3$ in D1 MSNs (Brami-Cherrier *et al.*, 2005; Bertran-Gonzalez *et al.*, 2008) (**Fig. 27**). As mentioned earlier, MSK1, an H3 kinase, has been demonstrated to mediate this cocaine-induced increase in H3 phosphorylation (Brami-Cherrier et al., 2005). Furthermore MSK1 KO results in reduced locomotor responses to the drug, consistent with a role for histone phosphorylation events in drug-induced behavioral sensitivity. Our laboratory demonstrated that nuclear translocation of DARPP-32 could regulate these changes though the consequent inhibition of PP1 in the nucleus of the D1 expressing MSNs (Stipanovich *et al.*, 2008).

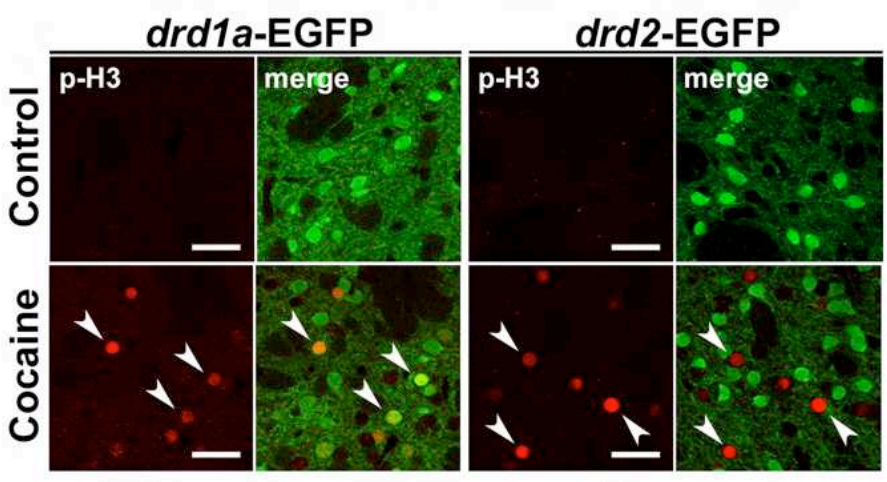

Figure 27: Acute cocaine induces histone H3 phosphorylation only in D1 MSNs

p-H3 (red) immunofluorescence and EGFP fluorescence (green) in the dorsal striatum of *drd1a*- and *drd2*-EGFP mice, 15 min after cocaine treatment. Arrowheads show p-H3 immunoreactivity in D1 MSNs (left) and excluded from D2 MSNs (right). Single confocal sections are shown. Scale bars, 40 μm (Bertran-Gonzalez *et al.*, 2008).

7.2.1.3. COCAINE AND HISTONE METHYLATION

Histone methylation is also regulated by drugs of abuse. Global levels of me_1K_9H3 and me_2K_9H3, both euchromatin marks, are reduced in the NAc after chronic cocaine exposure. Maze *et al*. (2010) identified an essential role for the lysine dimethyltransferases G9a/GLP in cocaine-induced structural and behavioral plasticity. Repeated cocaine administration reduced global levels of me_2K_9H3 in the NAc mediated through the repression of G9a and GLP.

Furthermore, NAc-specific knockdown of G9a mimicked the effects of chronic cocaine exposure, increased the dendritic spine plasticity, and enhanced drug-associated reward behaviors, whereas G9a overexpression blunted this response. G9a could thus have a crucial role in the responsiveness to cocaine.

The heterochromatic mark me_3K_9H3 displays a highly dynamic fluctuation in the NAc in response to cocaine exposure (Maze *et al.*, 2011). Acute cocaine increases me_3K_9H3 levels within 30 min with a return to basal levels in 24 h. Repeated treatment of the drug, in contrast, induced a delayed increase in me_3K_9H3 expression at 1h, followed by a decrease 24 h after the last injection, this down-regulation lasting for a week. This suggests a potential role for heterochromatin regulation in the long-term actions of cocaine. Besides, it is not known whether such events occur only within specific cells or represent more global phenomena within particular circuits.

As with acetylated histones, Renthal *et al*. (2009) performed a genome-wide chromatin changes study and showed that chronic cocaine induced an increase in H3 dimethyl-K9/K27 for the majority of the genes studied, such as protein phosphatase 2 or adenylate cyclase 3. This finding suggests that cocaine dampens the induction of many genes, despite the predominant effect of cocaine being gene activation (McClung and Nestler, 2003). Interestingly, they found that relatively few cocaine-regulated genes were associated with reductions of histone acetylation or methylation, suggesting that the most common mechanisms of cocaine-induced gene regulation in the NAc involve increases in histone acetylation for gene activation (rather than demethylation) or histone methylation for gene repression (rather than deacetylation).

The changes of histones marks induced by acute and chronic cocaine treament ar summarized below in **Table 1**.

Table 1: Summary of cocaine effect on histone PTMs

Histone	Residues	PTM	Function	EFFECT OF COCAINE acute	EFFECT OF COCAINE chronic	Ref.
PHOSPHORYLATION						
H3	H3S10	ph	permissive	↑		1
	H3S10K14	ph ac	permissive	↑(*c-fos* only)		2,3
ACETYLATION						
H3	H3K9+K14	ac	permissive	←→	↑(*FosB; Cdk5; Bdnf*...)	2,3
H4	H4K5	ac	permissive	↑		4
	H4K5+K8 +K12+K16	ac	permissive	↑(*c-fos, FosB*)	↓(*c-fos*)↑(*Cart*; *Grm3*...)	2,5,3
METHYLATION						
H3	H3K9	me1	permissive	↑		6,7
		me2	repressive	↑	↓	6
		me3	repressive	↑	↓	7
	H3K9+K27	me2	repressive		↑(*Kcnv2*...) ↓(*Ada*...)	3

LEGEND

↑ cocaine increases PTM
↓ cocaine decreases PTM
←→ no changes
↑(*c-fos*) cocaine increases PTM on *c-fos* promoter
permissive facilitates transcrition
repressive prevents transcription

Abbreviations: ac, acetylation; Ada, Adenosine deaminase ; bdnf, brain-derived neurotrophic factor; cart, cocaine and amphetamine regulated transcript; cdk5, cycline dependent kinase 5; ph, phosporylation; Grm3, metabotropic glutamate receptor 3; KcnV2, Potassium voltage-gated channel subfamily V member 2; me1/2/3, mono-, bi-,tri-methyltion, perm., permissive, repr., repressive, PTM, post translational modifications. References: 1 Bertran-Gonzales et al., 2008; 2 Kumar et al., 2005; 3 Renthal et al., 2009; 4 Brami-Cherrier et al., 2005; 5 Renthal et al., 2008; 6 Maze et al., 2010; 7 Maze et al., 2012.

7.2.2. COCAINE AND DNA METHYLATION

Accumulating evidence demonstrated that DNA methylation plays an important role in gene regulation induced by addictive drugs. Recent results have indicated that acute cocaine administration produces rapid changes in expression of DNMT isoforms within the NAc (Anier *et al.*, 2010; LaPlant *et al.*, 2010) suggesting a dynamic control of DNA methylation by drugs of abuse. Consistent with this observation, cocaine produces a hypermethylation at the promoter region of PP1c (the catalytic subunit of PP1) in the NAc, resulting in enhanced

MeCP2 binding to the PP1c promoter (Anier *et al.*, 2010). Conversely, cocaine decreases methylation at the *fosB* promoter, which coincides with the transcriptional upregulation of FosB and is consistent with the observed decrease in MeCP2 binding to *fosB* (Anier *et al.*, 2010). Importantly, systemic inhibition of DNA methyltransferase activity significantly impairs the development of locomotor sensitization induced by repeated cocaine administration (Anier *et al.*, 2010) and site-specific DNMT inhibition in the nucleus accumbens boosts the development of cocaine CPP (LaPlant *et al.*, 2010). In contrast, overexpression of the DNMT3a isoform within the nucleus accumbens disrupts cocaine CPP (LaPlant *et al.*, 2010).

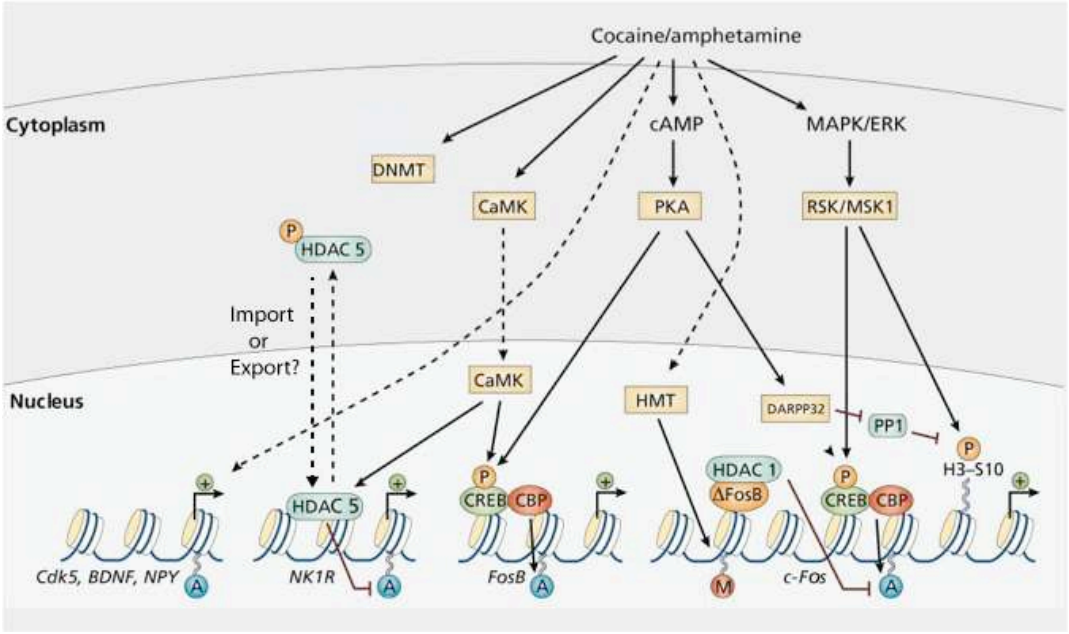

Figure 28: Regulation of chromatin structure by psychostimulant.

In the striatum, psychostimulants regulates the phosphorylation of H3-Ser10 through two distinct pathways. The first one involves MSK1 (mitogen- and stress-activated protein kinase 1) activation, through the MAPK (mitogen activated protein kinase) cascade, which phosphorylates CREB (cAMP response element-binding) and histone H3 on serine 10[1]. The second one occurs through cAMP/ PKA (protein kinase A) cascade activation that leads to DARPP-32 nuclear accumulation and the consequent inhibition of protein phosphatase-1 (PP1), which normally dephosphorylates H3-Ser10[2]. Besides, PKA phosphorylates CREB and induces its association with the histone acetyltransferase, CBP, to acetylate histones and facilitate gene activation[3]. This takes place on many genes including *fosB* and *c-fos*[3]. Chronic psychostimulant exposure upregulates DeltaFosB, recruits HDAC1 on *c-fos* promoter and repress its expression. This repression also involves increased repressive histone methylation, which is thought to occur via the induction of specific Histone methyltransferases[4]. Also, DeltaFosB activates CDK5 and BDNF expression[5]. Finally, cocaine regulation of HDAC5 is a matter of debate[6,7]. On the one hand, cocaine exposure could induce HDAC5 export consequently to its phosphorylation by CaMK and increase histone acetylation on HDAC5 target genes[6] (e.g NK1R). On the other hand, cocaine and cAMP could promote HDAC5 nuclear import through PP2A dephosphorylation. However, it seems that HDAC5 limits in both case cocaine reward[7]. Adapted from Renthal and Nestler ,2009a. References: 1 Brami-Cherrier *et al*., 2005; 2 Stipanovich *et al*., 2008; 3 Levine *et al*., 2005; 4 Renthal *et al*., 2008; 5 Kumar *et al*., 2005; 6 Renthal et al., 2007; 7 Taniguchi *et al*., 2012.

CHAPTER V

PROFILING D1 AND D2 MSNS

Ever since Santiago Ramon y Cajal discovered the structure of the neuron, scientists have attempted to classify them into discrete groups. As seen in the previous chapters, striatonigral and striatopallidal neurons are highly segregated, conferring to dopamine the ability to exert opposite functional modulation of the two pathways. With the heterogeneous striatal composition, analyzing lysates from pooled neuronal populations would average the responses and low signals could be masked or changes in opposite directions in the two populations would cancel each other. That is why it has been crucial to develop tools to identify and classify neuron families in order to study specific changes for a better and more subtle description of striatal signaling.

In this section, we review the development of models and techniques allowing the selective targeting of D1 and D2 populations to understand their specific roles in the context of drug of abuse. In the first part, we present some examples of cell-type specific genetic manipulation that contributed to investigate the implication of a precise gene/protein in each cell types as well as the interplay between the two pathways. In the second part, we review recent high throughput technologies that allow a more extensive profiling of D1 and D2 MSNs signaling pathways.

1 IDENTIFYING D1 AND D2 MSNS FOR DIFFERENTIAL STUDIES

1.1. INITIAL APPROACHES

Early studies contributing to describe the different composition in neuropeptides and GPCRs between D1 and D2 MSNs were the starting point of our ability to profile the two cell types and to progress in our understanding in their signaling differences (Gerfen and Young, 1988; Le Moine *et al.*, 1990; Le Moine *et al.*, 1991; Schiffmann and Vanderhaeghen, 1993). Pharmacological modulation of striatal subpopulation activity by drugs is commonly used but

remains difficult because drugs have multiple targets widely distributed throughout the brain. Likewise initial approaches to target striatonigral/pallidal populations using toxic compounds lacked specificity (Harrison *et al.*, 1990; Herve *et al.*, 1993; Roberts *et al.*, 1993).

1.2. CLASSICAL TRANSGENESIS STRATEGIES

Genetic targeting is the most promising approach to target a specific neuronal population, either by inactivating a specific gene within the cells of interest or by ablating them, given the large number of available tools (inducible systems, specific recombination) and regulatory sequences. We provide below some examples of genetic manipulations performed specifically in the striatonigral or/and the striatopallidal pathway that contributed to determine some precise molecular events upon cocaine-exposure and related to cocaine-induced behaviors. A summary of these strategies is presented in **Table 2** at the end of this section.

The first striatal cell population successfully targeted by classical transgenesis were the striatonigral neurons. Nestler's group generated two lines of bitransgenic mice that can inducibly overexpress ΔFosB selectively in striatal regions under the control of the tetracycline gene regulation system (Chen *et al.*, 1998; Kelz *et al.*, 1999; Werme *et al.*, 2002). Mice that overexpressed ΔFosB selectively in striatonigral neurons showed an increased response to the rewarding and locomotor effects of cocaine (Kelz *et al.*, 1999; Colby *et al.*, 2003) as well as to the rewarding effects of morphine (Zachariou *et al.*, 2006). Voluntary running was increased in these mice, whereas mice that overexpressed ΔFosB predominantly in striatopallidal neurons ran considerably less. This supports the importance of studying the two pathways independently and the opposite role of striatonigral and striatipallidal neurons in natural and drug reward and locomotion.

Another group assessed the implication of striatonigral neurons in drug reward, by generating a knock-in, i.e. the insertion of a transgene into the native locus of the gene of interest by homologous recombination. For example, a mouse line in which D1 MSNs cells express an inactive form of the NR1 NMDA receptor subunit was generated (Heusner and Palmiter, 2005; Beutler *et al.*, 2011). These mice failed to display locomotor sensitization to repeated cocaine administration and have a decreased ability to form a conditioned place preference to cocaine. These results suggest that NMDA receptor signaling in the striatonigral pathway is required for the manifestation of behaviors associated with repeated drug exposure. Likewise, Beutler *et al*. (2011) demonstrated that unbalanced loss of NMDAR signaling in D1 MSNs alone prevented amphetamine sensitization. Interestingly, they found that the balanced loss of NMDARs from both D1 and D2 MSNs had no effect on amphetamine sensitization.

1.3. BAC STRATEGIES

Using the GPCRs and neuropeptides as reporter genes, it became possible to develop a high throughput genetic labeling of the distinct neuronal populations with the advent of bacterial artificial chromosome (BAC) transgenic mice pioneered by Nat. Heintz and the Gensat from the Rockefeller University (Yang *et al.*, 1997; Heintz, 2001; Gong *et al.*, 2003). BACs are large fragments of genomic mouse DNA (100-250 kb) that contain almost all the regulatory sequences necessary for an accurate expression *in vivo*. Furthermore, BAC-driven expression of tagged proteins allows an easy and reproducible identification of specific neuronal populations.

To tag the neuronal populations of the striatum the two main transgenes used were GFP and/or d Tomato, as well as Cre recombinase (review in Valjent *et al*., 2009) (**Fig. 29**). *drd1a*-EGFP and *chrm4*-EGFP BAC mice express EGFP in striatonigral cells and their axonal projections to the GPi and SN, whereas *drd2a*-EGFP BAC mark the striatopallidal neurons and their projections to the GPe (Gong *et al.*, 2003; Lobo *et al.*, 2006; Bertran-Gonzalez *et al.*, 2008; Matamales *et al.*, 2009). Also, the use of the red fluorescent protein Tomato in *drd1a*-tdTomato BAC, crossed with the *drd2a*-GFP lines (Gong *et al.*, 2003; Shuen *et al.*, 2008), allowed the visualization of the two populations in the same animal. These reporter mice provide an extremely important tool for deciphering the anatomical, electrophysiological and molecular differences of D1 and D2 MSNs.

1.3.1. DARPP-32 PHOSPHORYLATION IN D1 AND D2 MSNS

Bateup *et al*., 2008, analyzed the differential effects of psychostimulants and antipsychotics on DARPP-32 phosphorylation in the MSNs of the two pathways. As mentioned in chapter II, the phosphorylation of DARPP-32 at threonine 34 is essential for mediating the effects of both psychostimulant and antipsychotic drugs. The authors used a double BAC transgenic mice expressing a Flag-tagged DARPP-32 (and Venus) under the control of the D1R promoter and a Myc-tagged DARPP-32 (and EGFP) under the control of the D2R. Immunoprecipitation with anti-flag and anti-Myc antibodies allowed the direct comparison of DARPP-32 phosphorylation in the two neuronal populations. As a result, the authors finally resolved the apparent paradox that psychostimulants and antipsychotics cause the same biochemical changes in the striatum though having opposing behavioral and clinical effects. Indeed, they found that cocaine increased DARPP-32 T34 phosphorylation selectively in striatonigral neurons, whereas the antipsychotic haloperidol increased T34 phosphorylation

only in striatopallidal neurons. Thus, the two drugs exert differential effects on DARPP-32 phosphorylation in the two neuronal populations that can explain their opposing behavioral effects.

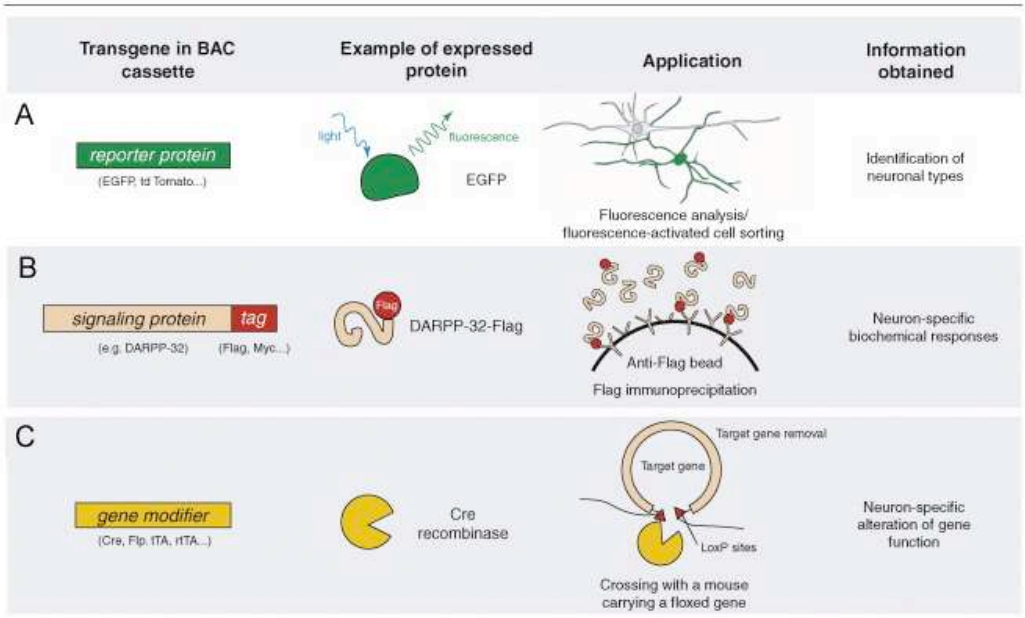

Figure 29 : Various *in vivo* functional applications of BAC transgenic mice

A. Reporter genes (e.g. EGFP or any other fluorescent protein) allow distinguishing different neuronal types in tissue preparations **B.** Epitope-tagged signaling proteins (e.g. Flag, Myc, etc.) reveal neuron-specific biochemical responses after immunoprecipitation with tag-specific antibodies **C.** Gene modifiers (e.g. Cre recombinase) permit neuronal population-specific alteration of gene function after crossing with a mouse line carrying target genes flanked by appropriate sequences (Valjent *et al.*, 2009).

1.3.2. DARPP-32 KNOCK-OUT IN D1 AND D2 MSNS

The function of the striatonigral and striatopallidal pathways was investigated by conditionally deleting DARPP-32 using the D1R and D2R promoters to drive cell type–specific Cre recombinase expression (Bateup *et al.*, 2010). The authors found that the loss of DARPP-32 in striatonigral neurons results in reduced basal locomotor activity, consistent with the interpretation that activation of the direct pathway normally exerts a stimulatory effect on locomotion. Conversely, selective deletion of DARPP-32 in striatopallidal neurons results in increased locomotor activity, suggesting that the indirect pathway exerts a basal

inhibitory tone on locomotion that is disrupted by a loss of DARPP-32. In conclusion, the loss of DARPP-32 resulted in a loss of function in each neuronal population. This is in agreement with a recent report showing that ablation of striatopallidal neurons using a toxin resulted in increased locomotor activity (see also section **3.1)** (Durieux *et al.*, 2009).

1.3.3. GLUCOCORTICOID RECEPTORS KNOCK-OUT IN D1 MSNS

The glucocorticoid receptor is an ubiquitous transcription factor mediating adaptation to environmental challenges and stress. Its implication in cocaine addiction was investigated generating mice D1 MSNs lacking the glucocorticoid receptor gene, Nr3c1. Ablation of Nr3c1 in dopaminoceptive D1R-expressing neurons, but not in dopamine-releasing neurons, showed a decrease in cocaine self-administration and a decrease in VTA DA neurons firing (Ambroggi *et al.*, 2009). In these studies, the Cre expression appears not to be restricted to striatonigral neurons but to involve also striatopallidal neurons, resulting in a deletion of the transgene in the majority of MSNs (Lemberger *et al.*, 2007).

1.3.4. MGLUR$_5$ RECEPTORS KCNOCK-DOWN IN D1 MSNS

Novak *et al*. (2010) used cell type-specific RNA interference and generated a novel mouse line with a selective knock-down of the metabotropic glutamate receptor 5 mGluR$_5$ in D1 MSNs. These mutant mice show impairment in the reinstatement of cocaine-seeking induced by a cocaine-paired stimulus, as well as deficits in specific incentive learning processes. The study suggests that glutamate signaling in the D1 neurons through mGluR$_5$ is necessary for incentive learning processes that contribute to cue-induced reinstatement of cocaine-seeking and which may underpin relapse in drug addiction.

2 SELECTIVE MANIPULATION OF STRIATONIGRAL & STRIATOPALLIDAL PATHWAYS

2.1. SELECTIVE ABLATION OR BLOCKADE OF D1/D2 MSNS

Durieux *et al*. (2009) selectively ablated D2 MSNs by Cre-mediated expression of a diphtheria toxin receptor and diphteria toxin injection using the *adora2a* gene that is

expressed exclusively in this subpopulation. The authors found that ablation of D2 MSNs in the entire striatum induced hyperlocomotion, whereas ablation in the ventral striatum increased amphetamine conditioned place preference. Thus, striatopallidal neurons are critical for both the control of motor behavior and inhibition of drug reinforcement.

Another study aimed at targeting striatonigral and striatopallidal neurons to block their transmission using viral vector and study their role in reward and aversive behavior (Hikida *et al.*, 2010). The authors developed a reversible neurotransmission blocking technique (Yamamoto *et al.*, 2003; Wada *et al.*, 2007), in which transmission of each pathway is selectively blocked by specific expression of transmission-blocking tetanus toxin in a doxycycline-dependent manner. This investigation demonstrates that transmission blockade of either of the two pathways abolishes the psychostimulant-induced acute responses, suggesting that the coordinated modulation of the two pathway is required. This modulation, however, shifts to the predominant roles of the direct pathway in reward learning and cocaine sensitization and the indirect pathway in aversive behavior.

2.2. SELECTIVE ACTIVATION OF D1/ D2 MSNS

Optogenetics, as the name implies, involves a combination of light optics and genetic expression of light-sensitive proteins that can be targeted to living mammalian cell types and controlled with light on a spatiotemporal scale. The most extensively used light-activated proteins that can respectively activate or silence neurons are the channelrhodopsin-2 (ChR2) cation channel and the halorhodopsin (NpHR) chloride pump (Boyden *et al.*, 2005; Zhang *et al.*, 2007). Blue light induces a conformational change that open the ChR2 pore which rapidly and reversibly depolarizes the cell membrane and triggers action potential (Boyden *et al.*, 2005), while yellow light activate NpHR, which generates a chloride flow and causes rapid and reversible cell membrane hyperpolarization that prevent action potential (Zhang *et al.*, 2007) (**Fig. 30**). Additionally, optogenetic control of a given intracellular signaling cascade is now possible with the development of light-sensitive signaling molecules. These proteins, referred as to optoXRs (Airan *et al.*, 2009), are GPCR opsin chimeras in which the intracellular loops of rhodopsin are replaced with a specific GPCR.

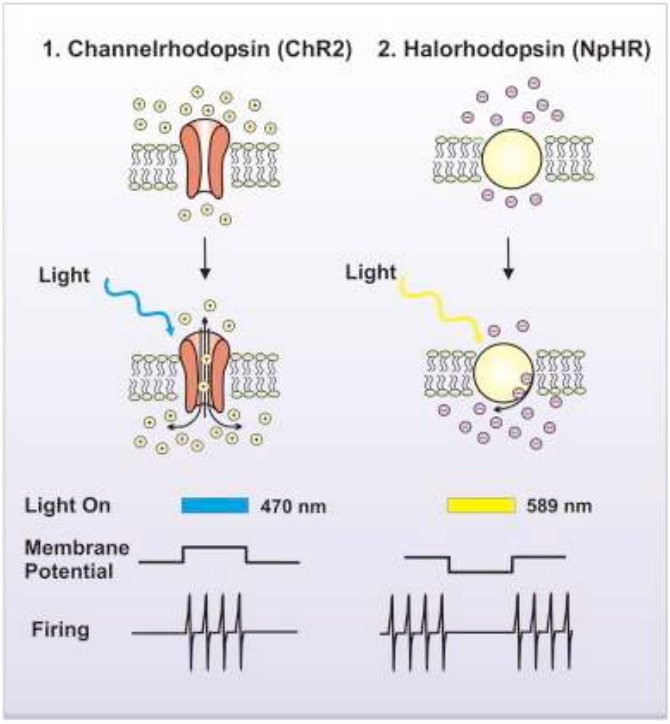

Figure 30: Optogenetic tools for controlling neuronal activity.

Channelrhodopsins (ChR2) display an intrinsic light-gated cationic conductance and can be used to depolarize neurons upon emission of blue light. Halorhodopsin (NpHR) is a light-driven ion pump, specific for chloride ions. It produces hyperpolarizing currents by translocating chloride ions into the cell. Membranes are represented with the cytoplasmic side toward the bottom.

The most recent advances in this domain have been obtained by combining optogenetics with BAC-Cre expressing mice and adeno associated virus (AAV) cell-type delivery, allowing neuron-type control of neuronal activity with high temporal and spatial resolution (Kravitz *et al.*, 2010; Lobo *et al.*, 2010). Lobo *et al*. (2010) used this system to examine for the first time the relationship between neuronal activity of specific neuronal populations and animal behavior. They investigated the role D1 and D2 specificity in NAc by specifically pairing ChR2 activation with cocaine CPP. In particular, they used Cre reporters lines (D1-Cre or D2-Cre) combined with a conditional ChR2 AAV virus containing a double-floxed inverted open reading frame encoding a fusion of channelrhodopsin-2 and enhanced yellow fluorescent protein (ChR2-EYFP), in which ChR2 is only expressed in the presence of Cre-recombinase. Functional ChR2–EYFP is transcribed only in neurons containing Cre, thus restricting expression to either D1 or D2 MSNs. They used herpes simplex virus -ChR2-mCherry to activate all neurons in the NAc. They showed that optogenetic control of total NAc and D1 MSNs neuronal firing potentiated place preference for cocaine, whereas optogenetic control of D2 MSNs firing attenuated cocaine place preference. They also showed that deletion of TrkB, selectively in D1 or D2 neurons, oppositely affected cocaine reward and proposed a role for D2 MSNs in mediating BDNF-TrkB signaling in the NAc on cocaine reward.

Likewise, Kravitz *et al*. (2010) obtained the selective optogenetic control of the direct and indirect pathways *in vivo* using a Cre-dependent viral strategy. In a first series of

experiments, the authors illustrated the dual function of the two pathways in the regulation of motor control and used optogenetic activation of direct pathway neurons to rescue parkinsonian motor deficits (Kravitz *et al.*, 2010). More recently, they investigated the specific role of these pathways in mediating reinforcement (i.e. causes a behavior to occur with greater frequency) and punishment (i.e. cause a behavior to occur with less frequency) (Kravitz *et al.*, 2012). They found D1 MSNs neuronal firing to be sufficient for reinforcement and D2 MSNs firing sufficient for punishments although the duration of these two behaviors differed. Reinforcement appeared to persist for long durations (at least 30 min), whereas punishment was transient (30–45 s).

Table 2: Strategies to target D1 and D2 MSNs

Cell-type	Gene/function manipulated	Method	Effect mediated by cocaine/psychotimulant	References
D1	ΔFosB	Overexpression with tetracycline inducible D1 mouse line	Enhanced cocaine sensitization and CPP	Zacchariou (2006), Keltz (1999)
D1	NR1(NMDA) subunit	mutated knocekd int the D1 locis	Diminished cocaine sensitzation and CPP	Heusner and Palmiter (2005)
D1	NR1(NMDA) subunit	D1-Cre knockout	Diminished amphetamine sensitization	Beutler (2011)
D1	mGlur5	mGlur5 shRNA driven under D1 BAC	Diminished cue-undiced cocaine seeking	Novak (2010)
D1	Glucocorticoid R nuclear receptor 3c1 (Nr3c1)	D1-Cre knockout	Diminished cocaine self-administration	Ambroggi (2009)
D2	Tetanus toxin light chain inhibited synaptic transmission	Overexpressed with AAV virus with enkephalin promoter	No change in CPP, slight decrease in sensitization	Hikida (2010)
D2	Inducible diptheria toxin receptor – ablates cells	Overexpressed with A_{2A}-Cre	Enhanced amphetamine CPP	Durieux (2009)
D1	BDNF receptor (TrkB)	D1-Cre knockout	Enhanced cocaine sensitization and CPP	Lobo (2010)
D2	BDNF receptor (TrkB)	D2-Cre knockout	Dimished cocaine sensitization and CPP	"
D1	ChR2, light-activated neuronal activity	Conditional AAV viruses+D1-Cre	Enhanced cocaine CPP	"
D2	ChR2, light-activated neuronal activity	Conditional AAV viruses+D2-Cre	Diminished cocaine sensitization	"
D1	DARPP-32	D1-Cre knockout	Diminished cocaine sensitization	Bateup (2010)
D2	DARPP-32	D2-Cre knockout	Enhanced cocaine sensitization	"
D1	DARPP-32	D1-DARPP-32/Flag	cocaine increases phospho-T34, decreases phospho-T75	Bateup (2008)
D2	DARPP-32	D2-DARPP-32/Myc	cocaine decreases phospho-T34, increases phospho-T75	"

3. GENETIC PROFILING OF D1 AND D2 MSNS

Together with the methods, large-scale profiling of D1 and D2 MSNs has been developed to investigate in more details their respective differences in gene expression.

3.1. FACS-ARRAY

Lobo *et al*. (2006) utilized the GFP BAC mice to perform high throughput gene expression in the two MSN subtypes developing the FACS-array approach. It consists on isolating live EGFP neurons using fluorescence-activated cell sorting (FACS) (**Fig. 31**), and purifying RNA from the sorted neurons for microarray profiling. They showed that RNA can be obtained from neurons of juvenile and adult ages. They found the 6 known D1 and D2 markers to be segregated in the correct population together with a new set of genes differentially enriched in each MSN subtypes critical for their respective function. For example, in the D1 MSNs, they identified and confirmed the selective expression of *Slc35d3*, a gene encoding an uncharacterized nucleotide sugar transporter. This result indicates that cell type–specific protein glycosylation may have a key role in the function of the striatonigral neurons. Also, Ebf1, a D1-enriched lineage-specific transcription factor, was found essential for the maturation of D1 but not for D2 MSNs. In a second study, G-protein coupled-receptor 6 (*Gpr6*) was found to be enriched in the striatopallidal neurons and important for mediating cAMP signaling in the context of instrumental learning (Lobo *et al.*, 2007).

In the line of the FACS-array paper, Guez-Barber *et al*. (2011), used FACS to purify adult striatal neurons but for the first time, based on their activation state, as defined by their *c-fos* promoter induction (Guez-Barber *et al.*, 2011; Guez-Barber *et al.*, 2012). They used transgenic *cfos-lacZ* rats so activated neurons could be labeled with an antibody against β-galactosidase, the protein product of the lacZ gene. They isolated activated neurons by acute and repeated cocaine injection and compared their unique patterns of gene expression with those in the non-activated majority of neurons. The authors found higher expression of the D1 neuronal marker gene prodynorphin in activated neurons, and lower expression of the D2 neuronal marker genes D2R and A2AR in activated neurons. Also, with their procedure they found that IEGs were induced only in activated neurons, whereas IEG expression was unchanged or even decreased in the non-activated majority of neurons. Since many of these IEGs and neural activity markers are also transcription factors, it is likely that very different patterns of gene expression are subsequently induced within these activated neurons that may contribute to the physiological and behavioral effects of cocaine.

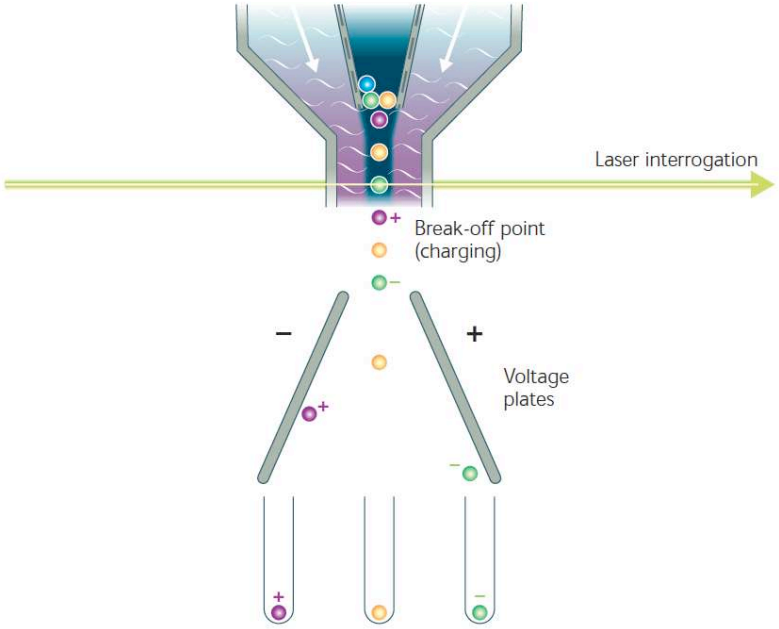

Figure 31 : Flow cytometry principles

Flow cytometry is a laser-based technology that measures and analyzes the optical properties of mono-dispersed single particles, such as cells, nuclei, bacteria, microbeads, other similarly-sized particles, passing in a single file through a focused laser beam. The laser can excite fluorophores that have been used to mark various molecules. The use of fluorophores with different fluorescence characteristics, multiple lasers and multiple photo-detectors allows flow cytometers to measure many characteristics of each particle simultaneously. An important feature is that large numbers, for example thousands of particles per second, are analyzed and therefore provide a statistically significant picture of a specimen's physical and biochemical make-up. Physical properties, such as size (represented by forward angle light scatter) and internal complexity (represented by right-angle scatter) can resolve certain cell populations. In particular, FACS (fluorescence-activated sorting) allows the physical separation of particle of interest from a heterogeneous population in addition to collect data. Particles (ie cells, nuclei etc) are held into a stream of fluid which breaks up into droplets, each of them containing one particle. They are introduced into the laser beam for interrogation. As the droplets pass through the laser, a decision is made whether to sort that event. If the event is sorted, typically based on fluorescent labeling, the droplet will be charged either positive or negative by a charging electrode and travel toward positively or negatively charged platinum plates into the appropriate collection tube. Separated fractions can be then analyzed independently and used for downstream applications.

However, an important caveat of both approaches is the time needed to isolate the mRNAs and the damages caused by homogenization and physical shearing of the neurons. Indeed, the entangled nature of mature neurons makes it difficult to separate cells without causing cellular damage or death, thereby introducing artefactual mRNA responses. Moreover, a long incubation in the presence of proteases is needed to dissociate the cells. This procedure is

likely to favor the occurrence of changes in RNA or protein levels, and even more so in post-translational modifications. Another technology, the BacTRAP, has been developed to overcome these limitations.

3.2. BACTRAP TECHNOLOGY

BacTRAP (translating ribosome affinity purification) allows for both spatial specificity of the cell population profiled and a very short post-mortem interval. It consists in a rapid affinity purification strategy for the isolation of translated mRNA from genetically targeted cell types (**Fig. 32)**. A fusion of EGFP to the N terminus of the large subunit ribosomal protein L10a is inserted under the control of the promoter of either *drd1a* or *drd2* (or other promoters) in BAC transgenic mice (**Fig. 33)** (Doyle *et al.*, 2008; Heiman *et al.*, 2008). EGFP-tagged polysomes from brain homogenates can be immunoprecipitated with anti-GFP coated beads allowing their selective enrichment in the neuron of interest and purified mRNA analyzed by microarray.

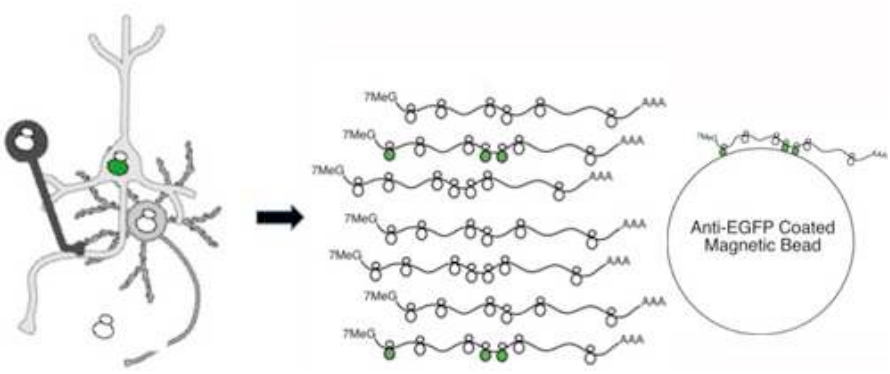

Figure 32: BacTRAP principles

Schematic drawing of a mixed neuronal populations and their polysomes. Polysomes from neurons expressing the EGFP-L10a transgene (green) can be mmunoprecipiated with an anti-GFP coated beads for isolation and analysis.

Microarray analysis revealed that all well characterized, differentially expressed MSN markers were enriched with this approach and further identified more than 300 genes differentially expressed between the two cells. Some of them were also found by Lobo and colleagues but this approach revealed many more differences between the two neuronal populations than those observed with the FACS-array. Furthermore, they evaluated the expression changes in the two populations after acute and chronic cocaine treatments. They

showed differential expression of genes known to be regulated by cocaine such as *FosB*, *Homer1*, *Vamp2*, *Kcnd2* and *Zfp64* (Hope *et al.*, 1992; Brakeman *et al.*, 1997; McClung *et al.*, 2004). As an evaluation of the possible physiological relevance of the approach, they demonstrated that, following chronic cocaine administration, the GABA signaling pathway was specifically enhanced in D1 MSNs. Recordings from striatal slices confirmed these findings by disclosing the existence of an increased frequency of miniature inhibitory post synaptic currents (mIPSCs) only in striatonigral MSNs.

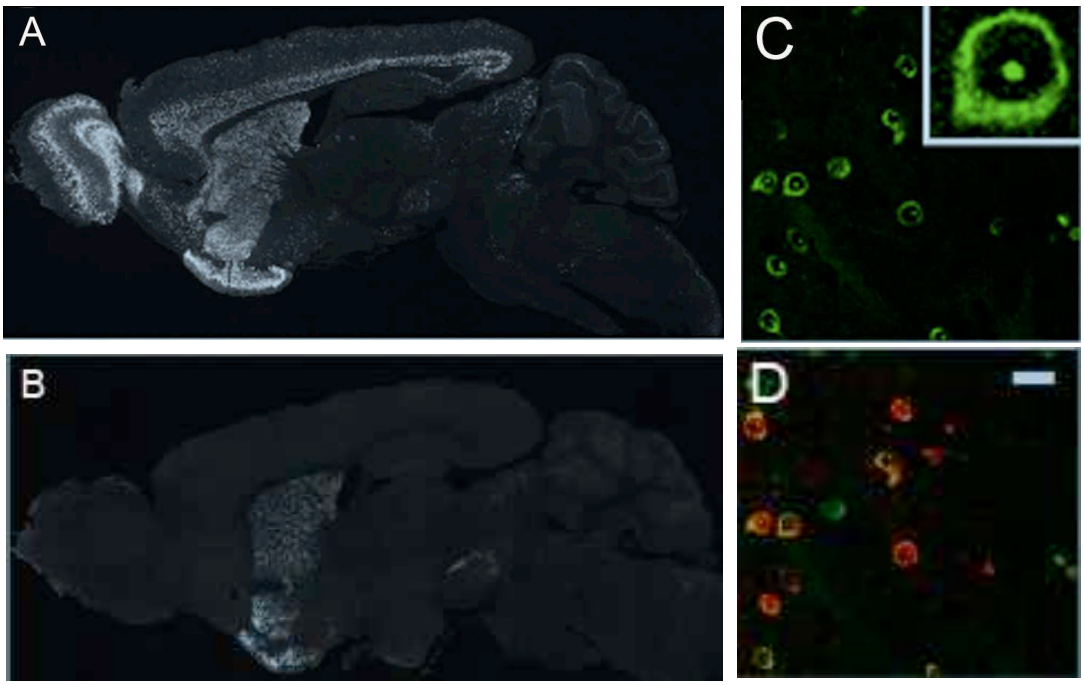

Figure 33: Expression of EGFP-L10a in *drd1a* and *drd2* BacTRAP lines

A. Immunohistochemistry to EGFP in adult sagittal sections from the *drd1a* line. Highest staining in dorsal and ventral striatum, olfactory bulb, olfactory tubercule and cortical layer 5 and 6. **B.** Immunohistochemistry to EGFP in adult sagittal sections from the *drd2a* line. Highest staining in dorsal and ventral striatum, olfactory tubercule, hippocampus, substantia nigra, VTA because of D2 autoreceptor expression in dopaminergic cells. **C-D.** Characterization of *drd2* BacTRAP line striatal MSN cells. **C**. direct EGFP immunofluorescence with high magnfication image insert on the top right). **D.** Direct EGFP immunofluorescence merged with enkephalin immunohistochemical staining. Scale bar is 20 µm. (Heiman *et al.*, 2008).

4. NEW DIRECTIONS

BacTRAP technology has been proved to be extremely useful to study accurate expression profile of targeted neuronal populations but it does not provide information on others levels of regulation such as DNA and protein modifications. To study epigenetic and nuclear responses, FACS-sorted of nuclei of the neurons is the key strategy to obtain isolated structure such as nuclei without the drawbacks of FACsing entire neurons. In this perspective, Kriaucionis and Heintz (2009) exploited a characteristic of the bacTRAP mice and took advantage of the fact that ribosomal proteins, before being functional and cytoplasmic, are assembled in the nucleolus of the cells. As a consequence, the neurons of interest expressing L10a-EGFP, have not only fluorescent cytoplasms but also fluorescent nucleoli (**Fig. 33C**). It is then possible to sort neurons nuclei just as cells in suspension obtaining very pure preparation. This elegant approach combines the large-scale profiling of the FACS avoiding the harsh dissociation step of mature neurons and allows to focus on nuclear events. The authors successfully purified Purkinje and granule cell nuclei from the cerebellum and identified a novel DNA modification, the nucleotide 5-hydroxymethyl-2'-deoxycytidine. This methylation form is highly variable between cell lines, predominantly enriched in terminally differentiated cells and stem cells but not present in cancer cells, suggesting a role in epigenetic reprogramming in cancer and stem cells, as well as in the control of neuronal functions.

5. CONCLUSION

Chromatin remodeling and alterations in histone modification in response to drug of abuse take place striatum as seen in chapter III. So far, the studies of epigenetic mechanisms reveal important insights into chromatin modifications in whole striatal tissues. In this perspective, researchers have just started exploring cell-specific events induced by drug of abuse thanks to the many technologies developed to identify and isolate neuron families. We are still lacking data about cell-specific epigenetic modifications.

The present work is a logical follow-up and provides a novel methodology for the genetic profiling of the D1-expressing striatonigral and D2-expressing striatopallidal neurons. The approaches developed during my thesis allow to evaluate the cocaine-induced epigenetic modifications specifically in striatonigral and striatopallidal neurons that account for the dramatic differences in gene expression between the two cell-types.

EXPERIMENTAL RESULTS

The striatum is composed of multiple cell types, which exhibit different transcriptional responses to drugs of abuse and mediate distinct aspects of drug reward and addiction In particular, the D1 and D2 subtypes of MSNs are differentially modulated by dopamine and exhibit strikingly different cocaine-induced signaling cascades and transcriptional regulation. It is then crucial to be able to study their response in a subtype specific manner to get a precise understanding of drug action. In addition, little is known about any potential differences in histone modification in the two populations of MSNs.

My goal was to characterize the epigenetic status in these cell-types and understand their temporal regulation upon acute and chronic cocaine exposure. The main objective of my thesis was to develop an approach to isolate nuclei of D1 and D2 neurons *in vivo* and characterize their post-translational modifications (PTMs). We established the sorting of neuronal nuclei from the striatum, most importantly preserving the PTMs, guided by a previously published sorting technique using Fluorescence Activated Cell sorting (FACS-sort). Next, we developed an assay, based on flow cytometric analysis, to be able to study quantitatively the changes of PTMS in the two cell-types. This approach was used to investigate the difference in histone acetylation and methylation between D1 and D2 neurons, the temporal regulation of these PTMs and their correlation upon acute and chronic cocaine exposure. The results from this study are compiled in *Article I (manuscript in preparation)*.

The differential acetylation profiles for D1 and D2 MSNs were further characterized by analyzing the HDACs (Histone deacetylases) activity in these two cell types, and the results are presented in the section *complementary results 1*.

On the other hand in the last section of the results, a preliminary work not directly related to the sort of nuclei presented above is described. These data are useful in delineating precise cocaine targets as well as giving insight into the molecular mechanism underlying cocaine action in the striatum. Briefly, the role of DARPP-32/PP1 cascade in the nucleus of medium striatal spiny neurons was studied and new nuclear substrates of PP1 were explored. A model system was set up using striatal primary culture that allowed the study of PP1 action *in vitro*. A potent inhibitor of PP1 was used to identify the changes in PTMs of histones and other nuclear proteins compared to non-treated cells. A new indirect target of PP1 was identified. These data are presented in the section *complementary results 2.*

1. ARTICLE I

Cocaine induces specific patterns of histone post-translational modifications in the two populations of striatal medium-sized spiny neurons

(Manuscript in preparation)

Cocaine induces specific patterns of histone post-translational modifications in the two populations of striatal medium-sized spiny neurons

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ABSTRACT

Drugs of abuse such as cocaine induce changes in gene expression and epigenetic responses including alterations in histone post-translational modifications (PTMs) in striatal neurons. However, the striatum is comprised of multiple cell types, including two distinct populations of medium-sized spiny neurons (MSNs) and little is known concerning the cell-type specificity of epigenetic modifications. We used transgenic mice, in which expression of the EGFP-tagged ribosomal L10a subunit is driven by the D1 (*drd1a*-EGFPL10a) or D2 (*drd2*-EGFPL10a) dopamine receptor promoter. L10-EGFP labels specifically the cytoplasm and nucleoli and we took advantage of the nucleoli fluorescence to isolate D1R- or D2R-expressing MSN nuclei by flow cytometry. Using a FACS-analyzer we quantified the cocaine-induced changes in histone acetylation and methylation in isolated nuclei from each cell population. We found that D1- and D2-expressing MSNs displayed different patterns of epigenetic modifications in response to acute and chronic cocaine exposure. In particular, $acK_{14}H3$, $acK_{5}H4$, $acK_{12}H4$, and $me_{n}K9H3$ exhibited opposite patterns of regulation in the two cell types. Moreover, we found cell-type dependent correlations between histone PTMs that were altered by cocaine. Our data provide insights into the differential epigenetic responses to cocaine in D1 and D2 MSNs which may participate in the differential gene expression known to occur in these cell types. The method developed should have a general utility for studying nuclear modifications in different types of neurons in physiological or pathological conditions.

INTRODUCTION

In up to 20 % of humans and rodents, repeated self-administration of addictive substances leads to a state of addiction characterized by a loss of control over drug use with compulsive self-administration despite its severe negative consequences (Deroche-Gamonet *et al.*, 2004). The addictive state is persistent and can last an entire life time with drug craving and relapse occurring even after decades of abstinence. All addictive drugs share the ability to increase extracellular dopamine (DA) levels in the ventral striatum (Di Chiara and Imperato, 1988) and are thought to exert their long-lasting effects by "hijacking" the normal reward systems, including the mesolimbic-striatal DA pathway and the nucleus accumbens (NAc) (Koob and Le Moal, 2001; Wise, 2002).

A single exposure to psychostimulants triggers long-lasting alterations in behavior and synaptic physiology (Badiani *et al.*, 2011), revealing their powerful control on neuronal plasticity. Repeated exposures induce further changes which contribute to the occurrence of addiction (Nantwi and Schoener, 1993; Hyman and Malenka, 2001; Kalivas, 2004; Dietz *et al.*, 2009). Growing evidence suggests that long-lasting changes in the properties of striatal neurons involve epigenetic processes (Borrelli *et al.*, 2008). In particular, recent work shows that histone post-translational modifications (PTMs), including phosphorylation, acetylation, and methylation play a role in promoting transcriptional alterations following cocaine administration (Renthal *et al.*, 2008; Maze *et al.*, 2010). Acute exposure to cocaine induces rapid changes in histone phosphorylation and acetylation through a variety of signaling pathways activated downstream from DA and glutamate receptors (Brami-Cherrier *et al.*, 2005). Moreover, repeated cocaine regulates histone-modifying enzymes in the NAc, including histone deacetylases (HDACs), acetyl-transferases (HATS) and methyltransferases (Robison and Nestler, 2011).

The striatum is composed of multiple cell types, which exhibit different transcriptional responses to drugs of abuse and mediate distinct aspects of addiction (Lobo and Nestler, 2011). Most (95% in rodents) striatal neurons are GABAergic medium-sized spiny neurons (MSNs), which can be further differentiated into D1 DA receptor- (D1R-) and D2R-expressing neurons, broadly corresponding to neurons of the direct and indirect pathways respectively (Valjent *et al*., 2009). Each population of MSNs expresses its own subset of markers and has different projections (Gerfen, 1992). Cocaine-induced signaling cascades (Bateup *et al.*, 2008) and transcriptional regulation (Heiman *et al.*, 2008) are strikingly different in the two types of MSNs. However, little is known about any potential differences in histone modification in the two populations of MSNs.

Here we have developed a novel approach taking advantage of the specific labeling of the two types of MSNs through the expression of EGFP-tagged ribosomal L10a protein under the control of the D1R or D2R promoters in transgenic mice (Heiman *et al.*, 2008). The high enrichment of EGFP-L10a in nucleoli allows fluorescence-activated counting and sorting of nuclei (Kriaucionis and Heintz, 2009). Using this approach, we studied cocaine-induced histone PTMs in the two populations of MSNs separately. We found some similarities and many differences between the two populations, with distinct temporal regulations that are likely linked to specific patterns of gene expression.

MATERIAL AND METHODS

Animals

drd1a and *drd2* bacTRAP lines that express the transgene EGFP-L10a were generated as described in Heiman *et al*. (2008) and were maintained on a C57BL/6J background. For all experiments mice of either gender were between 8-12 weeks and had access to food and water *ad libitum* in their home cages. Lights were maintained on a 12 h light/dark cycle. Animal protocols were performed in accordance with National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Rockefeller University's Institutional Animal Care and Use Committee or in accordance with the guidelines of the French Agriculture and Forestry Ministry for handling animals (decree 87-848) under the approval of the "*Direction Départementale de la Protection des Populations de Paris*" (authorization number C-75-828, license B75-05-22).

Reagents

Mice were injected intraperitoneally with drug or saline and killed by decapitation. The head was dipped in liquid nitrogen for 5 sec, the brain removed, and striata were rapidly dissected on a piece of dry ice before being homogenized and subjected to nuclear fractionation. For the acute regimen, animals received one injection of 20 mg/kg cocaine-HCl (Sigma) or saline and were sacrificed 15 min or 24 h later. For the repeated (chronic) regimen, mice received one injection of cocaine-HCl or saline for 7 consecutive days and were sacrificed 24 h after the last injection. Injections were carried out in the home cage.

Nuclear fractionation

Nuclear fractionation was performed as described in Kriaucionis and Heintz (2009) with some modifications. Briefly, both striata from each mouse were homogenized in 500 µl of cold homogenization medium (0.25 M sucrose, 150 mM KCl, 5 mM $MgCl_2$, 20 mM tricine pH 7.8, 0.15 mM spermine, 0.5 mM spermidine, protease inhibitor cocktail (Protease Inhibitor set III, Calbiochem), phosphatase inhibitor cocktail (Phosphatase Inhibitor Cocktail II Calbiochem), using loose and tight glass-glass 2 ml Dounce homogenizer. The homogenate was briefly fixed with 1 % (weight/volume) paraformaldehyde (PFA) for 8 min in a rotating incubator at room temperature (RT) and quenched with 0.125 M glycine for 5 min in the same conditions. The cross-linked homogenate was supplemented with 500 µl of density medium (50% iodixanol [OptiPrep, Axis-Shield], 150 mM KCl, 5 mM $MgCl_2$, 20 mM tricine pH 7.8)

and overlaid on top of a 29 % (vol/vol) iodixanol cushion. The nuclei were collected by centrifugation at 10,000 x g for 30 min at 4°C in a swinging bucket rotor (Beckman TL-100 ultracentrifuge, TLS55 rotor). The nuclear pellet was resuspended in isotonic buffer (0.25 M sucrose, 25 mM KCl, 5 mM $MgCl_2$, 20 mM tricine pH 7.8) or PBS in a siliconized Eppendorf tube and complemented with spermine, spermidine, protease and phosphatase inhibitors cocktails at the same concentrations as in the homogenization (see above). Samples were then subjected to analysis using a fluorescence-activated sorter (FACS) or analyzer.

Flow cytometric analysis and sorting

Nuclear preparation using a FACS sorter

Striatal nuclei were resuspended in isotonic buffer supplemented with phosphatase and protease inhibitors, spermidine, spermine, 10 µM DyeCycle Ruby (Invitrogen), 1% donkey serum and incubated for 45 min on ice to allow good separation between single nuclei and aggregates. Nuclei were sorted using a BD FASCAria cell sorter equipped with 640 nm and 488 nm excitation lasers and a 85-µm nozzle. Sorted nuclei were collected in 100 µl of isotonic buffer, centrifuged for 15 min at 1000 x g, snap-frozen in liquid nitrogen and stored at -80ºC until analysis by immunoblotting.

Nuclear flow cytometry

The striatal nuclei pellet were resuspended in 1 ml cold PBS supplemented with phosphatase, protease inhibitors cocktail, spermidine, and spermine and permeabilized with Tween 0.005 % (vol/vol) for 4 min at 4ºC. Samples were blocked in PBS containing 1 % (weight/vol) BSA for 10 min and aliquoted into a conical 96 well-plate (50,000 nuclei per well). Striatal nuclei from each animal were permeabilized, blocked and aliquoted for single labeling with up to 10 different antibodies targeting histone post-translational modifications. From 50,000 total nuclei per well, 3,000 to 5,000 events corresponding to the D1- or D2- positive populations were recorded. Nuclei were incubated for 1 hr at RT with the following antibodies: anti-$pS_{10}H3$ (1/200), anti-$pS_{10}acK_{14}H3$ (1/1000); anti-acK_5H4 (1/7000), acK_8H4 (1/7000), $acK_{12}H4$ (1/5000) (Millipore); anti- me_nK_4H3 (1/500), meK_9H3 (1/700), me_2K_9H3 (1/200), me_3K_9H3 (1/200) (Abcam). Control samples were incubated with the appropriate isotype antibody (ChromoPure rabbit or mouse IgG; Jackson ImmunoResearch) to show background fluorescence. Nuclei were centrifuged and washed with PBS, then secondary antibody was added for 30 min at RT followed by centrifugation and a PBS wash. Nuclei were then resuspended in 60 µl of PBS containing Hoechst 33342 fluorescent DNA stain (1/10000) and incubated at RT for 1 hr before being analyzed/loaded on a LSRII (BD) FACS analyzer. Data were analyzed using FLOWJO software (Tree Star, San Carlos, CA, USA).

GFP-positive singlets were gated as the GFP population above the background assessed with a wild-type littermate mouse. Within the GFP-positive population, histone PTM indirect

fluorescence above isotype control fluorescence was quantified using the geometric mean of fluorescence of the appropriate channel and expressed as a ratio to the mean isotype control fluorescence. The data were normalized in each experiment by expression as a % of the mean values in saline-treated mice.

Image stream data acquisition

To assess the integrity and purity of the sorted nuclei, images were acquired (x 5,000 cells/sample, objective 40X) on an Amnis ImageStream system (Amnis, Seattle, WA) and data were analyzed with IDEAS 4.0 software.

Immunoblotting of sorted nuclei

Sorted D1 or D2 nuclei lysates (1 µg) were separated by SDS/PAGE, and proteins transferred to PVDF membranes. Membranes were blocked for 1 h in 5% BSA (weight/vol) and incubated overnight at 4°C with the following primary antibodies: anti-$pS_{10}H3$ (1/1000), anti-$pS_{10}acK_{14}H3$ (1/1000), (Millipore) or H3 total (1/5000) (Abcam). Antibody binding was revealed by HRP-conjugated secondary antibodies (1:500000) and the SuperSignal West Femto Substrate (ThermoScientific). Quantification of bands was performed by densitometry using ImageJ, version 1.42q .

Tissue preparation and immunofluorescence

drd1a-EGFP mice were anesthetized with pentobarbital (Sanofi-Aventis, France) and perfused transcardially with 4 % (weight/vol) paraformaldehyde in PBS (pH 7.4). Their brains were post-fixed overnight at 4°C. Thirty µm sections were cut with a vibratome (Leica, France) and stored in an ethylene glycol-glycerol-PBS mixture 30:30:40 at -20°C. Free floating sections were rinsed three times 10 min in PBS pH7.4, incubated for 20 min in PBS containing 50 mM NH_4Cl and then rinced as above before 5 min permeabilization with 10 % (vol/vol) methanol 0.3 % (vol/vol) Triton X-100 in PBS. After rinsing again and blocking for 30min with BSA 2 % (weight/vol), sections were incubated overnight at 4°C with the following primary antibodies in PBS containing 2 % BSA and 0.1 % Triton X-100: anit-meK9H3 (1/400, rabbit polyclonal, Abcam), anti-me_2K9H3 (1/1000, mouse monoclonal, Abcam), anti-me_3K9H3 (1/400, rabbit polyclonal, Abcam), anti-acK_5H4 (1/400, rabbit polyclonal, Millipore). The sections were rinsed and incubated for 45 min with goat-anti rabbit or mouse, Cy3-coupled secondary antibody (1/400, Jackson Laboratory). Finally, sections were rinsed again 10 min twice in PBS and in Phosphate buffer pH7.4 before being mounted in Vectashield Mounting Medium containing DAPI (Vector Laboratories).

Confocal microscopy and image analysis were carried out at the *Institut du Fer à Moulin* Imaging Facility. Double-labeled images were obtained using sequential laser scanning confocal microscopy (SP5, Leica), objective 100X.

Statistics

Results were expressed as means ± S.E.M. Statistical differences between groups were determined with two-tailed unpaired Student's t-test. Significance was attributed to a p value <0.05.

RESULTS

Fluorescence-activated purification of nuclei in *drd1a* and *drd2* EGFP-L10a transgenic mice

We used bacterial artificial chromosome- (BAC-) transgenic mice, which express the EGFP-L10a transgene, under the control of either the D1R (*drd1a*) or D2R (*drd2*) promoters (Heiman *et al.*, 2008). The L10a protein is a component of the large subunit of the ribosome and allows the immune affinity-purification of polysomes (Heiman *et al.*, 2008). Since ribosomes are assembled in the nucleoli before being exported to the cytoplasm, the nuclei of D1R- or D2R-expressing neurons are intensely fluorescent for EGFP-L10a in these mice, as previously reported for other types of neurons (Kriaucionis and Heintz, 2009) and can be isolated by fluorescence-activated sorting. Striata were dissected from the transgenic mice and after homogenization and fixation (see Methods), nuclei were prepared by subcellular fractionation and analyzed by flow cytometry (**Fig. 1A, B**). The nuclei were also labeled with Dye Cycle Ruby (DCR), an intercalating dye that binds quantitatively to DNA. The dot plots allowed distinguishing several populations of nuclei in transgenic mice according to the intensity of EGFP and DCR fluorescence. Some nuclei displayed a DNA fluorescence that was twice that of the majority of the others (**Fig. 1B**). These signals were considered to correspond to the aggregation of two nuclei (Kriaucionis and Heintz, 2009) and were not included in the analysis. Nuclear debris had lower DCR fluorescence. Only the EGFP-fluorescent nuclei with a "single" DNA signal (GFP singlets) were considered (**Fig. 1B**).

The total amount of GFP singlet nuclei isolated from the *drd1a*/L10a-EGFP mice was 16.4 +/-1.1 % of the total, while 7.1 +/- 0.7 % of GFP singlet nuclei were isolated from the *drd2*/L10a-EGFP mice (n= 18 experiments). FACS-purified nuclei were examined by fluorescence microscopy (data not shown) and flow imaging (**Fig. 1C**) to verify they were

intact, and correctly sorted. Purity (> 90%) was also assessed with a second round of flow cytometry (data not shown)

Histone modifications in nuclei from D1R- and D2R-expressing MSNs

In initial studies, we validated the FACS approach to isolate specific populations of nuclei and we assessed the stability of PTMs during the isolation procedure. Using immunoblotting of sorted nuclei, we examined the phosphorylation of H3 Ser10 ($pS_{10}H3$) isolated or in combination with acetylation of H3 K14 ($pS_{10}acK_{14}H3$). After a single injection of cocaine or repeated injections, these two modifications have previously been shown to be induced in MSNs expressing D1R, but not in D2R MSNs (Bertran-Gonzalez *et al.*, 2008). In the present study, nuclei were prepared from striata of EGFP-L10a mice, 15 min following a single injection of 20 mg/kg of cocaine or vehicle (saline). D1- or D2-MSN nuclei were isolated by FACS and the levels of $pS_{10}H3$ or $pS_{10}acK_{14}H3$ were analyzed by immunoblotting. As expected, we observed a robust cocaine-induced increase in phosphorylation and in phospho-acetylation of H3 in D1 nuclei, but no changes in the D2 MSN nuclei (**Fig. 2A and B**).

We carried out the same experiment but quantified the changes in $pS_{10}H3$ and $pS_{10}acK_{14}H3$ using immunolabeling of permeabilized nuclei combined with the use of a FACS-analyzer. When we plotted the number of immunoreactive, GFP-labeled nuclei as a function of the intensity of the immunofluorescence, we observed a rightward shift of the distribution for both $pS_{10}H3$ and $pS_{10}acK_{14}H3$ in the nuclei from D1 MSN population (**Fig. 2C, upper panel**). The fluorescence was quantified as the percentage of the mean of the fluorescence of the saline samples (**Fig. 2C**, **lower panel**). In contrast, in the D2 MSN nuclei cocaine did not induce any significant changes (**Fig. 2D**). These results demonstrate that both FACS approaches give similar results and can be used to measure histone PTMs in purified nuclear populations. Furthermore, these results show that cocaine-induced phosphorylation is preserved during the procedure and thus, can be used to study other PTMs, such as acetylation and methylation that are presumably as labile as or less labile than phosphorylation.

Differential effects of acute and chronic cocaine on histone acetylation in D1 and D2 MSN nuclei

Histone acetylation is considered to be a marker of actively transcribed genes. Acute and chronic cocaine administration have been shown to induce histone acetylation in the striatum (Kumar *et al.*, 2005; Levine *et al.*, 2005). To date, global acetylation of H3 and H4 has been investigated using polyacetylated lysine antibodies, whereas other studies have focused on specific gene promoters (Renthal *et al.*, 2008; Renthal *et al.*, 2009; Maze *et al.*, 2010). Little is known concerning the regulation of acetylation of specific histone residues in identified

neuronal cell types in the striatum. We examined the nuclear localization of acK_5H4 in striatal neurons *in vivo* and found that the immunoreactivity was mostly colocalized with the euchromatin (**Fig. 3, top row**), in support to its proposed role in gene activation. To determine which residues were affected 15 min after a single injection of cocaine, we examined acetylation changes on H3 K_{14}, K_{18}, and on H4 K_5, K_8, and K_{12} in D1 MSN and D2 MSN nuclei using flow cytometry. A single injection of cocaine rapidly increased acetylation of all studied lysine residues (H3 K_{14} and K_{18}, and H4 K_5, K_8, and K_{12}) in D1 MSN nuclei (**Fig. 4A**). The effect was much more pronounced for H4 K_5 (>3-fold increase) than for the other residues (<2-fold, **Fig. 4A**). In contrast, in D2 MSN nuclei, only H4 K_5 acetylation was moderately increased (**Fig. 4B**). Our data are consistent with the global increase in acK_5H4 immunofluorescence in striatal sections, measured 30 min after a cocaine injection (Brami-Cherrier *et al.*, 2005).

We next investigated the effects of repeated cocaine injections on lysine acetylation, 24 h after the last injection. In D1 MSN nuclei a strong increase (>3-fold) in H4 K_5 acetylation was observed, whereas the acetylation of other residues was either unchanged or moderately increased (**Fig. 4C**). Interestingly, the effects of repeated treatment with cocaine were different in D2 MSN nuclei, with an increase in H3 K_{14} acetylation and a decrease in H4 K_5 and K_{12} acetylation (**Fig. 4D**).

Since these changes were observed 24 h after the last injection of a repeated cocaine treatment, we evaluated the stability of the changes in H4 K_5 acetylation after a single injection of cocaine. We injected *drd1a*-EGFP-L10a mice with cocaine or saline and sacrificed them 24 h later. A robust increase of H4 K_5 acetylation was also observed in these conditions, showing the persistence of the histone modification (**Fig. 4E**). In parallel, we also measured acetylation of H4 K_8 and found a larger increase at 24 h than at the early time point of 15 min (**Fig. 4E)**. These data show that histone acetylation after a single injection cocaine is a persistent modification.

Flow cytometry reveals subsets of D1 and D2 MSN nuclei with different levels of histone acetylation

Flow cytometry allows a quantitative analysis of heterogeneous samples providing a detailed profile of complex cells or nuclei populations that cannot be achieved with other methods such as immunoblotting. While the results shown in Figure 3 were based on comparison of mean fluorescence values, the analysis of the distribution of the fluorescence intensity showed that acK_5H4 immunofluorescence was not homogeneously distributed in control (saline-treated) mice, but that two distinct populations of nuclei were identifiable with low and high fluorescence intensity (**Fig. 5A**, **left panel**). This not observed for other marks such as pS_{10}H3 or pS10acK_{14}H3 (see **Fig. 2C, D upper panels**). A similar bimodal distribution of acK_5H4

immunofluorescence was also observed in the D1 and D2 nuclei from the various groups of saline-treated mice (**Fig. 5B-D**, **left panels**). Following an acute or repeated chronic cocaine injections, the distribution of fluorescence in D1 nuclei was dramatically altered, with a shift towards a single population of highly fluorescent nuclei (**Fig. 5A, C**, **middle and right panels**). A similar change was observed in D2 nuclei from acutely cocaine-treated mice (**Fig. 5B**, right panels). In contrast, in D2 nuclei from mice treated with chronic cocaine, the two populations were not changed as compared with saline-treated mice (**Fig. 5D, middle and right panels**), in agreement with the lack of increase in total fluorescence (**Fig. 4D**).

D1 and D2 MSN nuclei display opposite profiles of histone H3 methylation after acute and chronic cocaine

While considered to be relatively stable, histone methylation is emerging as a target for complex regulation (Maze and Nestler, 2011). Histone methylation has been correlated with either gene activation (e.g. me_2K_4H3, meK_9H3) or repression (me_2/me_3K_9H3) (Barski *et al.*, 2007). Recent work has demonstrated that cocaine-regulated histone methylation in the striatum is associated with changes in gene transcription (Maze *et al.*, 2010; Maze *et al.*, 2011), but it is not known in which cell type this post-translational modification takes place. Interestingly, we found that the localization of the immunoreactivity for the three types of modifications of the same residue (mono, di and trimethylation of H3 K9) was different in striatal neurons (**Fig. 3**). meK_9 immunoreactivity colocalized with euchromatin whereas me_3K_9 labeling was almost exclusively found in heterochromatin consistently with their respective correlation with transcriptional activation and repression. me_2K_9 immunoreactivity was observed in both euchromatin and heterochromatin.

We then examined the effect of a single injection of cocaine on four methylated marks me_nK_4H3, and the mono-, bi-, and tri-methylated forms of H3 K_9. Cocaine induced rapid and robust changes in histone methylation as early as 15 min after a single injection of cocaine. In D1 MSN nuclei an increase in H3 K9 trimethylation was observed, whereas the mono- and di-methylated forms were unchanged (**Fig. 6A**). In contrast, in D2 MSN nuclei, the mono-, bi-, and tri-methylated forms of H3 K9 were increased (**Fig. 6B**). In both types of nuclei methylation of H3 K4 was unchanged (**Fig. 6A, B**). Interestingly 24 h after the last of 7 daily injections of cocaine, the effects on histone methylation were different in D1 and D2 MSN nuclei. The mono-, di-, and tri-methylated forms of H3 K9 were increased in D1 MSN nuclei (**Fig. 6C**), whereas in D2 MSN nuclei no difference between cocaine-treated and saline-treated mice was observed (**Fig. 6D**). We examined specifically the H3K9 di and tri-methylation 24 h after a single injection of cocaine and found it was persistently increased (**Fig. 6E**). These results show that the levels of H3 K9 methylation are differentially

dynamically altered in D1 and D2 nuclei in response to cocaine and that these alterations are persistent.

Comparison of multiple post-translation histone modifications in MSN nuclei

It is well established that the multiple histone PTMs can influence each other and their interaction with other chromatin components function to dictate dynamic transitions between transcriptionally active or transcriptionally silent chromatin states (Fischle *et al.*, 2003). We examined the relationship between the PTMs that were analyzed in the same animals, and looked for any correlation between the levels of histone marks in saline- and cocaine-treated condition and in both cell-types. We found that some marks were strongly correlated and that their correlations depended on both treatment and cell-type. Figure 7 is a representative example where mono-methyl and tri-methyl H3 K9 were significantly correlated but with opposite signs in the two cell populations. In D1 nuclei the correlation was negative, suggesting that tri-methylation occurred at the expense of the mono-methylated form (**Fig. 7A**). In contrast, in D2 nuclei the correlation was positive (**Fig. 7B**) suggesting that a global methylation was taking place, with increased activity of mono, bi and trimethylases, in agreement with the increase in the three forms of methylated H3 K9 (**Fig. 7B**). We also found that $pS_{10}H3$ was positively correlated with me_nK_4H3 in D1 nuclei 15 min after a single cocaine injection but not in saline-treated mice (**Fig. 7C-D**). These data are consistent with the fact that pS_{10} is permissive to me_nK_4 (Fischle *et al.*, 2003).

DISCUSSION

To gain better insight into the cell type-specific epigenetic marks induced by cocaine, we adapted and validated an innovative FACS procedure for isolating specific neuronal nuclei and analyzing rapidly and quantitatively histone PTMs. We showed that genetically labeled nuclei of D1 and D2 DA receptor-expressing neurons were intact and pure after FACS-sorting and that labile cocaine-induced histone phosphorylation was preserved during the procedure. We then used flow cytometry to study histone PTMs in immunolabeled nuclei. This approach revealed the time-dependent specificity of cocaine effects on histone acetylation and methylation in D1 and D2 MSNs nuclei.

The use of FACS in neuroscience provides a high-throughput means to study the phenotypic and functional heterogeneity of brain cells but is hampered by the intricate cell processes and interactions. Cells with few processes, such as those in embryonic tissues, and synaptosomes have been sorted with some success (St John *et al.*, 1986; Wolf and Kapatos, 1989; Arlotta *et al.*, 2005). More recently, adult neurons have been isolated by FACS, taking advantage of genetically encoded fluorescent probes, and used for gene expression profiling studies (Lobo *et al.*, 2006; Guez-Barber *et al.*, 2011). However, the entangled nature of mature neurons makes it difficult to separate cells without causing cellular damage or death, thereby introducing a risk of artifactual responses. In addition to severing processes, the difficulty and duration of the procedure used to dissociate adult neurons are particularly problematic to study reversible post-translational modifications. To overcome these limitations, we took advantage of a recently developed approach (Kriaucionis and Heintz, 2009) and used FACS to purify the nuclei of identified neuronal populations. This procedure was developed to study DNA methylation. Moreover, we developed a rapid quantification approach using immunolabeling and fluorescence-activated cell counting, to obtain information about the dynamics of histone PTMs induced by cocaine in the two striatal MSNs subtypes.

Among the acetylated residues analyzed, acK_{14}H3, acK_5H4, acK_{12}H4 had a remarkable temporal regulation in response to cocaine, different in D1 and D2 nuclei. Fifteen min following a single injection, acK_{14}H3 was activated in D1 exclusively, whereas in chronically-treated mice it was activated in D2 nuclei. In contrast, acK_5H4 was increased in D1 neurons after both acute and chronic treatment with cocaine. Additional differences were noted for acetylation of H4 on K8 and K12. The steady state of acetylation is controlled by HDACs and HATs, which have a broad range of specificity, with multiple enzyme isoforms being able to target the same residues. Acetylation of H4 K5 and H4 K12 is increased in the

hippocampus of HDAC2 knock-out mice (Guan *et al.*, 2009), suggesting its possible role. Interestingly, HDACs isoforms expression has been found to be differentially modulated in D1 and D2 neurons after cocaine (Heiman *et al.*, 2008) and could explain the differential regulation of H4 acetylation. For instance, HDAC4 expression is increased by about 45% specifically in D2 neurons after chronic cocaine (Heiman *et al.*, 2008). Moreover, chronic cocaine up-regulates the class III HDACs, sirtuins, in the NAc (Renthal *et al.*, 2009) although it is not known in which cell-type.

The signaling pathways mediating the changes in histone acetylation are not known. In D1 neurons activation of the cAMP/PKA cascade could influence histone acetylation, although direct evidence is lacking. In dividing cells, protein phosphatase 1 (PP1) influences histone acetylation, through its ability to form a complex with HDAC1 and to recruit it to chromatin (Canettieri *et al.*, 2003). PP1 also interacts *in vitro* with HDAC1, 6, and 10 (Brush *et al.*, 2004). Besides, PP1 inhibits HDAC1 and HDAC2 by dephosphorylation (Galasinski *et al*., 2002). In adult hippocampal neurons, specific inhibition of PP1 in the nucleus induced a decrease in acK_5H4, $acK_{14}H3$, and acH2B (Koshibu *et al.*, 2009). DARPP-32, a potent PP1 inhibitor, activated by the cAMP/PKA cascade, can translocate to the nucleus (Stipanovich *et al.*, 2008) and could modulate histone acetylation regulation through PP1 regulation.

We found that acK_5H4 is a persistent mark, still increased 24 h after a single injection of cocaine in D1 neurons. The increase of acK_8H4 was delayed, being observed 24 h after the injection but not at an early time. These late modifications were in agreement with previous reports (Martin *et al.*, 2012). It is important to note that the turnover of most acetyl groups is high, with a half-life of a few min for the most part (Barth and Imhof, 2010). The apparent stability of some acetylation *in vivo* is therefore likely to result from a different steady state level of acetylation, possibly related to stable changes in acetylation and deacetylation rates.

We observed a strong difference in acK_5 regulation by cocaine in D1 and D2 MSNs, which may be related to the differences in cocaine-induced transcriptional effects in these two populations (Heiman *et al.*, 2008). The FACS analysis showed that acK_5H4 immunoreactivity allowed the separation of 2 populations of striatal neurons in both D1 and D2 MSN subpopulations, with high and low fluorescence. This bimodal distribution was not observed for all PTMs (e.g. distribution of $pS_{10}H3$ or me_nK_4H3 immunoreactivity was unimodal) The distributions of fluorescence for the others acetylated and methylated marks were very variable and could not be easily monitored. Cocaine administration shifted the low fluorescence population towards the levels of immunoreactivity of the high fluorescence population, without increasing the latter. These data suggests the existence of discrete functional populations of nuclei, sensitive to cocaine regulation.

Methylation marks are generally considered to be relatively stable, with a slower turnover than phosphorylation and acetylation and half-lives of about a day for $me_{2,3}K_9H3$ and 7 h for meK_9H3 (Barth and Imhof, 2010). Nevertheless, we observed a rapid increase in methylation as early as 15 min after a single cocaine injection, suggesting that this PTM is as reactive as the others. D1 and D2 nuclei displayed almost opposite profiles of histone methylation change after acute and chronic cocaine. Previous reports showed that a single exposure to cocaine increases the three methylated forms of H3 K9 in the NAc (Maze *et al.*, 2010; Maze *et al.*, 2011). Interestingly, we found a significant increase of these three forms in D2 nuclei from acutely-treated mice, whereas in D1 nuclei only me_3K_9H3 was increased. This observation highlights the independent regulation of meK_9H3 and me_2K_9H3 versus me_3K_9H3. Moreover we found a positive correlation between the levels of meK_9H3 and me_3K_9H3 in D2 nuclei, whereas the correlation was inverse in D1 nuclei, further supporting different types of regulation. Methylation steady state is controlled through the action of methyltransferases and demethylases. G9a/GLP is a methyltransferase that forms specifically meK_9H3 and me_2K_9H3 whereas me_3K_9H3 is produced by SUV39H (Lachner *et al.*, 2003). Our results are compatible with an activation of SUV39H in both D1 and D2 neurons and of G9a only in D2 neurons. SUV is upregulated after chronic amphetamine (Renthal *et al.*, 2008), but at the earliest investigated time point (15 minutes), its regulation is unlikely to result from changes in expression levels, but is more probably due to post-translational mechanisms. In contrast, repeated injections of cocaine have been shown to down-regulate G9a expression in the NAc, but not SUV39H, via the transcription factor ΔFosB, which is induced in D1 neurons. However, we observed an increase in all three methylated forms of H3 K9 in D1 but not in D2 neurons, suggesting the existence of other regulations. Differential regulations in D1 and D2 nuclei may explain apparent contradictions in the literature. For example, chronic cocaine increases $me_2K_9/K_{27}H3$ associated with the majority of the genes studied (Renthal *et al*., 2009), whereas a global decrease in me_2K_9H3 was reported (Maze *et al*., 2010). This underlines the importance of studying histone PTMs in specific populations.

Our method combining FACS and flow cytometry analysis of nuclei provides novel results on the specific regulations of histone PTMs in the two populations of striatal MSNs. It shows the specificity, time dependence and persistence of these modifications. Thus it provides a strong framework to understand the short-term and long-lasting regulations of transcription in the two neuronal populations. Moreover, the FACS sorting of nuclei will allow to apply to cell type specific nuclei the high-throughput technologies currently used to study histone modifications on a genome-wide scale (Huebert and Bernstein, 2005), which have been so far investigated in the whole striatum only (Pedrosa *et al.*, 2009; Maze *et al.*, 2011; Zhou *et al.*, 2011). Similar approaches to investigate DNA methylation (Lister *et al.*, 2009) or hydroxymethylation (Kriaucionis and Heintz, 2009) are also being investigated in our

laboratories. The combination of these methods with flow cytometry for dynamic studies as reported here, should provide a better understanding of epigenetic regulations in the physiology of the basal ganglia and the long term actions of drugs of abuse.

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FIGURES & LEGENDS

Figure 1: Fluorescence-activated purification of nuclei in *drd1a* and *drd2* EGFP-L10a transgenic mice.

A. Outline of the procedure to study epigenetic changes in isolated neuronal population. **B.** Typical flow cytometry dot plot and gating of D1 or D2 MSN nuclei. Scatter of GFP fluorescence against Dye Cycle Ruby (DCR) fluorescence of WT mouse and *drd1a* and *drd2* EGFP-L10a transgenic mice. DCR binds quantitatively to the DNA and allows discrimination of singlets versus aggregated nuclei. The data from WT mice was used to set a threshold for the GFP signal background. **C**. After sorting, nuclei are pure and intact as verified with Amnis Image Stream analysis. Representative results for pre- and post-sort fractions from *drd1a* EGFP-L10a mice. Bright-field and GFP channels are merged, original magnification ×40.

FIGURE 1

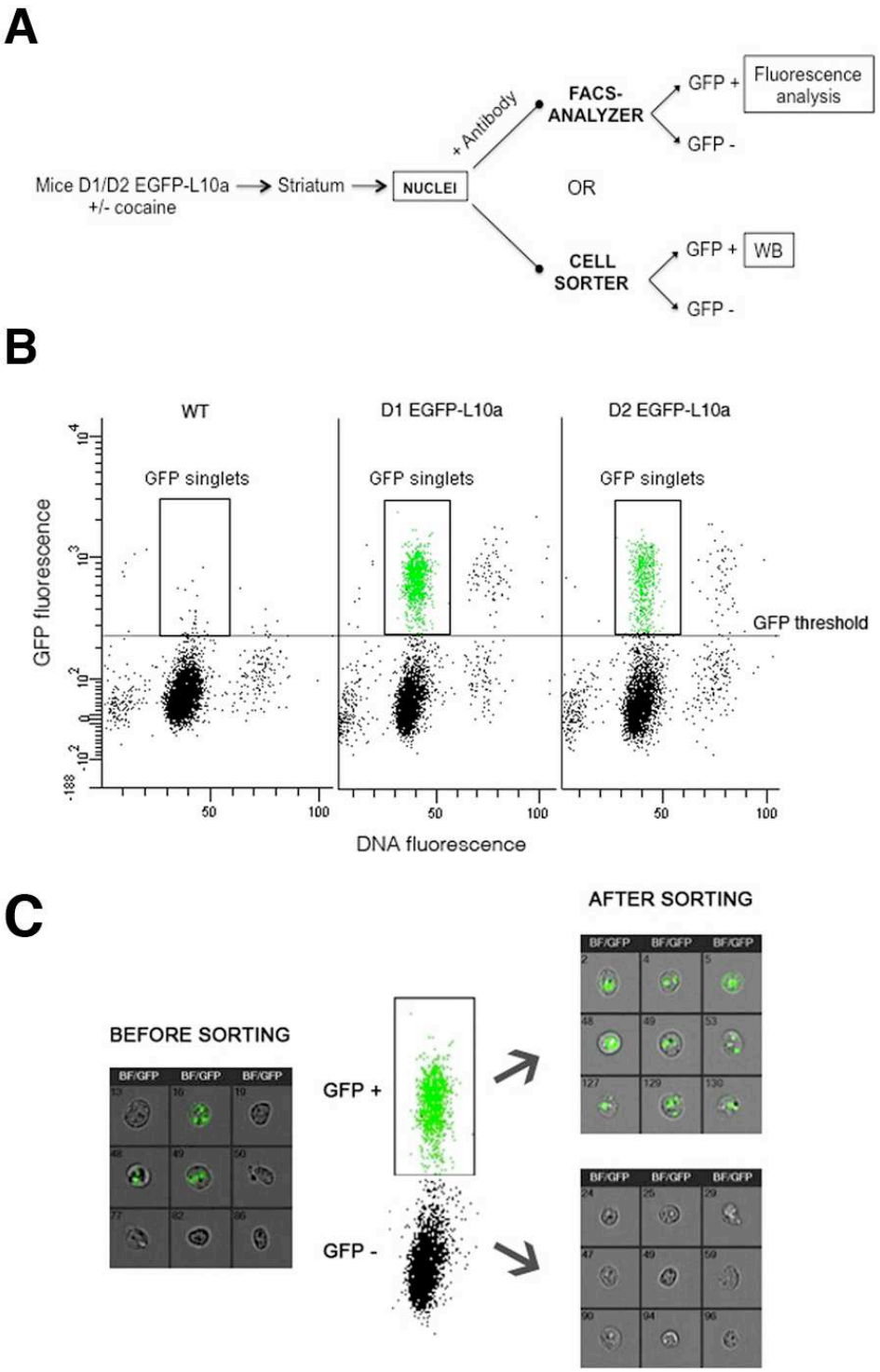
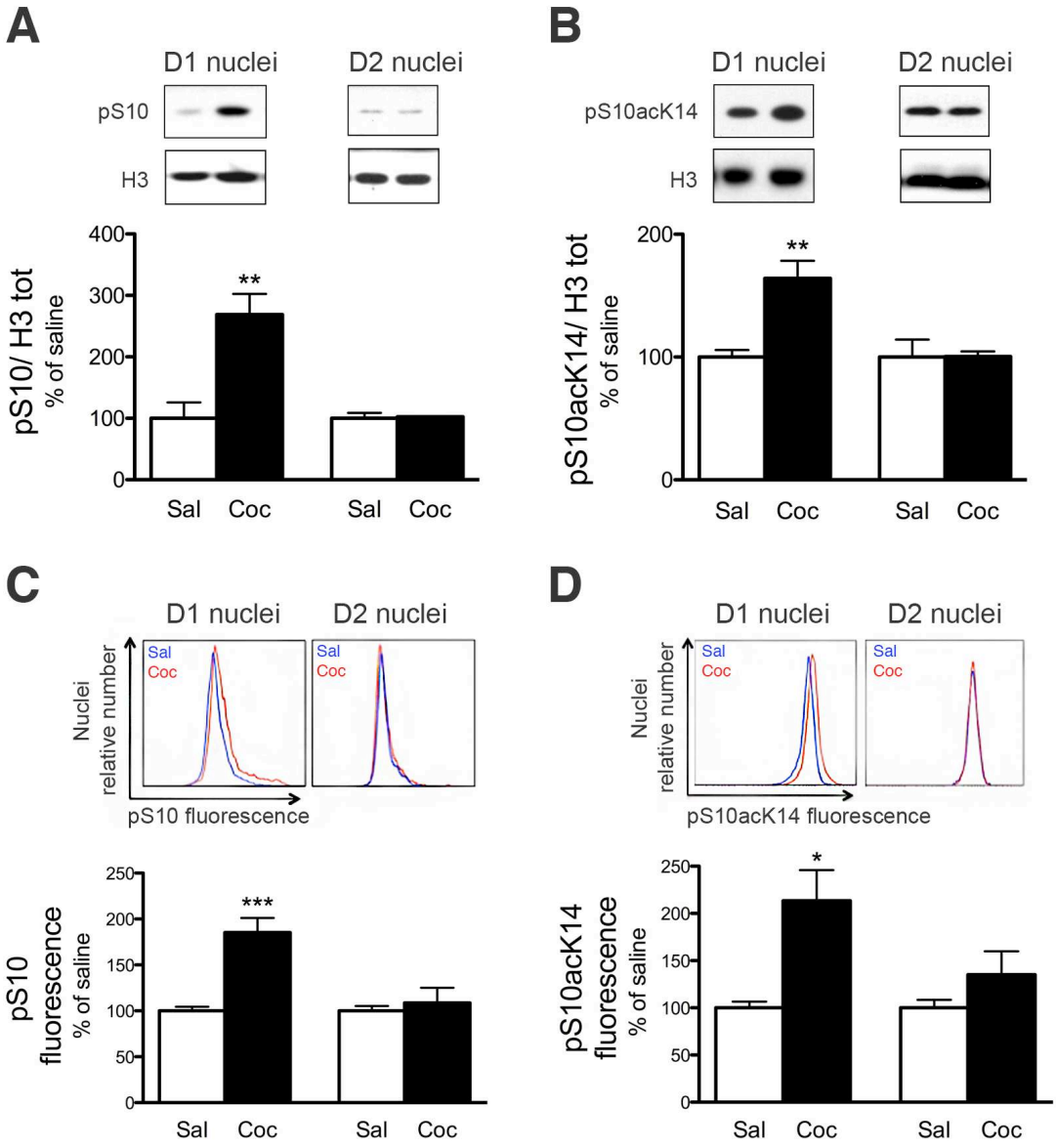

A
Mice D1/D2 EGFP-L10a
+/- cocaine
Striatum
NUCLEI
+ Antibody
FACS-ANALYZER
GFP +
GFP -
Fluorescence analysis
OR
CELL SORTER
GFP +
GFP -
WB
B
WT
D1 EGFP-L10a
D2 EGFP-L10a
GFP singlets
GFP fluorescence
DNA fluorescence
GFP threshold
C
BEFORE SORTING
AFTER SORTING
GFP +
GFP -
BF/GFP

Figure 2: Comparison of cocaine-induced histone H3 phosphorylation by nuclear flow cytometry and immunoblotting of sorted nuclei.

Mice expressing EGFP-L10a in D1 or D2 MSN neurons were treated with saline or cocaine 20 mg/kg for 15 min. **A, B**. Immunoblot analysis of phospho-Ser10 ($pS_{10}H3$, **A**.) and H3 phospho-Ser10 acetyl-Lys14 ($pS_{10}acK_{14}H3$, **B**) in lysates from FACS-sorted D1 or D2 MSN nuclei. All results were normalized to the total level of H3. Top panels show representative blots obtained using antibodies against $pS_{10}H3$, $pS_{10}acK_{14}H3$ and total H3. Bottom panels show quantification as % of saline-treated controls, mean +SEM (n=5–8; $^{**}p<0.01$ Student t-test). **C, D**. Flow cytometry analysis of $pS_{10}H3$ (**C**) and $pS_{10}acK_{14}H3$ (**D**) immunofluorescence in the GFP positive fraction corresponding to D1 or D2 MSN nuclei. Top panels show representative flow cytometry histograms after saline (blue line) or cocaine (red line) treatment. Bottom panels show the quantification of flow cytometry data as % of saline-treated controls, mean +SEM (n=8–9; $^{*}p<0.05$, $^{***}p<0.001$, Student t test).

FIGURE 2


A
D1 nuclei
D2 nuclei
pS10
H3
pS10/ H3 tot
% of saline
400
300
200
100
0
**
Sal
Coc
Sal
Coc
B
D1 nuclei
D2 nuclei
pS10acK14
H3
pS10acK14/ H3 tot
% of saline
200
100
0
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Sal
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Sal
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D2 nuclei
Nuclei
relative number
Sal
Coc
Sal
Coc
pS10 fluorescence
pS10
fluorescence
% of saline
250
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D2 nuclei
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relative number
Sal
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pS10acK14 fluorescence
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250
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Figure 3: Immunolocalization of histone PTMs in the nuclei of MSNs.

Immunolocalization of FACS analysis of acK_5H4 (top row) and of the three methylated forms of H3K9 (bottom rows) was studied by high magnification confocal microscopy in the mouse striatum. The immunolocalization was compared to the DNA staining with DAPI. Intense staining corresponds to heterochromatin (usually less actively transcribed). Scale bar: 5 µm.

FIGURE 3

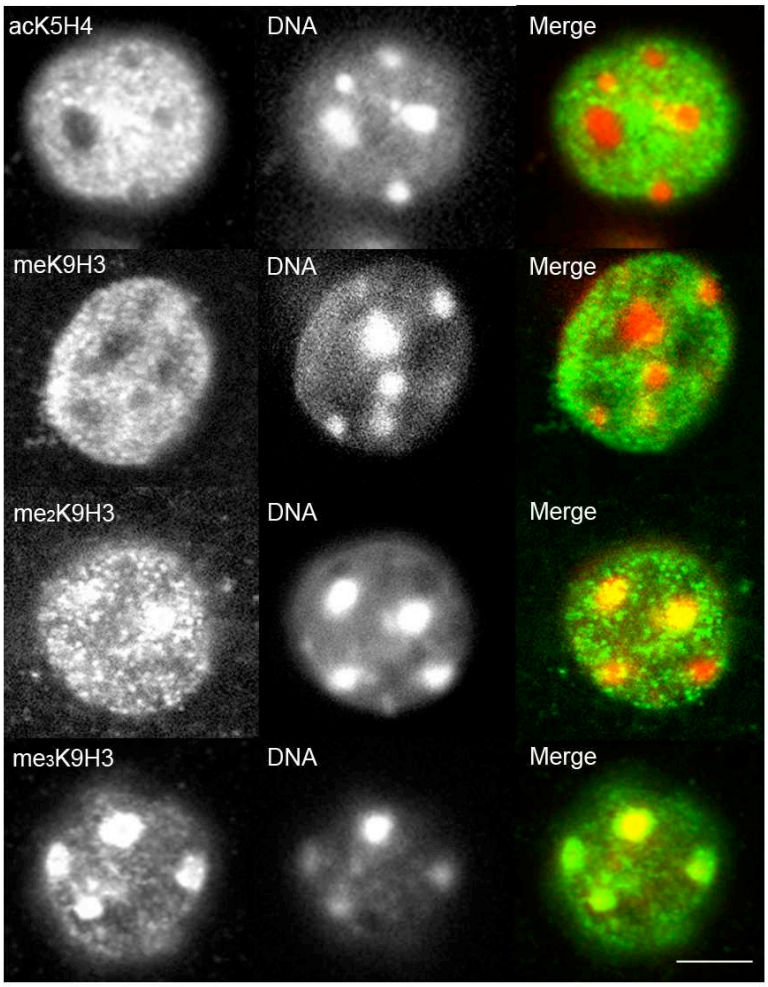

acK5H4
DNA
Merge
meK9H3
DNA
Merge
me2K9H3
DNA
Merge
me3K9H3
DNA
Merge

Figure 4: Acute and chronic cocaine induce distinct patterns of histone H3 and H4 acetylation in D1 or D2 MSNs.

Flow cytometry analysis of H3 acetyl-lysine14 (acK_{14}) and acetyl-lysine18 (acK_{18}), and of H4 acetyl-lysine5 (acK_5), acetyl-lysine8 ($H4acK_8$), and acetyl-lysine12 (acK_{12}) fluorescence in the GFP positive nuclear fraction from D1 (**A, C, E**) or D2 (**B, D**) MSNs. EGFP-L10a mice were injected with saline or cocaine 20 mg/kg and were killed 15 min (**A, B**) or 24 h (**E**) later. Other mice received a daily injection of 20 mg/kg or cocaine or saline during 7 days and were killed 24 h after the last injection (**C, D**). Quantification of flow cytometry data is represented as % of saline-treated controls, mean ± SEM (n=8–14; $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, Student t-test).

FIGURE 4

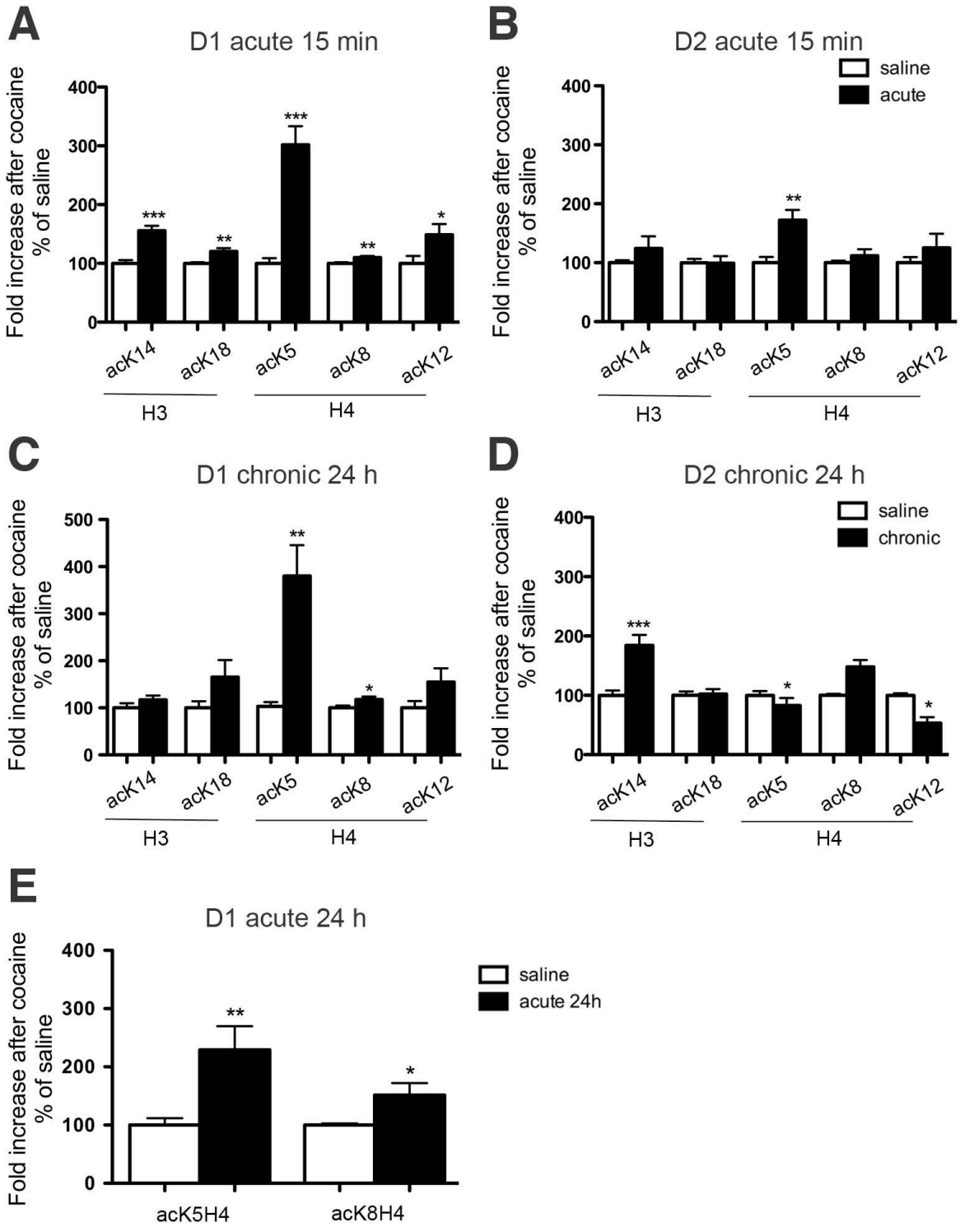

A
D1 acute 15 min
Fold increase after cocaine
% of saline
400
300
200
100
0
***
**
***
**
*
acK14
acK18
acK5
acK8
acK12
H3
H4
B
D2 acute 15 min
saline
acute
Fold increase after cocaine
% of saline
400
300
200
100
0
**
acK14
acK18
acK5
acK8
acK12
H3
H4
C
D1 chronic 24 h
Fold increase after cocaine
% of saline
500
400
300
200
100
0
**
*
acK14
acK18
acK5
acK8
acK12
H3
H4
D
D2 chronic 24 h
saline
chronic
Fold increase after cocaine
% of saline
400
300
200
100
0
***
*
*
acK14
acK18
acK5
acK8
acK12
H3
H4
E
D1 acute 24 h
Fold increase after cocaine
% of saline
400
300
200
100
0
**
*
acK5H4
acK8H4
saline
acute 24h

Figure 5. Flow cytometry reveals the apparent existence of specific subsets of D1 or D2 MSN nuclei with different levels of histone H4 lysine-5 acetylation.

acK_5H4 immunofluorescence distribution in D1 (**A, C**) and D2 (**B, D**) MSN nuclei after a single or repeated cocaine injections. For each condition. representative examples of flow cytometry plots of acK_5H4 immunofluorescence intensity in saline treated (blue line, left panel), cocaine 20 mg/kg treated (red line, middle panel) or superimposition of the 2 plots (right panel). On each row the bar graph on the right panels shows quantification of the % of total nuclei with high acK_5H4 fluorescence in saline and cocaine-treated mice. The separation of the two populations is indicated on the representative plots. Data are means + SEM (n=8–12; $^{**}p<0.01$, Student t-test).

FIGURE 5

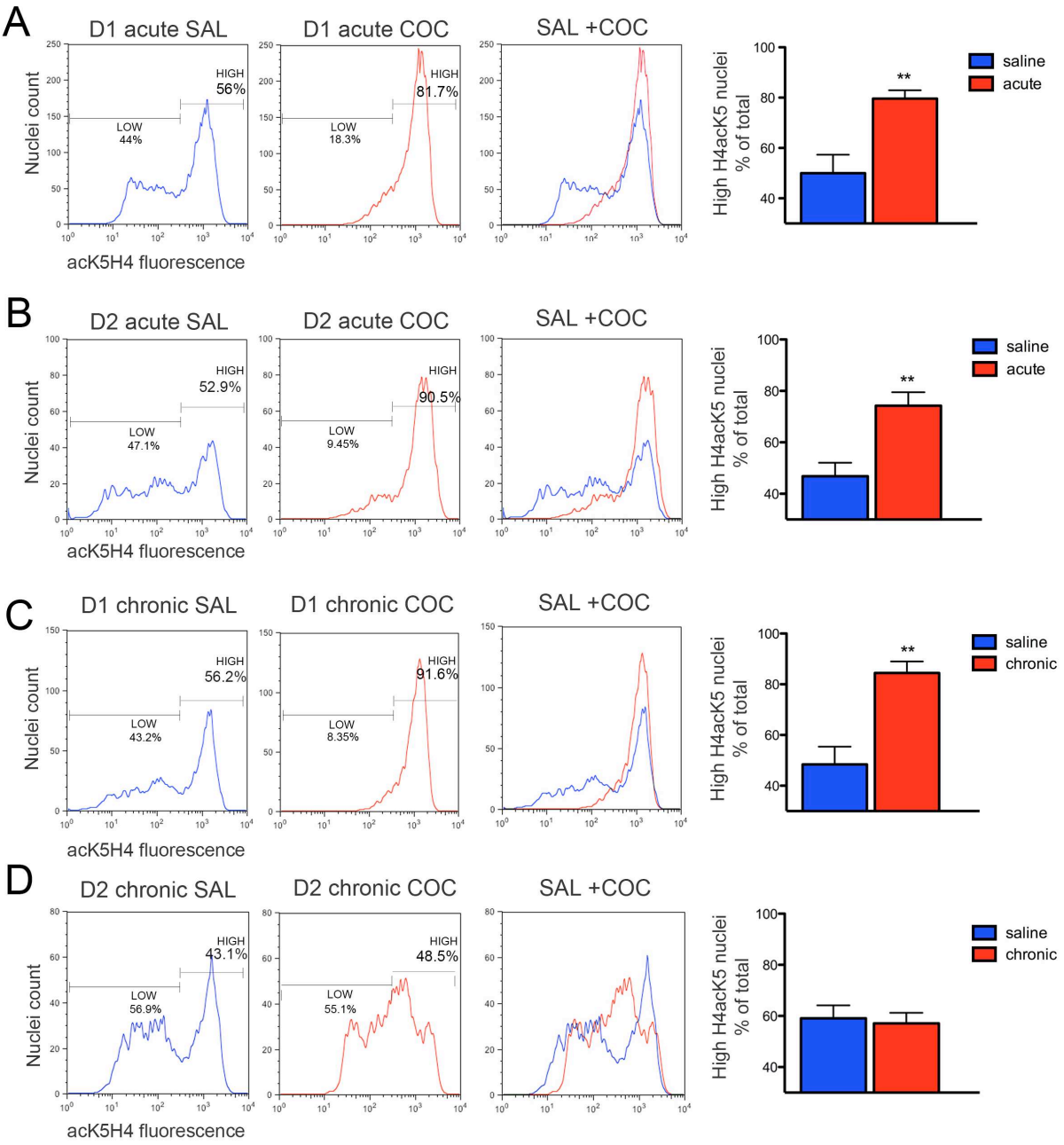

A
D1 acute SAL
D1 acute COC
SAL +COC
Nuclei count
HIGH
56%
LOW
44%
HIGH
81.7%
LOW
18.3%
acK5H4 fluorescence
High H4acK5 nuclei
% of total
**
saline
acute
B
D2 acute SAL
D2 acute COC
SAL +COC
HIGH
52.9%
LOW
47.1%
HIGH
90.5%
LOW
9.45%
C
D1 chronic SAL
D1 chronic COC
SAL +COC
HIGH
56.2%
LOW
43.2%
HIGH
91.6%
LOW
8.35%
chronic
D
D2 chronic SAL
D2 chronic COC
SAL +COC
HIGH
43.1%
LOW
56.9%
HIGH
48.5%
LOW
55.1%

Figure 6: Acute and chronic cocaine induce distinct patterns of histone H3 methylation in D1 or D2 MSNs.

FACS analysis of H3 methyl-lysine4 (me_nK4H3), H3 monomethyl-lysine9 (meK_9H3), H3 bimethyl-lysine9 ($H3me_2K9$) and H3 trimethyl-lysine9 ($H3me_3K9$) fluorescence in the GFP positive nuclei from either (**A, C, E**) or D2 (**B, D**) MSNs. EGFP-L10a mice were injected with saline or cocaine 20 mg/kg and were killed 15 min (**A, B**) or 24 h (**E**) later. Other mice received a daily injection of 20 mg/kg or cocaine or saline during 7 days and were killed 24 h after the last injection (**C, D**). Quantification of flow cytometry data is represented as % of change from saline control, mean ±SEM *(n=8–14; $^{*}p<0.05$, $^{**}p<0.01$ $^{***}p<0.001$)*.

FIGURE 6

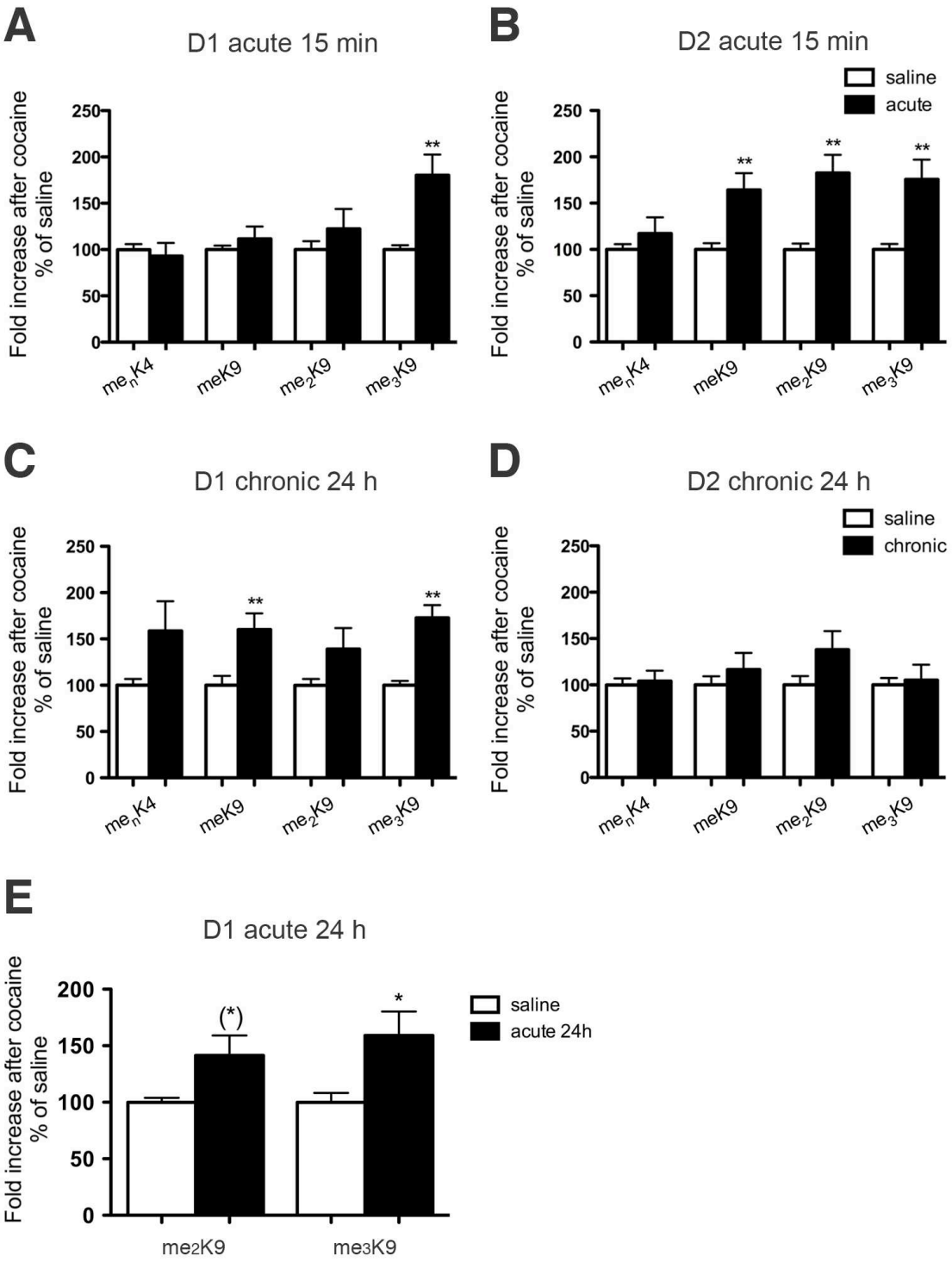
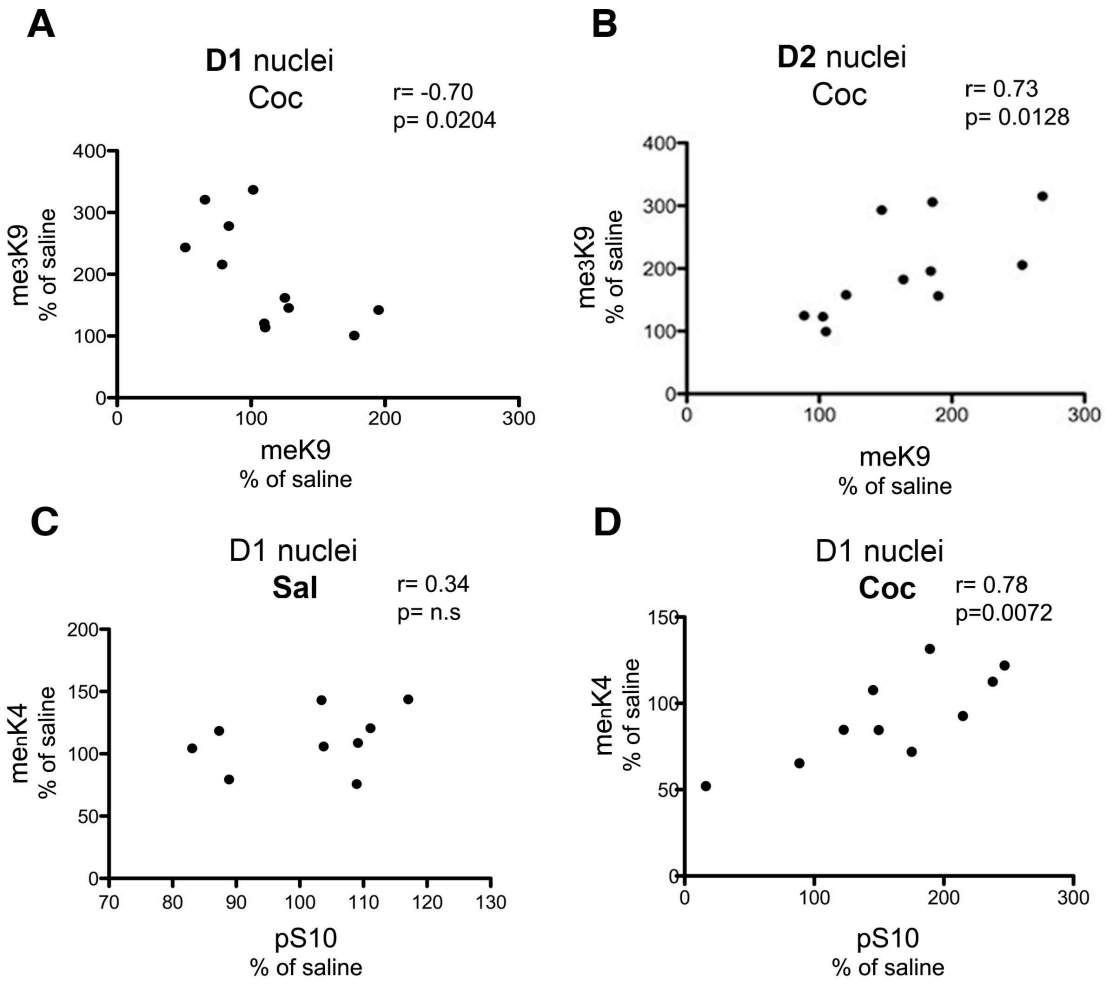

A
D1 acute 15 min
Fold increase after cocaine % of saline
250
200
150
100
50
0
**
me$_n$K4
meK9
me$_2$K9
me$_3$K9
B
D2 acute 15 min
saline
acute
**
**
**
C
D1 chronic 24 h
D
D2 chronic 24 h
saline
chronic
E
D1 acute 24 h
(*)
*
saline
acute 24h
me2K9
me3K9

Figure 7: Correlations between distinct histone post-translational modifications in MSN nuclei.

When several histone PTMs were studied in the same animals we examined whether they were correlated. Two types of interesting correlations are shown. (**A, B**) The levels of meK9H3 and me_3K9H3, 15 minutes after a single injection of 20 mg/kg cocaine and expressed as a % of saline, were negatively correlated in D1 nuclei (**A**) and positively correlated in D2 nuclei (B). (**C, D**) A significant correlation between the levels of pS_{10}H3 and meK_4H3 was observed in D1 nuclei 15 minutes after a single injection of 20 mg/kg cocaine (**D**) whereas no correlation was observed in saline treated mice (**C**). Pearson correlation coefficient (r) and its associated p-value are shown.

FIGURE 7


A
D1 nuclei
Coc
r= -0.70
p= 0.0204
me3K9
% of saline
meK9
% of saline
B
D2 nuclei
Coc
r= 0.73
p= 0.0128
me3K9
% of saline
meK9
% of saline
C
D1 nuclei
Sal
r= 0.34
p= n.s
menK4
% of saline
pS10
% of saline
D
D1 nuclei
Coc
r= 0.78
p=0.0072
menK4
% of saline
pS10
% of saline

2 COMPLEMENTARY RESULTS

2.1 REGULATION OF HDAC ACTIVITY BY ACUTE AND CHRONIC COCAINE EXPOSURE

We also were interested in studying the temporal regulation of the HDACs activity in the D1 and D2 MSNs upon acute and chronic cocaine.

2.1.1 ACUTE EFFECT

Numerous studies support the role of particular HDACs isoforms (HDAC4, HDAC5) in cocaine addictive behaviors (or in cocaine reward). However, the regulation of HDAC activity by cocaine and the relative contribution of the striatonigral and stritaopallidal pathway in this regulation are not known. Since HDAC regulation depends on their classification, we investigated members of class I: HDAC1 and 3 and class II: HDAC 4, 5, and 7. We excluded from our screen HDAC2 as mRNA expression data showed that it was not expressed in the NAc, HDAC6 as it is mainly a cytoplasmic protein as well as HDAC8 as it is not studied in the brain.

Phosphorylation of class I HDACs has been shown to promote their enzymatic and catalytic activities (Pflum *et al*., 2001; Sun *et al*., 2007). To determine whether HDAC1 and HDAC3 phosphorylation was affected 15 min after a single injection of cocaine, we purified D1 MSN and D2 MSN nuclei and examined phosphorylation at Ser421 or Ser424 using flow cytometric analysis. In D1 MSN nuclei, phosphorylation of both HDAC isoforms were not affected (**Fig. 34A**). In D2 MSN nuclei, pHDAC1 was not affected either but we observed a significant increase in pHDAC3 (**Fig. 34B**). These results suggest that cocaine regulates the phosphorylation of specific HDAC isoform differentially between the two cell types.

Together with phosphorylation, subcellular localization is another way to control HDAC activity. HDAC3 and Class II HDACs are known to shuttle in and out the nucleus upon diverse stimuli. We investigated the quantity of total HDACs present in D1 or D2 nuclei at the same time point than above. In D1 MSNs, we observed a significant reduction of nuclear HDAC3 (30% decrease, **Fig. 34C**) following acute cocaine exposure. In D2 MSNs, total HDAC3, HDAC5, and HDAC7 were also significantly reduced (40-60% decrease, **Fig. 34D**). Since 15 min is an early time, a reduction in total HDAC is unlikely due to a down-regulation of mRNA or protein turnover and is likely to imply an export of the protein out of the nucleus. These data suggest that HDAC regulation of subcellular localization is very

dynamic and can occur in response to a single injection of cocaine. known to shuttle in and out the nucleus in response to diverse stimuli. We investigated the quantity of total HDACs present in D1 or D2 MSN nuclei at the same time point than above. In D1 MSN nuclei, we observed a significant reduction of nuclear HDAC3 (30% decrease, **Fig. 34C**) following acute cocaine exposure. In D2 MSN nuclei, total HDAC3, HDAC5, and HDAC7 were also significantly reduced (40-60% decrease, **Fig. 34D**).

The mechanism of Class II HDAC export is well described (McKinsey *et al*., 2000a). It occurs upon the phosphorylation of a conserved serine motif comprised of 3 or 4 serines. All serines must be phosphorylated to induce a nuclear export. We analyzed the phosphorylation of one serine of the motif, Ser632 HDAC4, Ser498 HDAC5 and Ser318 HDAC7, after acute cocaine exposure. We found overall a moderate increase in phosphorylation, although this effect was somewhat more pronounced in D2 MSNs nuclei (**Fig. 34 A,B**). However, we could not conclude on the direct effect of phosphorylation on export as all serines must be analyzed. Also, the fact that we still observe the presence of phospho-HDAC in the nucleus could either mean that the export is yet to occur since fully phosphorylated HDACs presumably would be cytoplasmic.

Next, we normalized the fluorescence values of phosphorylated HDACs with their respective total HDAC. As expected from the above results, the normalized phospho-HDACs were overall slightly increased (< 15%) except for phospho-HDAC1 in D1 MSN nuclei (**Fig. 34E**). Conversely, we found a dramatic increase in the normalized phospho-HDAC3 level (3.5-fold increase) and phospho-HDAC7 level in D2 MSN nuclei (2.9-fold increase) (**Fig. 34F**). These results suggest that acute cocaine action is not global and acts principally in D2 MSN in terms of HDAC3 and HDAC7 regulation. In addition, it seems than HDACs are regulated differentially whether they are located in D1 or D2 MSN nuclei.

2.1.2 CHRONIC EFFECT

We next investigated the effects of repeated cocaine injections on HDACs phosphorylation and HDACs nuclear localization, 24 hr after the last injection. In D1 MSN nuclei, a moderate but significant increase in phosphorylation was observed on all HDAC isoforms (**Fig. 35A**). D2 MSN nuclei displayed the same profile with a moderate increase in phospho-HDAC1, 3, 4 (< 30 % increase, **Fig. 35B**) and a more pronounced effect on phospho-HDAC7 (> 60% increase).

We looked again at the total HDAC abundance in the nuclei at the same time point and observed a decrease in HDAC7 in D1 MSN nuclei (30% decrease; **Fig 35C**). Conversely, in D2 MSN nuclei, no changes were observed (**Fig. 35D)**.

When we normalized the phosphorylation fluorescence values with their respective

fluorescence value for the total protein, we found that phospho-HDAC1, phospho-HDAC5, phospho-HDAC7 were the three isoforms significantly modified by chronic exposure to cocaine in D1 MSN nuclei (**Fig. 35E**). In accordance with the decrease of total HDAC7, the effect on phospho-HDAC7 was much more pronounced (> 2-fold increase, **Fig. 35E**) compared to phospho-HDAC1, phospho-HDAC5 (50-60% increase). In D2 MSN nuclei, phospho-HDAC5 and phospho-HDAC7 were also increased (40-75%, **Fig. 35F**) but phospho-HDAC1 was unaffected.

Figure 34 : Differential effects of acute cocaine on HDACs in D1 or D2 MSN nuclei.

Effect of cocaine on the regulation of HDACs in D1R- and D2-R expressing MSNs. EGFP-L10a mice were treated with saline or cocaine (20 mg/kg for 15 min). FACS analysis of un-normalized HDAC1, 3, 4, 5, and 7 phosphorylation levels (**A, B**) and total HDAC levels (**C, D**) in the GFP positive fraction corresponding to D1 or D2 MSN nuclei. Values of normalized fluoresence data corresponding to the fold increase of phospho-HDACs/Total HDACs are presented in **E** (D1 MSN nuclei), **F** (D2 MSN nuclei). Quantification of flow cytometry data represented as % of change from saline control, mean ±SEM. *(n=8–16; Student t-test :* $^{*}p<0.05$, $^{**}p<0.01$ $^{***}p<0.001$*)*

ACUTE

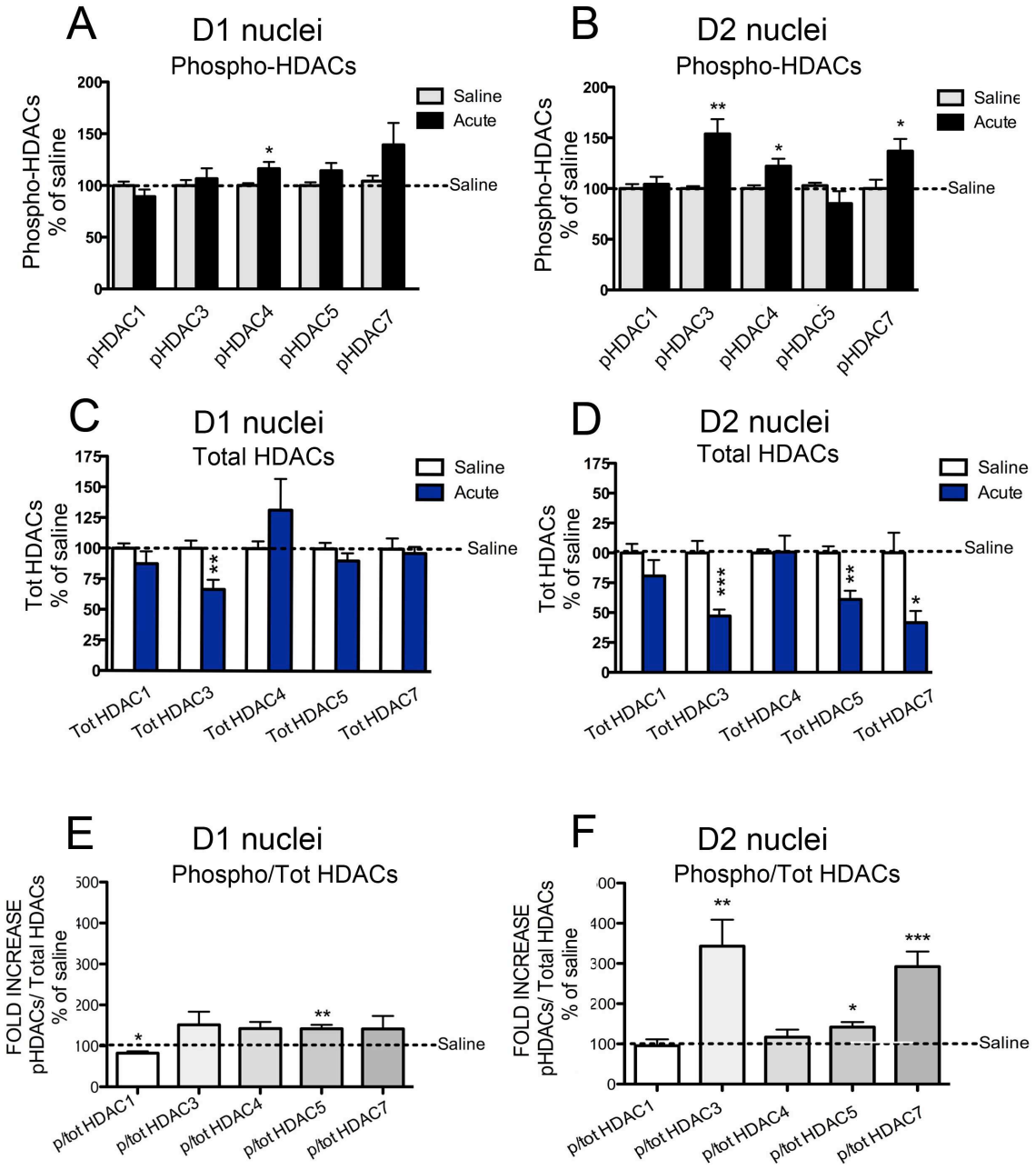
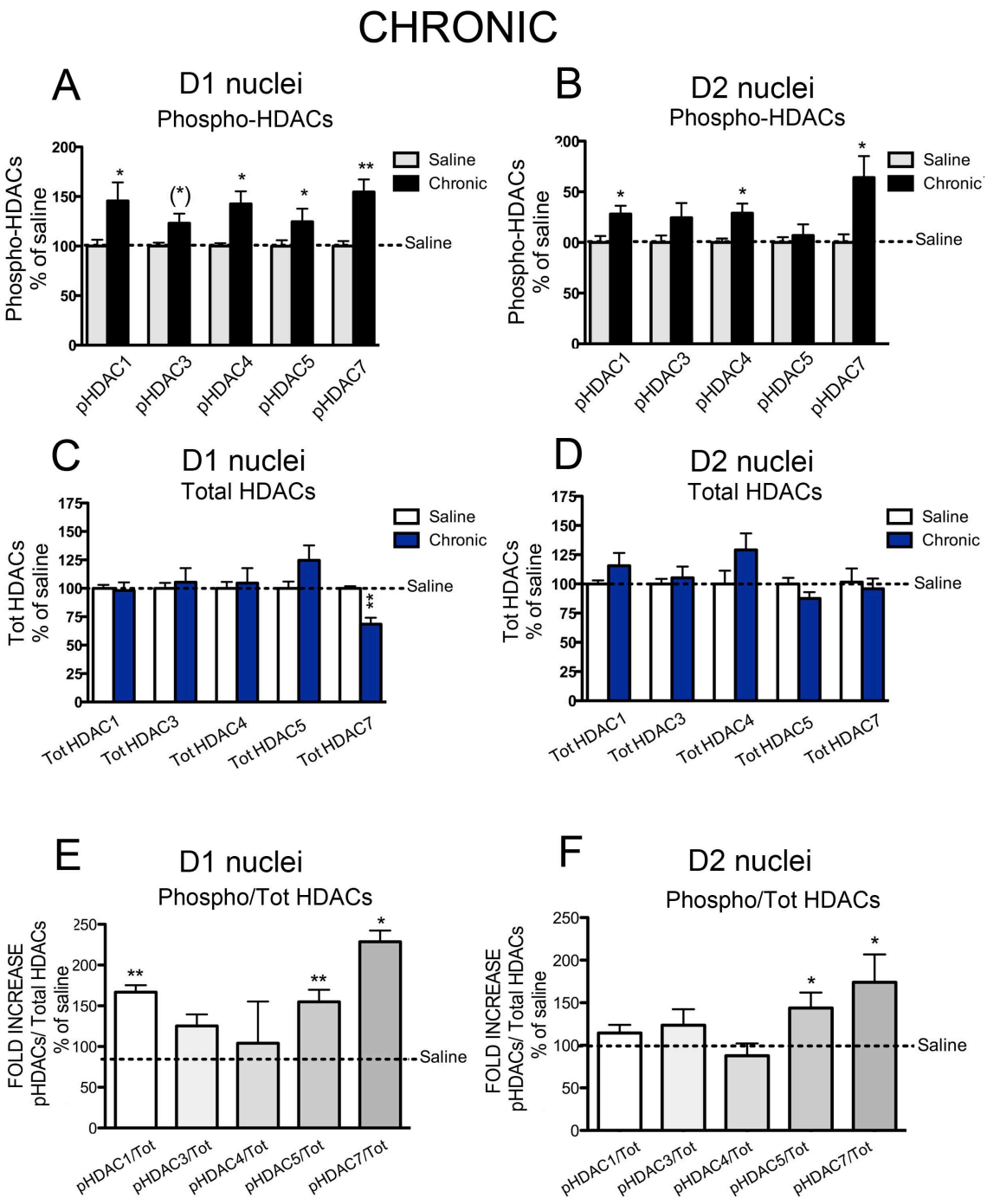

A
D1 nuclei
Phospho-HDACs
Phospho-HDACs % of saline
Saline
Acute
pHDAC1
pHDAC3
pHDAC4
pHDAC5
pHDAC7
B
D2 nuclei
Phospho-HDACs
C
D1 nuclei
Total HDACs
Tot HDACs % of saline
Tot HDAC1
Tot HDAC3
Tot HDAC4
Tot HDAC5
Tot HDAC7
D
D2 nuclei
Total HDACs
E
D1 nuclei
Phospho/Tot HDACs
FOLD INCREASE pHDACs/ Total HDACs % of saline
p/tot HDAC1
p/tot HDAC3
p/tot HDAC4
p/tot HDAC5
p/tot HDAC7
F
D2 nuclei
Phospho/Tot HDACs

Figure 35 : Differential effects of chronic cocaine on HDACs in D1 or D2 MSNs nuclei.

Effect of cocaine on the regulation of HDACs in D1R- and D2-R expressing MSNs. EGFP-L10a mice were treated daily with saline or cocaine (20 mg/kg for 7 days) and sacrificed 24 hr after the last injection. FACS analysis of HDAC1, 3, 4, 5, and 7 phosphorylation levels (**A, B**) and total HDAC levels (**C, D**) fluorescence in the GFP positive fraction corresponding to D1 or D2 MSN nuclei. Values of normalized fluoresence data corresponding to the fold increase of phospho-HDACs/Total HDACs are presented in **E** (D1 nuclei), **F** (D2 nuclei). Quantification of flow cytometry data represented as % of change from saline control, mean ±SEM. *Student t-test : (n=8–9;* $^{*}p<0.05$, $^{**}p<0.01$ $^{***}p<0.001$*)*


CHRONIC
A
D1 nuclei
Phospho-HDACs
Phospho-HDACs % of saline
Saline
Chronic
pHDAC1
pHDAC3
pHDAC4
pHDAC5
pHDAC7
B
D2 nuclei
Phospho-HDACs
C
D1 nuclei
Total HDACs
Tot HDACs % of saline
Tot HDAC1
Tot HDAC3
Tot HDAC4
Tot HDAC5
Tot HDAC7
D
D2 nuclei
Total HDACs
E
D1 nuclei
Phospho/Tot HDACs
FOLD INCREASE pHDACs/ Total HDACs % of saline
pHDAC1/Tot
pHDAC3/Tot
pHDAC4/Tot
pHDAC5/Tot
pHDAC7/Tot
F
D2 nuclei
Phospho/Tot HDACs

2.2 IDENTIFICATION OF NEW NUCLEAR SUBSTRATES OF PP1

In another line of though, we were interested in studying the role of DARPP-32 and PP1 in strital neurons. Our laboratory discovered that in striatal neurons, treatment of mice with various drugs including cocaine triggered a rapid nuclear accumulation of the physiological PP1 inhibitor, DARPP-32 through a D1R-dependent mechanism, suggesting a nuclear role for DARPP-32 (Stipanovich *et al*., 2008). PP1 has multiple substrates and functions in the nucleus and it is likely that the regulated inhibition of its activity through DARPP-32 translocation can in turn influence many others nuclear processes. Our laboratory showed that DARPP-32 amplifies phosphorylation of Histone H3 serine 10 by inhibiting PP1. It is likely that inhibition of nuclear PP1 has other targets, either direct (e.g phosphoproteins) or indirect (e.g on acetylated residues). Hence, the goal of this study is to find new PP1 nuclear substrates in order to decipher the role of nuclear DARPP-32

In this context, we cultured embryonic (E15.5) murine striatal neurons with a trophic support from a ring of surrounding cortical neurons for 10 days DIV (**Fig. 36**) (Fath *et al*., 2009). Both neural types were prepared at the same time from the same embryos. Classic primary culture of striatal cells is limited by the fact that striatal neurons are poorly differentiated and express low level of DARPP-32 when they are grown in defined media. In our co-culture model, cortical cells presumably synthesize neurotrophic factors such as BDNF that support striatal neuron differentiation. BDNF has been shown to increase DARPP-32 expression in culture of striatal neurons, greatly improving their maturation and maintenance (Ivkovic and Ehrlich, 1999). That is the reason why we chose this model over the simple striatal primary culture. It is important to note that cortical cells are not mixed with the striatal neurons (**Fig. 36A and B**). Also, they do not make any physical contact with striatal cells (data not shown). Some microscope fluorescence pictures of the culture are presented illustrating the good expression of PP1 and DARPP-32 **(Fig. 36 C-E)**.

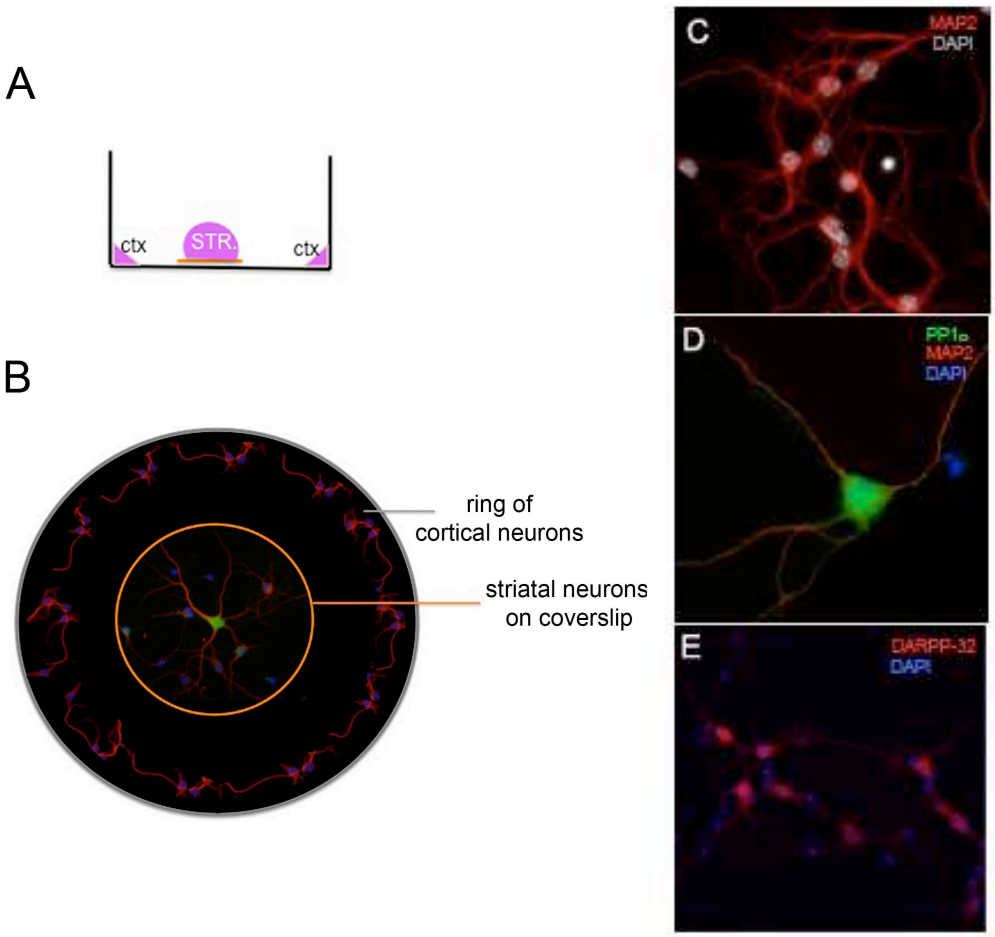

A
ctx
STR
ctx
B
ring of
cortical neurons
striatal neurons
on coverslip
C
MAP2
DAPI
D
PP1α
MAP2
DAPI
E
DARPP-32
DAPI

Figure 36: Development of a model of embryonic striatal cells cultured with a cortical support ring.

Schematic of the procedure of coating **(A, B) A.** Side view of a well of a 12 well-plate: coating is done on both the surrounding "ring" and the coverslip. Striatal cells (Str.) are plated onto the coated coverslip (represented as an orange line) and the cortical support cells (ctx) are plated as a "ring" along the inner edges of the well. **B.** View from the top. "photoediting" showing cortical cells surrounding the striatal neurons plated on the coverslip. The coverslip can be removed for immunofluorescence and microscope visualization. Characterization of the striatal primary neurons in co-culture with cortical neurons (not seen on picture), DIV10. (**C-E**) Overlaid immuostainings of striatal neurons with: **C.** MAP2 (red) and DAPI (grey) **D.** PP1α (green), MAP2 (red) and DAPI (blue) **E.** DARPP-32 (red) and DAPI (blue). All images were taken with a direct immunofluorescence microscope and 20X objective.

Since cocaine blocks the DAT dopamine transporter located presynaptically, the drug cannot be applied on DIV 10 striatal cultures that are lacking of synapses. To mimic cocaine-induced nuclear PP1 inhibition, we chemically inhibit PP1 by incubating the culture with tautomycetin (5 μM, for 1 hr at 37ºC). Tautomycetin is a potent PP1 inhibitor that selectively inhibits PP1 over PP2A (IC50 values of 1.6 and 62 nM respectively) (Mitsuhashi *et al*., 2001). In parallel, control cultures were incubated with vehicle (DMSO) and subjected to the same procedure.

All cultures were pretreated with glutamate (1-3 μM) for 10 min prior to the tautomycetin or vehicle incubation, as we observed that this stimulation was required to see the effect of PP1 inhibition of its known substrates. After the incubation with tautomycetin or vehicle, cells were fixed with PFA (4%) and striatal cells were examined by immunofluoresence microscopy. To validate our model, we first looked at residues that are known to be direct substrates of PP1 such as $pS_{10}H3$, $pS_{10}acK_{14}H3$ and S133-pCREB and we checked whether PP1 inhibition would actually increase their phosphorylation. An arbitrary fluorescence threshold was set and neurons were counted positive when the staining fluorescence for a particular marker was above the threshold. Neurons were counted automatically using an in-house metamorph program. This avoided any user-bias in the quantification. 70 to 100 neurons nuclei were counted with the nuclear DAPI staining and the neuronal-specific MAP2 staining. Positive neurons for the marker of interest (FITC) were expressed as a % of the mean of control.

As expected, we found that tautomycetin-treated neurons displayed a significant increase of phosphorylation of Ser10H3 (**Fig. 37A)** and $S_{10}acK_{14}H3$ (**Fig. 37B**) as well as of S133-CREB (**Fig. 37C**). Interestingly, the effect was much more pronounced for pS10 (>10-fold increase) than for pCREB and $pS_{10}acK_{14}$ (< 2-fold increase). This is likely due that there

were almost no positive nuclei for pS10 in the controls cells whereas basal levels of pCREB and $pS_{10}acK_{14}$ were substantially higher.

Additionally, PP1 has been shown to interact with HDACs (HDAC1, 6,10) (Canettieri *et al*., 2003; Brush *et al*., 2004) and is able to regulate HDAC1 and HDAC2 enzymatic and catalytic activities through dephosphorylation of particular serine residues (Galasinski *et al*., 2002). Hence, PP1 can indirectly regulate the acetylation states of histones through the regulation of HDAC activity. In this perspective, we investigated whether tautomycetin-induced inhibition of PP1 modified the acetylation level of H4 K5, H4 K8, H3 K14. Interestingly, H4 K5 acetylation was significantly increased in treated cultures (**Fig. 37D**), whereas no changes were observed for H4 K8 or H3 K14 (**Fig. 37 E and F**). These data are consistent with our previous data on the *in vivo* cocaine-induced increase of acK_5H4 in D1 and D2 MSN nuclei measured by FACS analysis as well data obtained from slices in cocaine-treated mice (Brami-Cherrier *et al*., 2007).

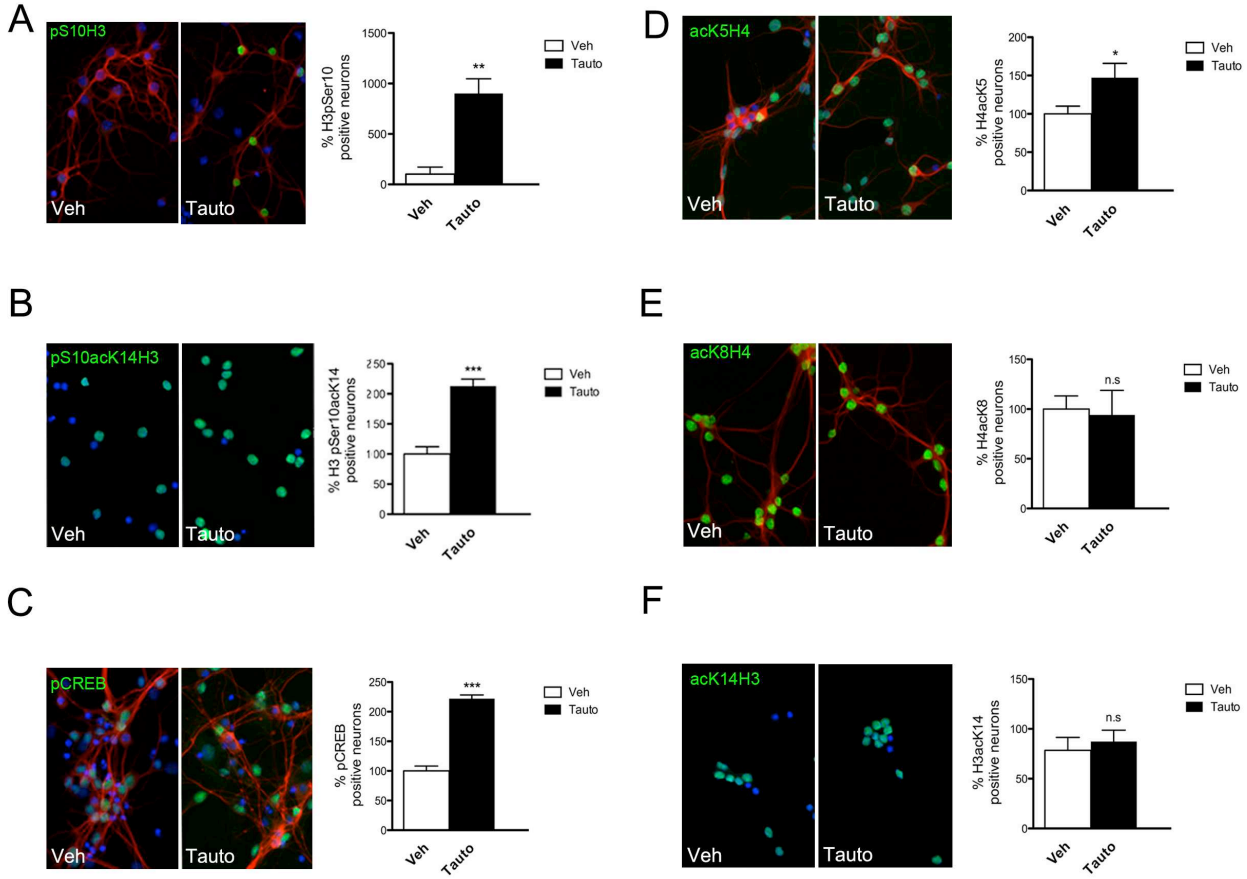


Figure 37: Effect of PP1 inhibition on nuclear protein PTMs in striatal neurons.

Left panel show representative immunofluorescence micrographs of striatal neurons treated with vehicle (Veh) or tautomycetin (Tauto) for 1 hr; Overlaid staining of MAP2 (red), DAPI (blue) and **A.** $pS_{10}H3$ (green), **B.** $pS_{10}acK_{14}H3$ (green), **C.** pCREB (green), **D.** acK_5H4 (green), **E.** acK_8H4 (green), **E.** $acK_{14}H4$ (green). Right panel show the quantification of positive neurons expressed as a % of control. Mean +/- SEM. n= 3 independent cultures and experiments. *Student t-test,* $^{*}p<0.05$, $^{**}p<0.01$ $^{***}p<0.001$, *n.s non significant*

GENERAL DISCUSSION

1. FLOW CYTOMETRIC ANALYSIS OF NUCLEI

Traditional biochemical studies have mainly reported changes in overall striatal signaling or function in response to different factors. While these studies are informative, they report an average of signaling events which obscure differences in responses between direct and indirect pathway neurons. Researchers have just started exploring cell-specific events induced by drug of abuse thanks to the technologies allowing to identify neuron families (Valjent *et al.*, 2009). However, except for the regulation of $pS_{10}H3$, we are still lacking data about cell-specific epigenetic modifications. In this perspective, guided by a previously published sorting technique using Fluorescence Activated Cell sorting (FACS-sort) (Kriaucionis and Heintz, 2009), we established a method to isolate and study the epigenetic profile of D1 and D2 MSNs. Our approach display many advantages and yet, has some limitations.

1.1 ADVANTAGES AND LIMITS

The main interest of our cell-specific approach is that we were able to detect changes induced by the drug that could not have been detected in a mixed population of cells either because they were too low or because they were opposing and canceling each other. For example, we observed moderate but significant increases of acetylation on K14 H3 and K12 H4 in D1 nuclei or on K5 H4 in D2 nuclei, following a single exposure of cocaine. Being inferior to 50%, these changes would have been averaged with the response of the other striatal cells. Even more striking is the opposite profile of methylation of histone H3 in D1 and D2 MSNs after both acute and repeated cocaine that we were able to reveal and that is likely to underlie different gene transcription or response to the drug. Finally, the divergent regulation of acK_5 H4 in the two cell types upon repeated exposure of cocaine would have been masked and thus, we would not have highlighted this discrepancy and the possible functional opposite regulation of this residue.

We wondered whether we could reproduce our data on the regulation of histone PTMs obtained with flow cytometric analysis with a more "traditional" method such as immunofluorescence (IF) of striatal section using *drd1a*-EGFP mice (experiments in collaboration with Lucile Marion-Poll). It turned out that the IF method had limitations for this particular study. There was some variability in overall fluorescence levels between animals and basal levels of fluorescence nuclei stained with either anti- acK_5H4, and mono, bi or tri meK_9H3 were too strong and heterogeneous to reliably identify drug-induced increase in fluorescence. Nevertheless a comparison of the most intensely fluorescent nuclei in the D1 (GFP-positive) and D2 (GFP-negative) neurons showed changes that were in good agreement

with the results obtained by flow cytometry. Therefore the reproducibility and high sensibility of our approach make it a method of choice for the quantitative exploration of cocaine-induced changes in histone PTMs.

One drawback of sorting cell-specific nuclei is that it delivers low yield in term of nuclear protein amount (1-3 µg of total nuclear proteins per animal depending on mouse strain, corresponding to 30 000- 90 000 nuclei). This makes downstream analysis very challenging. We managed nonetheless to perform some WB but we realized that this approach was not suitable for a screen using multiple antibodies as it would have use too many mice. That is the reason why we developed a semi high-throughput approach using FACS-analyzer that allows to get correct statistical representation of the samples with only 5000 nuclei. We could investigate 10 residues from the nuclei of one single animal.

While our screen for histones marks has provided numerous indications on cocaine-induced modifications of acetylation and methylation marks, at different time points, it is obviously not exhaustive. The combinatorial nature of the histone code remains a challenge to analyze and it is limited by the number of site-specific antibodies one can possibly use, as well as technical obstacles related to immunological reagents (cross-reactivity, epitope occlusion, lack of specificity). Hence, a more exhaustive screen can be achieved by combining D1 or D2 nuclei sorting with mass spectrometry based proteomics providing the necessary amount of lysates (by pooling animal in the same sample for example). A lot of progress in the field of histone PTMs and mass spectrometry analysis have been made to detect and quantitate combination of histone PTMs, on abundant histones isoforms (Young *et al.*, 2010).

Finally, the screen could be extended to non-histone proteins such as transcription factors (pCREB) or important kinases (pERK1/2, pMSK1, pELK) that are key actors of the striatal response to cocaine

1.2 LIMITS OF D1 AND D2 COMPARISON

We were able to obtain about 16% of D1 MSN nuclei using the *drd1a* EGFP-L10a mice and about 7% D2 MSN nuclei using the *drd2* EGFP-L10a mice. This should be compared to the expected proportions of D1R and D2R expressing cells in the striatum, which was estimated to be around 35 % of the total number of cells (including glial cells) in striatal sections for both nuclei subtypes (Matamales *et al.*, 2009). The higher yield in *drd1a*/L10a-EGFP mice as compared to *drd2*/L10a-EGFP mice is linked to a consistently higher expression of the L10a-EGFP protein in the former line, as previously observed in polysome purification studies (Heiman *et al.*, 2008) or in studies using *drd1a*/ *drd2* BAC constructs (Bateup *et al.*, 2008). This can be explained by an intrinsic or insertional weaker *drd2* promoter activity and/or a lower BAC copy number. Indeed, when we checked for purity of the GFP negative fraction

by flow imaging with Image Stream X, we noticed the existence in the D2R line, of nuclei with a dimmer EGFP fluorescence, which were not distinguishable from the background. As a result, the analyses of fluorescence were not done on the exact same amount of nuclei (at least 5000 for D1 nuclei and 3000 for D2 nuclei) but this did not limit the analysis. The possibility that the cells with a high level of GFP expression formed a subset of cells with specific properties appears theoretical and unlikely but cannot be formally ruled out.

Another possible limitation of our study is that the D1 and D2 nuclei analyses were not performed at the same time. Hence, we lacked a good standard for quantitatively comparing the absolute fluorescence levels between experiments. Consequently we were not able to directly compare basal or treated nuclei responses between the two cell-types. It would have been very informative to assess the differences in nuclear protein content and in the basal level of acetylation or methylation. The generation of a double transgenic *drd1a* tdTomato-L10a/ *drd2* EGFP-L10a BacTRAP would allow the sorting of the two populations from the same animal and would be appropriate for such direct comparison.

Finally, it was reported that the *drd2*- EGFP BAC line had an altered phenotype with significant abnormalities in the release of DA and in the response to cocaine (Kramer *et al.*, 2011). These findings would have likely bias our epigenetic analysis of the D2 MSNs. However, two very recent papers corroborated these observations (Chan *et al.*, 2012; Nelson *et al.*, 2012) but showed that this effect was dependent upon mouse strain and transgene zygosity. In commonly used inbred strains of mice such as the one we used (C57BL/6) and in mice hemizygous for the *drd2*-EGFP BAC transgene, the authors showed that there were no significant changes in striatal gene expression, physiology, or motor behavior justifying the continued use of these mice as reliable tools.

2. Mechanisms and functional relevance of the effects of cocaine on the epigenetic profile of D1 and D2 MSNs

Modulation of gene expression is necessary in order to consolidate long-lasting changes during plasticity. Growing evidence suggests that long-lasting changes in the properties of striatal neurons involve epigenetic processes including post-translational modifications of histones. In this thesis, we investigated the effect of single and repeated cocaine exposures on the histone acetylation and methylation and the enzymes controlling them in the D1 and D2 MSNs. We chose three paradigms of injection to cover different aspects of the drug response. With the acute regimen (1 single injection, sacrifice 15 min later), we examined the reactiveness of the system to the drug, whereas the repeated injections regimen (7 daily injections, sacrifice 24 h later) allowed us to analyze the long-lasting biochemical responses. In some cases, we studied the effect of a single exposure (1 single injection, sacrifice 24 h

later) to decipher whether the changes observed were caused by the recurrent effect of the cocaine itself or whether it was due to the persistence of the PTM.

2.1 MECHANISMS OF PTMS REGULATION

The steady-state levels of acetylation and methylation of the core histones result from the balance between the opposing activities of the histone acetyltransferases (HATs)/ histone deacetylases (HDACs) and the histone methyltransferases / histone demethylases, respectively.

We investigated the regulation of HDACs phosphorylation and their nuclear abundance that are two important mechanisms in the control of HDAC activity, following acute or repeated cocaine exposure. The control of HDAC activity depends on their class. Members of class I (HDAC 1 and 3) are activated by phosphorylation whereas members of class II (HDAC 4, 5, and 7) are exported out of the nucleus upon phosphorylation of conserved serines.

A general observation that can be done is that cocaine regulates the phosphorylation of specific HDAC isoforms differentially between the two cell types. Unexpectedly, our screen revealed that several HDAC phosphorylations occurred in the D2 nuclei in response to acute or repeated cocaine (HDAC3 and HDAC7 in acute, HDAC5 in chronic). The majority of previous data on the effects of cocaine on MSNs described a clear-cut segregation of the signaling responses to cocaine between D1 and D2. For example, the phosphoproteins pMSK1, pERK, pH3 are exclusively activated in D1 MSNs upon acute cocaine injection in the home cage (Brami-Cherrier *et al.*, 2007; Bertran-Gonzalez *et al.*, 2008). Conversely, these phosphoproteins are activated in D2 MSNs following D2R blockade by antipsychotic administration (Bertran-Gonzalez *et al.*, 2009). These phosphorylations depend on the activation of the cAMP/PKA cascade induced by dopamine D1R stimulation or by D2R inhibition. Hence, the presence of phospho-HDAC in D2 MSNs suggests that their regulation may be controlled through other signaling cascade(s). Examples of signaling cascades activated in D2 neurons include the Akt pathway (Brami-Cherrier *et al.*, 2002) and Cdk5 (Bateup *et al.*, 2008) and additional ones may be also regulated. Thus, our data, as many others in the literature, imply that striatal signaling in response to cocaine do not occur exclusively in striatonigral neurons and that both cell types are subjected to plasticity although their persistence may differ (Lee *et al.*, 2006).

In addition, we observed a reduction in the nuclear enrichment of HDAC3 in D1 MSNs nuclei and in HDAC3, HDAC5 and HDAC7 in D2 nuclei MSNs following a single injection of cocaine. Since 15 min is an early time point, a reduction in total HDAC is unlikely to be due to a down-regulation of mRNA or protein turnover and is likely to imply an export of the protein out of the nucleus. These results suggest that HDAC regulation of

subcellular localization is very dynamic. Accordingly, another group demonstrated that cocaine induced HDAC5 nuclear export in the NAc 30 min after chronic cocaine exposure (Renthal *et al.*, 2007). Once again, a moderate export occuring in specific cell-type may not have been detected in total NAc. We did not observe any export of HDAC5 in our chronic paradigm probably because it is not a long-lasting phenomenon. Export of HDACs induces a reduction in histone deacetylation and an increase in gene expression of the putative HDAC targets. In regard to the specificity of D1 and D2 MSNs, export of HDAC5 and HDAC7 in D2 MSNs exclusively may result in the expression of specific subset of genes.

Along the same line of thought, HDAC3 and HDAC7 isoforms were strongly modulated by acute cocaine in D2 MSNs. The two isoforms both displayed an increased phosphorylation concomitant with the initiation of their export out of the nucleus. Phosphorylation of HDAC3 at Ser424 promotes its enzymatic and catalytic activity suggesting that at 15 min D2 nuclei contained a very active form of the repressor, although that increased activity is somehow counteracted by its export. In any case, these two isoforms seem to be the hallmark of D2 MSNs and may contribute to specific gene expression.

One interesting observation in the chronic effect of cocaine is the long-lasting increase of phosphoHDAC1 in D1 exclusively. The specificity of HDAC1 regulation in D1 MSNs may be correlated to HDAC1 implication in the desensitization of immediate early genes (IEGs). HDAC1 has been shown to interact with ΔFosB and desensitize the IEG *c-fos* upon repeated injections of amphetamine (Renthal *et al.*, 2008). Here, we found that HDAC1 is phosphorylated in D1 MSNs only upon repeated injections of cocaine but not after an acute exposure. Phosphorylation of HDAC1 at Ser421 promotes it enzymatic and catalytic activity, as well as its capacity to interact with other partners. This temporal regulation matches with the desensitization of IEG and the cell-type specificity of IEG induction. ΔFosB is expressed in D1 nuclei subtypes, and could bind HDAC1 when phosphorylated only, i.e in chronic condition only. An interesting experiment would be to perform a chromatin immnoprecipitation (ChIP) against pHDAC1 in D1 nuclei and examine whether HDAC1 binds IEG promoter genes.

A fundamental question raised by this study is the regulation HDACs kinases and phosphatases in D1 and D2 nuclei MSNs. We found that, in acutely-treated mice, the normalized phosphorylation of HDAC3 was strongly increased in D2 but not D1. This could suggest that the set of kinase/phosphatase controlling the steady-state of HDAC3 phosphorylation is differentially activated between D1 and D2 nuclei MSNs. CK2 phosphorylates HDAC3 at Ser424 while PP4 dephosphorylates it. This could suggest that, CK2 is activated in D2 and not D1 nuclei and/or that PP4 is inactivated following 15 min of cocaine exposure. Likewise, pHDAC7 was strongly increased in D2 and not in D1 nuclei suggesting that protein kinase D (PKD), the HDAC7 Ser318 kinase, and PP2A, the corresponding phosphatase could be differentially regulated between the two cell types. These

two hypotheses could be confirmed by investigating the nuclear enrichment and, when it is detectable by changes in immunofluorescence, the activation state of these proteins using our flow cytometric analysis approach.

Finally, HDACs have relatively low substrate specificity by themselves. Their specificity depends on interaction with transcription factors that target them to specific site of the chromatin (Kuo and Allis, 1998). Hence, it was not possible based on our data to know which particular lysine could be affected by the changes in HDAC regulation observed. The only way to investigate this issue would be to study HDAC KO mice and to examine the changes in lysine acetylation (Guan *et al.*, 2009). On the other hand, HAT specificity appears to be higher (Kuo and Allis, 1998) but we could not investigate HAT changes following cocaine administration because of the lack of available antibodies suitable for flow cytometry. All together, our results provide evidence that cocaine action on HDACs is highly isoform specific. In addition, they show that HDACs are regulated differentially depending on their location in D1 or D2 MSN nuclei, perhaps contributing to their divergent profile of gene expression.

2.2 Effects of cocaine-induced alterations in histone PTMs

Research by Nestler's group found that relatively few cocaine-regulated genes were associated with reductions in histone acetylation or methylation, suggesting that the most common mechanisms of cocaine-induced gene regulation in the NAc involve increases in histone acetylation for gene activation (rather than demethylation) or an increase in histone methylation for gene repression (rather than deacetylation) (Renthal *et al*., 2009). This is consistent with what we observed in our screen, since we generally found an increased acetylation and an increased methylation.

A relevant question for understanding the long-lasting effects of cocaine on striatal plasticity is the identification of persistently modified histone residues. We found that acK_5H4 and me_3K_9H3 are persistent marks since their acetylation and methylation was still increased 24 h after a single injection of cocaine in D1. It will be extremely interesting to determine whether these marks can last even longer (work in progress). The main biological point is that a sustained K5 acetylation or K9 methylation may have effective consequences on gene expression, inducing gene responsible for long-lasting behavioral changes and/or putting the chromatin in a different state of responsiveness to new stimuli.

Post-translational modifications of histones may increase or decrease the rate of transcription of genes involved in plasticity in MSNs. Since we observed an opposite profile of global H3 K9 methylation as well as a strong difference in acK_5H4 regulation by cocaine between D1 and D2 MSNs, we can hypothesize that these differential regulations participate in the specificity of transcriptional changes in D1 and D2 MSNs (Heiman *et al.*, 2008). An

interesting experiment to do would be to pull-down the genes that are enriched in acK5 or in K9 methylation by ChIP. Since acetylation is associated with active transcription, we could examine whether the genes promoter enriched in acK_5 are up-regulated in D1 and down-regulated in D2 based on cell-specific expression data (Heiman *et al.*, 2008) (Heiman *et al*., 2008 and unpublished data).

Last, the identification of unique histone marks correlating with a specific behavioral output could be very informative. Peleg *et al* (2010) identified H4K12 acetylation as a key residue in learning and memory. However, it seems naïve to expect that a specific histone residue modification would correspond to a particular behavior. It is more likely that multiple changes in the patterns of chromatin modifications in various neurons contribute to the altered behavioral responses through changes in neuronal and synaptic properties.

3. ROLE OF DARPP-32/PP1 CASCADE IN THE NUCLEUS

The presence of PP1 immunoreactivity in the nucleus of MSNs strongly suggests that this phosphatase may play an important role in the control of nuclear proteins activity (Ouimet et al., 1995). Furthermore, cocaine-induced nuclear accumulation of pThr-34 DARPP-32, the physiological PP1 inhibitor, appears to be important for the increase in phosphorylation of serine10 H3 in D1 MSNs (Stipanovich *et al.*, 2008). Additionally, PP1 has been shown to interact with HDACs (HDAC1, 6, and 10) (Canettieri *et al.*, 2003; Brush *et al.*, 2004) and is able to regulate HDAC1 and HDAC2 catalytic activities through dephosphorylation of particular serine residues (Galasinski *et al.*, 2002). Hence, PP1 can indirectly regulate the acetylation states of histones through the regulation of HDAC activity.

We investigated the effects of PP1 inhibition by tautomycetin on acK_5H4, acK_8H4 and $acK_{14}H3$. H4 K5 acetylation increased significantly suggesting that PP1 is able to regulated indirectly acetylation. However, the increase of acK5 was moderate compared to the increase of our control, phosphoSer10, suggesting there are other pathways controlling the steady state of K5 acetylation like other HDAC not interacting with PP1. Moreover, it is important to underline that phosphatase inhibition is expected to potentiate the action of activated kinases. In the experimental conditions we used it is possible that this activation was not complete, as compared to adult striatum. Nevertheless, our data support a role for nuclear D32 and PP1 in regulating other histone PTMs than phosphorylation. Furthermore, they provide evidence to test the implication of DARPP-32 in the regulation of histones PTMs by cocaine. To examine this question we have prepared BacTRAP mice lacking DARPP-32. Since DARPP-32 activation is a D1R- dependent mechanism, D1 and D2 epigenetic profiles are very likely to be differentially altered by the absence of DARPP-32. Determining how this overall regulation directly affects neuronal function will shed light on the complex nuclear processes that mediate neuronal plasticity.

4. CONCLUSION

Our study provides important insights into the molecular response to cocaine of the two neurons subtypes that comprise the direct and indirect pathways, underlying their gene expression specificity. The changes induced in one or the other population of striatal neurons may modify the balance of striatonigral and striatopallidal pathways activity, and thereby contribute in the long-term effects of psychostimulants and presumably other drugs of abuse.

The development of efficient tools to study cell-type specific PTMS of nuclear proteins will allow studying them in models of addiction, such as drug self-administration. Thus, the characterization of discrete epigenetic marks could have clinical implications and allow the identification of new therapeutic targets, in order to prevent the maintenance of long-term drug-induced modifications, and relapses from occurring.

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