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MOLECULAR MECHANISMS UNDERLYING THE ACTIONS OF PSYCHOACTIVE DRUGS IN THE BASAL GANGLIA: FOCUS ON CANNABINOIDS AND MORPHINE

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Cover: Striatal medium spiny neurons. Immunohistochemistry with a monoclonal antibody detecting DARPP-32.

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ABSTRACT

This thesis is centered on the identification of the molecular mechanisms involved in the psychomotor effects of cannabinoids and morphine. These drugs share the ability of acting at the level of the basal ganglia, a group of subcortical structures involved in the control of locomotion, as well as in cognitive and motivational aspects of motor function.

In Paper I and II, we have examined the involvement of the dopamine- and cAMP-dependent phosphoprotein of 32 kDa (DARPP-32) in the motor depressant effect produced by activation of the neuronal CB1 receptor (CB_1R). DARPP-32 is highly expressed in the medium spiny neurons of the striatum, which is the largest component of the basal ganglia. We found that administration of CP55,940, a selective CB_1R agonist, or Δ^9-tetrahydrocannabinol (Δ^9-THC), the active component of marihuana or hashish, increased the state of phosphorylation of DARPP-32 at the cAMP-dependent protein kinase site (PKA), Thr34. Similar increases were observed with AM404, a blocker of the reuptake of endogenous cannabinoids (e.g. anandamide and 2-arachidonyl glycerol), or URB597, an inhibitor of the enzyme fatty acid amide hydrolase (FAAH), which is responsible for the degradation of endocannabinoids. The motor depressant effect (catalepsy) produced by CP55,940, was attenuated by genetic inactivation of DARPP-32. Point mutation of Thr34 on DARPP-32 produced a similar reduction in the effect of the CB_1R agonist. Genetic inactivation either of dopamine D_2 receptors (D_2Rs) or of adenosine A_{2A} receptors ($A_{2A}Rs$) reduced the phosphorylation of DARPP-32 at Thr34 and the motor depression produced by CP55,940. These data indicated that a considerable proportion of the psychomotor effect of cannabinoids is accounted for by a signaling cascade involving PKA-dependent phosphorylation of DARPP-32, achieved via CB_1R-mediated modulation of D_2R and $A_{2A}R$ transmission.

In Paper III, we have examined the involvement of DARPP-32 in the short- and long-term effects of morphine. We found that acute administration of morphine increased DARPP-32 phosphorylation at Thr34 in both dorsal striatum and ventral striatum (nucleus accumbens). The ability of morphine to stimulate Thr34 phosphorylation was prevented by blockade of dopamine D_1 receptors (D_1Rs). Genetic inactivation of DARPP-32 or point mutation of Thr34 reduced the hyperlocomotor response to a single injection of morphine. In contrast, DARPP-32 mutant mice developed behavioral sensitization to morphine comparable to that of wild-type controls and displayed normal morphine conditioned place preference. These results demonstrated that dopamine D_1R-mediated activation of the cAMP/DARPP-32 cascade in striatal MSNs is involved in the psychomotor action, but not in the rewarding properties, of morphine.

Exposure to cues previously associated with intake of substances of abuse can promote drug related responses. In Paper IV, we have examined the effects of exposure to a drug-associated context on the psychomotor response to morphine. We found that the psychomotor sensitization produced by repeated administration of morphine was markedly increased in mice examined 4 weeks after the last drug injection. In addition, the withdrawal period was able to confer to the environment paired with morphine the ability to increase ERK phosphorylation in a specific compartment (i.e. the shell) of the nucleus accumbens. Using transgenic mice with enhanced green fluorescent protein (EGFP) expression under the control of the D_1R (*drd1*a-EGFP) or D_2R promoter (*drd2*-EGFP), we showed that context-dependent ERK phosphorylation was restricted to D_1R-expressing MSNs. Furthermore, we found that this effect depended on D_1R activation. This study showed that, following repeated morphine injections, a drug free period induced context-dependent phosphorylation of ERK in a discrete group of neurons within the nucleus accumbens shell. This activation was associated with enhanced psychomotor sensitization and could be implicated in context-elicited drug seeking induced by repeated exposure to drugs of abuse.

LIST OF PUBLICATIONS

I. Andersson M, Usiello A, **Borgkvist A**, Pozzi L, Dominguez C, Fienberg AA, Svenningsson P, Fredholm BB, Borrelli E, Greengard P, Fisone G. Cannabinoid action depends on phosphorylation of dopamine- and cAMP-regulated phosphoprotein of 32 kDa at the protein kinase A site in striatal projection neurons. J. Neurosci., 2005, 25(37):8432-8

II. **Borgkvist A**, Usiello A, Greengard P, Fisone G. Regulation of cAMP/DARPP-32 by Δ^9-tetrahydrocannabinol. Neuropharmacology, 2008, 54(1):31-5

III. **Borgkvist A**, Usiello A, Greengard P, Fisone G. Activation of the cAMP/PKA/DARPP-32 signaling pathway is required for morphine psychomotor stimulation but not for morphine reward. Neuropsychopharmacology, 2007, 32(9):1995-2003

IV. **Borgkvist A**, Valjent E, Santini E, Hervé D, Girault JA, Fisone G. Delayed, context- and dopamine D1 receptor-dependent activation of ERK in morphine-sensitized mice. Submitted Manuscript

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LIST OF ABBREVIATIONS

2-AG	2-arachidonyl glycerol
Δ^9-THC	Δ^9-tetrahydrocannabinol
A_1R / $A_{2A}R$	Adenosine receptor 1 / Adenosine receptor 2A
ADHD	Attention-deficit/hyperactivity disorder
AEA	Anandamide
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole proprionic acid
ATP	Adenosine-5'-triphosphate
B-Raf	B-Raf proto-oncogene serine/threonine protein kinase
cAMP	3'-5'-cyclic adenosine monophosphate
CB_1R / CB_2R	Cannabinoid receptor 1 / Cannabinoid receptor 2
Cdk5	Cyclic-dependent kinase 5
CK1 / CK2	Casein kinase 1 / Casein kinase 2
CREB	3'-5'-cyclic adenosine monophosphate response element-binding
D_1R / D_2R	Dopamine D1 receptor / Dopamine D2 receptor
DA	Dopamine
DARPP-32	Dopamine- and cAMP-regulated phosphoprotein of 32 kDa
eCB	Endocannabinoid
ERK1/2	Extracellular signal-regulated kinase 1 and 2
FAAH	Fatty acid amide hydrolase
GABA	γ-aminobutyric acid
GluR1	Glutamate receptor 1 (AMPA receptor subunit)
GPCR	G-protein coupled receptor
GPe / GPi	Globus Pallidus pars externa / Globus pallidus pars interna
GTP	Guanosin-5'-triphosphate
MAP	Mitogen activated protein
MAPK	Mitogen activated protein kinase
MDMA	1-(3,4-metylendioxifenyl)-2-metyletyl-amin, (Ecstasy)
MEK1/2	MAP/ERK kinase 1 and 2
MEKK	MEK kinase
MKP3	MAP kinase phosphatases-3
mRNA	Messenger ribonucleic acid
MSK1	Mitogen- and stress-activated kinase 1

NAcb	Nucleus accumbens
NMDA	*N*-methyl-D-aspartate
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PKB	Protein kinase B (or Akt)
PP1	Protein phosphatase 1
PP-2A	Protein phosphatase 2A
PP-2B	Protein phosphatase 2B (or calcineurin)
RSK	Ribosomal protein S6 kinase
SNpc	Substantia nigra pars compacta
SNpr	Substantia nigra pars reticulata
STN	Subthalamic nucleus
VTA	Ventral tegmental area

1 INTRODUCTION

1.1 THE BASAL GANGLIA

The basal ganglia are a group of subcortical nuclei that give rise to a functional system involved in various aspects of initiation and control of movement. The main inputs to the basal ganglia originate in the cerebral cortex, limbic structures and thalamic nuclei, which innervate the striatal complex [caudate-putamen and nucleus accumbens (NAcb)] (Alexander et al., 1986; McHaffie et al., 2005). The corticostriatal pathway is organized in parallel, segregated circuits, so that specific cortical areas innervate subregions of the basal ganglia, which feed back on the same cortical areas. These cortico-basal ganglia-cortical pathways are critically involved in execution of selected motor programs (Alexander et al., 1986).

The striatum is the main receiving area of the basal ganglia, and ~95% of all striatal neurons consist of GABAergic medium spiny neurons (MSNs) (Gerfen, 1992b). Although the striatum lacks a distinct cytoarchitectural definition, its uniform structure can be divided into a functional gradient following a dorsolateral – ventromedial axis on the basis of cortical, thalamic and limbic innervations (Voorn et al., 2004). For example, the dorsolateral striatum receives primarily somatotopically arranged sensorimotor-related inputs from motor and sensory cortices (Brown et al., 1998), whereas the ventral striatum (which corresponds to the NAcb) is predominantly innervated by limbic structures such as infralimbic cortex, the basal amygdala complex and the hippocampal formation (de Olmos and Heimer, 1999). It must be pointed out, however, that since overlap exists among the different corticostriatal projections, the striatal territories should be viewed more as a continuum rather than subdivisions with strict boundaries (Voorn et al., 2004)

1.1.1 Functional organization of the dorsal striatum

The MSNs are functionally divided in two populations of approximately equal number of cells (Kawaguchi et al., 1990) according to their projections to the output structures of the basal ganglia, the substantia nigra pars reticulata (SNpr)/globus

pallidus pars interna (GPi; entopeduncular nucleus in rats) (Fig 1). The direct pathway, or striatonigral pathway, projects from the striatum directly to the SNpr/GPi, whereas the indirect pathway, or striatopallidal pathway, projects to the SNpr/GPi via the globus pallidus pars externa (GPe) and the subthalamic nucleus (STN).

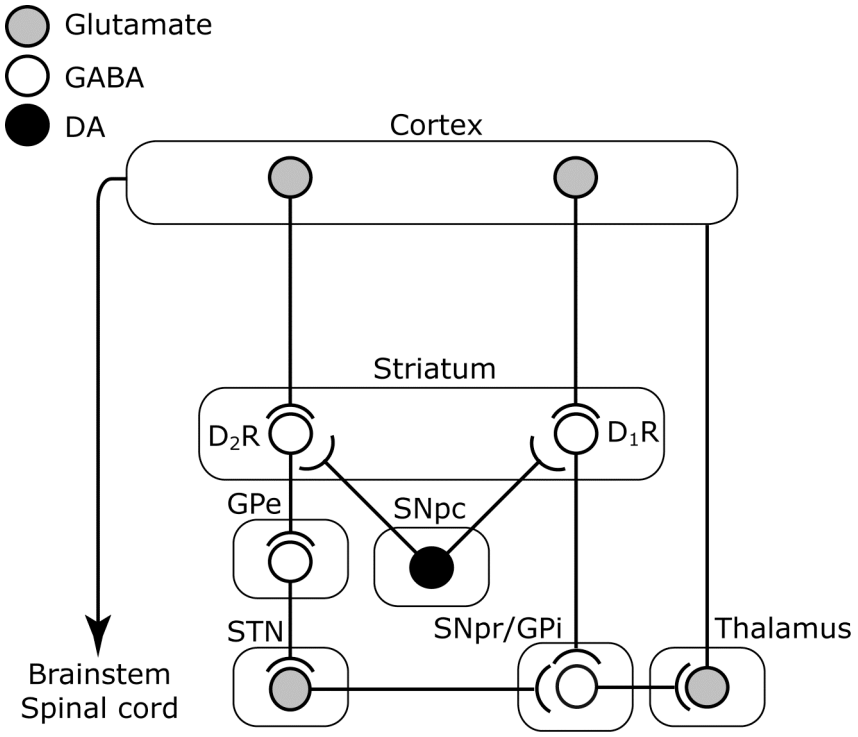

Fig 1. Schematic illustration of the organization of the basal ganglia. The nigrostriatal dopaminergic pathway originates from the SNpc and projects to the GABAergic MSNs in the striatum. These neurons receive glutamatergic innervation from the cortex. MSNs form two distinct pathways: the striatonigral pathway, which projects directly to the SNpr, and the striatopallidal pathway that projects indirectly to SNpr, via GPe and STN. The striatonigral MSNs express D_1Rs and synthesize substance P and dynorphin, whereas the striatopallidal neurons express D_2Rs together with enkephalin.

The basal ganglia process stimuli into behavioral outputs via modulation of the activity of thalamocortical projections of the ventral thalamus. Several lines of evidence indicate that these neurons are inhibited by the output signal from basal ganglia neurons in resting conditions and that there is loss of inhibition during basal ganglia associated behavior (Kawaguchi, 1997). The different wiring of the direct and indirect circuits results in two pathways with antagonistic effects on the firing rate of output neurons. Thus, corticostriatal excitation of the direct pathway results in

inhibition of SNpr/GPi neurons and subsequent disinhibition of thalamocortical projections. In contrast, stimulation of the indirect pathway results in disinhibition of STN neurons, excitation of SNpr/GPi neurons, and hence inhibition of thalamocortical neurons (Smith and Bolam, 1990; Gerfen, 1992a; Bolam et al., 2000).

In addition to rely on GABAergic transmission, striatonigral and striatopallidal neurons contain different sets of neuropeptides and dopamine (DA) receptors. Thus, striatonigral neurons of the direct pathway contain dynorphin and substance P and express DA D_1 receptors (D_1Rs). In contrast, striatopallidal neurons contain enkephalin and express DA D_2 receptors (D_2Rs) (Gerfen et al., 1990). DA activates the striatonigral neurons, through D_1Rs, and inhibits the striatopallidal neurons, via D_2Rs. These opposite regulations result in a disinhibition of thalamocortical neurons and increased motor activity (Fig 1).

1.1.2 Functional organization of the nucleus accumbens

The NAcb, which represents the major part of the ventral striatum, is generally divided in core and shell and is the most heterogenous area of the entire striatal complex. The distinction between core and shell is preferentially observed by calbindin immunohistochemistry. Whereas the medial, ventral and lateral parts of the NAcb are moderately immunoreactive for calbindin and are considered to constitute the shell, the central and dorsal parts show stronger immunoreactivity and belong to the core. The core shares with the dorsal striatum the patch-matrix compartmental organization [the patches are islands of MSNs that express high levels of μ-opioid receptors, surrounded by matrix MSNs, which contain calbindin and somatostatin (Graybiel, 1990)], whereas the shell is composed of interspersed cell clusters and is conspicuously heterogeneous. In this sense and in agreement with a dorsolateral-ventromedial striatal gradient, the core is more homologous to the dorsal parts than the shell is. Indeed, the shell has been proposed to be part of a transitional extended amygdala complex (de Olmos and Heimer, 1999).

The accumbal MSNs can be distinguished, as in dorsal striatum, on the basis of neuropeptide expression. Thus, a subset of GABAergic MSNs contain enkephalin (c.f. striatopallidal neurons above) and another express dynorphin and substance P. As in the dorsal striatum, enkephalin MSNs are preferentially D_2R positive, while dynorphin and substance P MSNs are preferentially D_1R positive. In addition,

accumbal MSNs also contain high levels of DA D_3 receptors. In most cases these receptors are expressed in combination with D_2R, but are also observed in approximately 25% of the cells bearing D_1Rs. Interestingly, a similar proportion of D_1R positive cells express D_2R and among the enkephalin/D_2R containing neurons many express the D_1R. This would argue for a certain degree of colocalization between D_1R and D_2R within the NAcb (Meredith, 1999).

Afferents to NAcb arrive at both core and shell MSNs from the hippocampal region, amygdala, prefrontal cortex, midline and intralaminar thalamus, ventral pallidum, the DA neurons in the ventral tegmental area (VTA), the serotonergic median raphe nucleus and noradrenergic cells in the solitary tract. A clear-cut difference between the core and shell is particularly apparent at the level of output structures. Whereas the core projection neurons innervate the traditional striatal targets, that is, the pallidal and nigral complexes, the shell makes multiple contacts with neurons in several subcortical structures alongside the medial forbrain bundle, from the ventral pallidum, preoptic region, lateral hypothalamus, VTA, mesencephalic tegmentum to the periaqeductural gray and caudal brain stem nuclei. The striatopallido-thalamocortical connections of the accumbens core are basal ganglia loop-like with projections starting and ending at the level of dorsal prelimbic and agranular insular cortices. On the contrary, the shell is considerably feed-forward in nature with projections ending in the cortical areas that are innervating the core (de Olmos and Heimer, 1999; Zahm, 1999, 2000).

The NAcb processes various forms of reward-incentive stimuli (Cardinal et al., 2002) and has been suggested to represent a “limbic-motor interface” (Mogenson et al., 1980). On the basis of the anatomical differences between the core and the shell, and their relations with output structures, it has been suggested that these regions influence distinct aspects of behavioral responses to rewarding stimuli (Di Chiara, 2002). Thus, while the NAcb is critically involved in the generation of motor behaviors related to motivational stimuli, the expression of motor behavior in response to conditioned reinforcers seems to be dependent on the core (Everitt and Wolf, 2002; Everitt and Robbins, 2005), and the shell has been suggested to act by invigorating behavioral responding via its connections to the core (Zahm, 1999; Di Chiara and Bassareo, 2007).

1.2 DOPAMINE

DA is a catecholamine neurotransmitter involved in a wide range of physiological functions, such as motor behavior, cognition, emotion, food intake and endocrine regulation. Dysfunctions in the dopaminergic system are associated with severely disabling neurological diseases, including Parkinson's disease, schizophrenia, drug addiction, attention-deficit/hyperactivity disorder (ADHD) and depression.

DA neurons are located in the ventral mecencephalon and give rise to the mesotelencephalic DA systems. The DA neurons of the substantia nigra pars compacta (A9) give rise to the nigrostriatal system, which projects predominantly to the dorsolateral striatum. The DA neurons in the VTA (A10) give rise to the mesolimbic system, which innervates the ventral striatum, and to the mesocortical system, which projects to the cortex (Ungerstedt, 1971). The retrorubral DA neurons (A8) send axons to the median eminence in the hypothalamus and form the tuberoinfundibular system (Dahlström and Fuxe, 1964). The nigrostriatal neurons are involved in sensori-motor function, the mesolimbic DA system in motivated behavior and reward (Koob, 1992), and the mesocortical system in learning and memory (Le Moal and Simon, 1991). The tuberoinfundibular system regulates the release of prolactin, the hormone inducing milk secretion (Saiardi et al., 1998).

1.2.1 Dopamine receptors

Five different G-protein coupled receptors (GPCRs) for DA have been cloned and are traditionally divided into two families based on pharmacological and biochemical criteria. D_1R and D_5R receptors constitute the D_1-like family of receptors. These receptors activate Gs/Golf proteins and stimulate adenylyl cyclase activity. D_2R, D_3R, and D_4R constitute the D_2-like family of receptors. These receptors activate Gi/Go proteins, which inhibit adenylyl cyclase and regulate the activity of ion channels, including potassium and calcium channeles (Stoof and Kebabian, 1981; Missale et al., 1998). In addition, alternative splicing of D_2R gene product generates two isoforms of the D_2R, $D_{2\ Long}$ (D_{2L}) and $D_{2\ Short}$ (D_{2S}) (Picetti et al., 1997). Behavioral (Usiello et al., 2000), electrophysiological (Centonze et al.,

2004), biochemical (Lindgren et al., 2003) and anatomical (Sesack et al., 1994; Khan et al., 1998b; Khan et al., 1998a) findings suggest that the D_{2S} isoform is a presynaptic DAergic autoreceptor and that the D_{2L} mediates postsynaptic responses to D_2R stimulation.

The expression pattern of the D_1Rs and D_2Rs in the central nervous system has been characterized thoroughly by *in situ* hybridization (Mansour et al., 1990; Mengod et al., 1992), ligand binding autoradiography (Mansour et al., 1990) and immunohistochemistry (Ariano and Sibley, 1994; Sesack et al., 1994). D_1Rs are the most abundant and widespread DA receptor type present in the brain. D_1R mRNA is found in the striatal complex and the olfactory turbercle. In addition, D_1R are detected in limbic areas, hypothalamus and thalamus. In cortical areas, D_1Rs are preferentially expressed in pyramidal neurons of prefrontal, premotor, cingulate and entorhinal cortex, the hippocampus and the dentate gyrus. D_2R expression is mainly restricted to the striatum, olfactory tubercle and the core of the nucleus accumbens. In addition, D_2R mRNA is present in the prefrontal cortex, cingulate, temporal and enthorinal cortex, the septal region, in the amygdala, and in the granule cells of the hippocampal formation (Missale et al., 1998).

1.3 SIGNAL TRANSDUCTION

1.3.1 DARPP-32 – Expression, regulation and function

The dopamine and cAMP regulated phospho-protein of 32 kDa (DARPP-32) is expressed in very high levels in dopaminoceptive areas, such as striatum, nucleus accumbens and olfactory tubercle (Fig 2) (Ouimet et al., 1984; Walaas and Greengard, 1984). Intense DARPP-32 immunoreactivity is also observed in terminal regions such as the GP and the SNpr (Ouimet et al., 1984). Other areas of the brain contain only a small number of DARPP-32 expressing neurons, whose localization appears to correspond with that of DA fibers (Ouimet et al., 1984). In the striatum, DARPP-32 is expressed by virtually all MSNs, striatopallidal as well as striatonigral, whereas no DARPP-32 is detected in interneurons, such as cholinergic or aspiny GABAergic neurons (Ouimet et al., 1998).

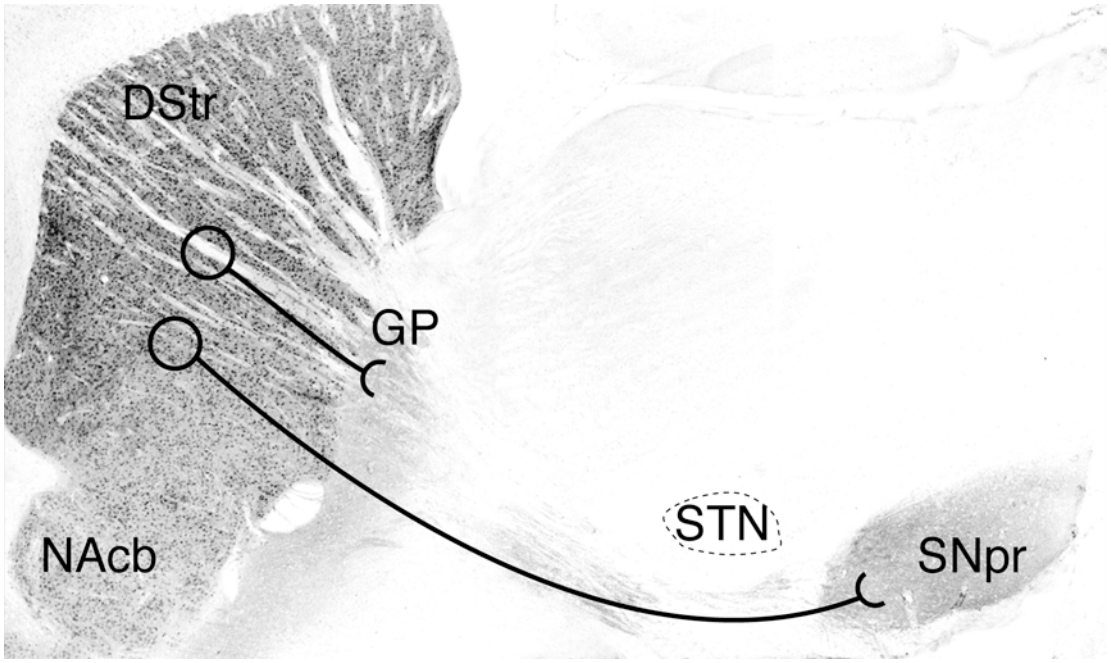

Fig 2. DARPP-32 immunohistofluorescence. The striatal projection neurons of the direct pathway (bottom) and indirect pathway (top) are depicted. MSNs express high levels of DARPP-32. The terminal areas (GP and SNpr) show diffuse immunostaining for DARPP-32.

DA, acting through cAMP and PKA, regulates the state of phosphorylation of DARPP-32 at a single threonyl residue in position 34 (Fig 3) (Walaas et al., 1983; Hemmings et al., 1984c; Hemmings et al., 1984b). This phosphorylation site is also targeted by D_2Rs that upon stimulation dephosphorylate DARPP-32 through either inhibition of adenylyl cyclase or activation of calcium/calmodulin-dependent protein phosphatase 2B (calcineurin; PP-2B) (King et al., 1984; Nishi et al., 1997; Lindskog et al., 1999a). Phosphorylation of DARPP-32 at Thr75 by the cyclin-dependent kinase 5 (Cdk5), on the other hand, converts DARPP-32 into an inhibitor of PKA and results in reduced phosphorylation of Thr34 (Bibb et al., 1999). The state of phosphorylation of DARPP-32 at Thr75 is regulated by protein phosphatase 2A (PP-2A (Nishi et al., 2000). DARPP-32 is also regulated by casein kinase II (CK2) and casein kinase I (CK1) via phosphorylation at Ser102 (Ser97 in the mouse) and Ser137, respectively (Girault et al., 1989; Desdouits et al., 1995b). The influence of CKs on DARPP-32 is preferentially to modulate the ability of PKA to catalyze phosphorylation at Thr34. Thus, CK2 phosphorylation facilitates the PKA stimulation of DARPP-32 by two-fold, whereas CK1 phosphorylation inhibits the dephosphorylation of Thr34 by PP-2B (Girault et al., 1989; Desdouits et al., 1995a).

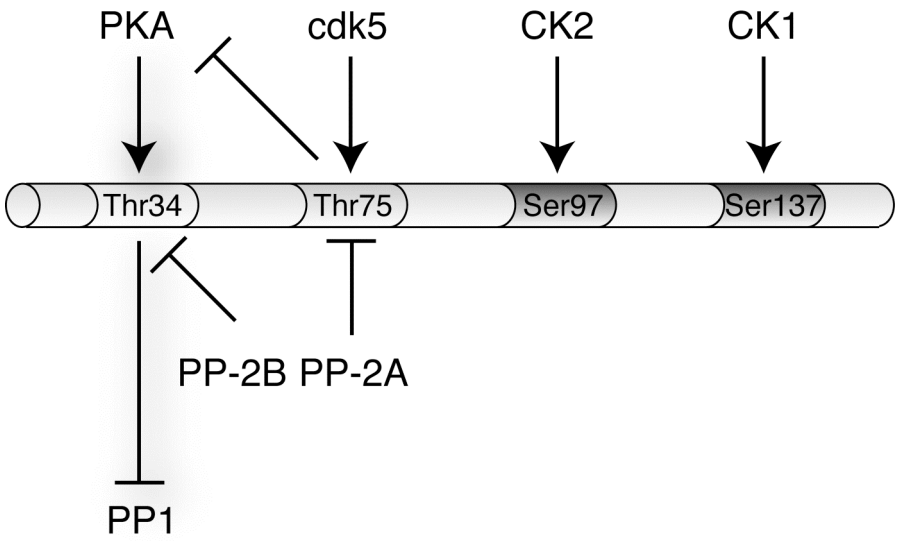

Fig 3. Regulation of DARPP-32 phosphorylation. The state of phosphorylation of DARPP-32 is regulated at multiple sites (see text). By far, the most studied is the Thr34, which is phosphorylated by PKA. Phosphorylation of DARPP-32 by CK1 and CK2 potentiates the phosphorylation on Thr34 (not shown). However, the functions of these two sites have not been extensively characterized *in vivo*. Arrows represents kinase phosphorylation.

Phosphorylation at Thr34 converts DARPP-32 into a potent inhibitor of protein phosphatase 1 (PP1) (Hemmings et al., 1984a). PP1 is a major multifunctional serine/threonine protein phosphatase that controls the state of phosphorylation and activity of numerous important cellular substrates, including neurotransmitter receptors, voltage-gated ion channels, ion pumps, and transcription factors (Greengard, 2001; Svenningsson et al., 2004). Thus, stimulation of DARPP-32 phosphorylation at Thr34 amplifies PKA mediated signaling by inhibiting the dephosphorylation of downstream target proteins. On the other hand, DARPP-32 also inhibits the activity of PKA when phosphorylation occurs at Thr75. These intricate modulations of the cAMP/PKA pathway positions DARPP-32 as a major integrator of signal transduction in the MSNs.

Activation of the D_1R alters the electrophysiological properties of MSNs through the cAMP/PKA/DARPP-32 cascade in several ways. For instance, D_1R stimulation decreases the activity of Na^+K^+-ATPase (Bertorello et al., 1991), decreases conductance in N/P-type Ca^{2+} channels (Surmeier et al., 1995), increases current through NMDA receptors (Flores-Hernandez et al., 2002), increases current amplitude and expression of AMPA GluR1 receptors (Price et al., 1999; Yan et al., 1999; Snyder et al., 2000; Mangiavacchi and Wolf, 2004), and reduces current

through $GABA_A$ receptors (Flores-Hernandez et al., 2000). Together, these effects suggest that the regulation of DARPP-32 phosphorylation participates in the control of the general excitability of MSNs and promotes the mechanisms by which DA modulate excitatory and inhibitory neurotransmission in the striatum (Fienberg et al., 1998; Greengard, 2001). Indeed, genetic inactivation of DARPP-32 results in loss of both excitatory and inhibitory synaptic plasticity in MSNs (Calabresi et al., 2000). Furthermore, loss of DA innervation to the striatum, induced by 6-hydroxydopamine lesions of midbrain DA neurons, leads to abnormalities in the regulation of DARPP-32 phosphorylation and concomitant pathological behavioral responses to DA agonists (Picconi et al., 2003; Santini et al., 2007).

In conclusion, the regulation of the state of phosphorylation of DARPP-32 represents a critical event in the integration and computational processing of cortical and subcortical inputs to the striatum, which are then transferred to basal ganglia output structures in order to elicit relevant behavioral activation in response to stimuli (Greengard, 2001; Svenningsson et al., 2004; Calabresi et al., 2007; Fisone et al., 2007).

1.3.2 DARPP-32 – Regulation by psychoactive drugs

The essential role of DARPP-32 as an integrator of neurotransmission in the striatum makes it attractive to study the regulation of this phosphoprotein in response to drugs that promote dopaminergic transmission, such as drugs of abuse (Di Chiara and Imperato, 1988). Cocaine and amphetamines increase the levels of extracellular DA by inhibiting the reuptake and disrupting the vesicular transport of monoamines. Indeed, systemic injections of cocaine (Svenningsson et al., 2000a; Bibb et al., 2001) and amphetamine (Valjent et al., 2005) increases phosphorylation of DARPP-32 at Thr34 in the striatum through a D_1R dependent mechanism. Furthermore, this effect on DARPP-32 phosphorylation seems critical in order for these drugs to produce hyperlocomotion (Fienberg et al., 1998; Valjent et al., 2005).

Essential steps in the response to addictive drugs are the effects on regulation of gene expression, protein synthesis and the concomitant changes in synaptic plasticity. These properties of drugs of abuse are considered to underlie their propensity to induce long-term changes in limbic brain areas, and to be critically implicated in establishment of drug addiction (Nestler and Aghajanian, 1997; Nestler, 2001, 2002).

One of these mechanisms is related to increased cAMP signaling, hyperphosphorylation of cAMP-response element binding protein (CREB) and accumulation ΔFosB, CREB and ΔFosB being two transcription factors regulated by repeated treatment with various drugs of abuse (Nestler et al., 2001; McClung and Nestler, 2003; Ulery et al., 2006). In line with a critical involvement of the cAMP/PKA/DARPP-32 cascade in these responses, mutation of DARPP-32 at Thr34 attenuates cocaine-induced expression of ΔFosB in the striatum (Zachariou et al., 2006). Furthermore, genetic inactivation of DARPP-32 greatly reduces the expression of immediate early genes, such as *c-fos* and *zif-268*, in the striatum in response to a single injection of a D_1R agonist (Svenningsson et al., 2000b). Thus, it appears that the inhibition of PP1 by DARPP-32 is critically involved in the mechanism by which D_1Rs are regulating gene expression.

Recent experiments in DARPP-32 mutant mice have shown that DARPP-32 is an essential modulator of cAMP signaling in striatal MSNs (Svenningsson et al., 2004). On the basis of the important role of DA transmission in mediating the reinforcing properties of drugs of abuse, the regulation of the state of phosphorylation of DARPP-32 is a critical step by which these drugs can produce long-term effects on striatal function and, hence, basal ganglia processing of rewarding stimuli.

1.3.3 The mitogen-activated protein kinase cascade

The extracellular signal-regulated kinase 1 and 2 (ERK1 and ERK2) are two ubiquitous protein kinases with multiple roles in activity-dependent regulation of neuronal function. The best example is their involvement in synaptic plasticity and critical role in long-term memory (Thomas and Huganir, 2004). The typical mitogen activated protein kinase (MAPK) activation cascade involves three steps of phosphorylation. Activation of ERK1 and ERK2 are mediated via phosphorylation at two amino acid sites, Thr202 and Tyr204, catalyzed by the dual specificity kinase MEK1/2 (MAP/ERK kinase). MEK1/2, in turn, are activated through phosphorylation by the MEKKs (MEK kinase) in the Raf family: Raf-1 and B-Raf (Rafs). The Rafs undergo activation at the cellular membrane after binding to the GTP-bound form of small G-proteins of the Ras family. The MEKK is also activated via phosphorylation by protein kinases, such as protein kinase C, Akt or Src (Pearson et al., 2001). Phospho-ERK1/2 is targeted by specific protein phosphatases, such as

the dual specificy phosphatases (MKP3) or the tyrosine-specific striatal-enriched protein tyrosine (STEP) (Camps et al., 2000; Keyse, 2000; Braithwaite et al., 2006).

Various drugs of abuse increase ERK1/2 phosphorylation in the NAcb and striatum (Valjent et al., 2004). This effect depends on concomitant stimulaton of glutamate and DA transmission (Girault et al., 2007). Importantly, ERK1/2 phosphorylation mediates a range of behavioral effects induced by repeated treatment with drugs. For instance, blockade of ERK1/2 phosphorylation decreases psychomotor sensitization to psychostimulants (Pierce et al., 1999; Ferguson et al., 2006; Valjent et al., 2006a), prevents conditioned place preference to Δ^9-THC, cocaine and MDMA (Valjent et al., 2001; Salzmann et al., 2003; Miller and Marshall, 2005), and decreases cue-induced cocaine seeking (Lu et al., 2004). Moreover, re-exposure to context and/or drug in the presence of an inhibitor of the ERK pathway, can erase previously acquired cocaine or morphine conditioned place preference (Miller and Marshall, 2005; Valjent et al., 2006b).

The fact that inhibition of ERK1/2 reduces these behaviors suggests that ERK1/2 participate in the mechanisms promoting long-term synaptic plasticity in brain areas involved in reward processing. Indeed, many of the downstream targets of ERK1/2 are regulatory proteins of the translational machinery or proteins that control gene expression. Thus, ERK1/2 has been shown to stimulate phosphorylation of the p90 ribosomal protein S6 kinases (RSK), which controls the translational activity through phosphorylation of ribosomal protein S6. Nuclear translocation of ERK1/2 can activate mitogen and stress activated kinase 1 (MSK1), which controls the expression of immediate early genes via phosphorylation and stimulation of CREB. In addition, MSK1 stimulates phosphorylation of histone H3, which participates in chromatin remodeling (Girault et al., 2007).

1.4 CANNABINOIDS

Two hallmarks in the field of cannabinoid pharmacology are first, the discovery that Δ^9-tetrahydrocannabinol (Δ^9-THC) (Gaoni and Mechoulam, 1964), the main psychoactive component of cannabis, acts through specific receptors (Matsuda et al., 1990; Munro et al., 1993) and second, that endogenous ligands (endocannabinoids, eCB) for these receptors are synthesized and released by mammalian tissue (Devane et al., 1992; Mechoulam et al., 1995). During the last decade, neuroanatomical,

neurochemical and electrophysiological studies revealed that cannabinoids are important modulators of many "classical" neurotransmitters (e.g. GABA, glutamate and DA, etc), and today cannabinoids are considered potential drugs for the treatment of vascular and inflammatory diseases, pain, obesity, depression and neurodegenerative disorders (Russo and Guy, 2006; Mangieri and Piomelli, 2007; Sagredo et al., 2007). However, the psychotropic effects [e.g. euphoria, working memory deficits and altered perception (Howlett et al., 2002)] of Δ^9-THC and its synthetic analogs have limited their use in medical therapy. In addition, cannabinoids fulfill most criteria attributed to compounds with reinforcing properties, and can therefore be considered as addictive substances (Maldonado, 2002; Maldonado and Rodriguez de Fonseca, 2002; Justinova et al., 2003).

1.4.1 Endocannabinoids

The two most studied eCBs are N-arachidonoylethanolamide (anandaminde, AEA) (Devane et al., 1992) and 2-arachidonoylglycerol (2-AG) (Mechoulam et al., 1995; Sugiura et al., 1995). These molecules are synthesized from membrane-derived lipid precursors and are released in response to increased intracellular Ca^{2+} levels. The mechanism of release is not entirely known, but it is suggested that eCBs, being lipophilic, diffuse through the post-synaptic membrane and acts on presynaptic receptors. Clearance of AEA and 2-AG is rapidly accomplished via carrier-mediated transport. Once taken up into cells, AEA is broken down by fatty acid amide hydrolase (FAAH) and 2-AG is degraded by monoacylglycerol lipase (Piomelli, 2003).

AEA is released in response to stimulation of D_2Rs and has been shown to antagonize D_2R dependent locomotor activity (Giuffrida et al., 1999; Beltramo et al., 2000; Giuffrida and Piomelli, 2000). It has also been found that eCBs are released from MSNs in response to high-frequency stimulation of corticostriatal neurons. This kind of protocol induces long-term depression in striatal neurons, and eCBs have been shown to play a central role in this particular form of synaptic plasticity (Chevaleyre et al., 2006).

1.4.2 Cannabinoid receptors

The first cannabinoid receptor (CB_1R) was discovered in 1988 by Devane *et al* (Devane et al., 1988) and was later found to be expressed in several brain regions including basal ganglia, cerebellum, hippocampus, cerebral cortex, olfactory bulb, amygdala, septum and a few brainstem nuclei (Herkenham et al., 1990; Pertwee, 1997; Howlett et al., 2002). Only a low concentration was observed in peripheral tissues and cells. A second cannabinoid receptor (CB_2R) (Munro et al., 1993), was found to be present mainly in cells of the immune and hematopoietic systems (Pertwee, 1997; Howlett et al., 2002).

The CB_1R is generally regarded as the brain cannabinoid receptor. However, CB_1Rs are also found in non-neuronal cells, including immune cells (Galiegue et al., 1995). In addition, CB_2Rs are expressed in neurons both within and outside the brain (Ross et al., 2001; Van Sickle et al., 2005; Wotherspoon et al., 2005; Beltramo et al., 2006; Gong et al., 2006; Onaivi et al., 2006), although their specific role has not been clarified (Degroot and Nomikos, 2007; Pertwee, 2008).

Although the CB_1R and the CB_2R are the "classical" receptor types, recent evidence suggests that there are additional cannabinoid receptors to be found. This hypothesis is supported by the presence of several non-CB_1R and non-CB_2R mediated effects of eCBs (Mackie and Stella, 2006), and in CB_1R (Ledent et al., 1999; Zimmer et al., 1999) or CB_2R (Jarai et al., 1999) knock-out mice. Three putative cannabinoid receptors are the vanilloid receptor type 1 ($TRPV_1$) ion channel (Begg et al., 2005) and the orphan receptors GPR55 and GPR119 (Brown, 2007; Ryberg et al., 2007), whereas the proposed "CB_3" receptor (Breivogel et al., 2001; Hajos et al., 2001) still needs to be cloned.

1.4.3 CB1 receptor signaling (I) – evidences from *in vitro* studies

The CB_1R is coupled to Gi/Go-proteins and inhibit the activity of adenylyl cyclase (Childers, 2006). In some circumstances, however, it has been found that stimulation of the CB_1R elicits cAMP accumulation (Rhee et al., 1998). In particular, it has been shown that concomitant stimulation of CB_1R and D_2R in cotransfected cells: 1) changes the $G\alpha$ coupling of CB_1R from pertussis toxin sensitive Gi/Go to

insensitive Gs (Jarrahian et al., 2004), 2) results in CB_1R-D_2R heterodimer formation (Kearn et al., 2005) and 3) activates $A_{2A}R$-dependent nuclear translocation of PKA (Yao et al., 2003).

In addition to controlling the activity of adenylyl cyclase, CB_1Rs have been found to decrease the permeability of N- or P/Q-type Ca^{2+} channels and increase conductance in voltage-dependent, GIRK (inward rectifying) and A-type K^+ channels (Twitchell et al., 1997; Shen and Thayer, 1998). It has also been reported that cannabinoids increase Ca^{2+} release from intracellular stores through a phospholipase C dependent mechanism (Netzeband et al., 1999). Furthermore, CB_1R have been implicated in the signaling mechanisms regulating cell growth, transformation and apoptosis via regulation of the MAPK and PI3K/PKB kinase families (Bouaboula et al., 1995; Gomez del Pulgar et al., 2000; Galve-Roperh et al., 2002).

1.4.4 CB1 receptor signaling (II) – evidences from *in vivo* studies

Previous *in vitro* studies have shown that CB_1R agonists regulate the activity of adenylyl cyclase in ways that depend on the isoform of adenylyl cyclase (Rhee et al., 1998) and with different efficacy in G-protein coupling (Howlett, 2004; Mukhopadhyay and Howlett, 2005). Thus, when studied *in vivo*, the effects of CB_1Rs in a particular neuronal population or brain region are influenced by the density and coupling efficiencies of the CB_1Rs, the expression of particular isoforms of adenylyl cyclase, the affinity of the exogenous agonist, the effects on the eCB system and, additionally, by the effects produced on other neurotransmitter systems (Howlett, 2004; Degroot and Nomikos, 2007; Pertwee, 2008).

The CB_1Rs are located on presynaptic terminals (Herkenham et al., 1991; Tsou et al., 1998; Egertova and Elphick, 2000), where they regulate the release of neurotransmitters such as GABA and glutamate in cortex, hippocampus, basal ganglia, and cerebellum (Shen et al., 1996; Katona et al., 1999; Schlicker and Kathmann, 2001; McAllister and Glass, 2002). This action depends on the stimulation of Gi/Go and inhibition of adenylyl cyclase, which modulate Ca^{2+} and K^+ channel conductions (Schlicker and Kathmann, 2001).

DA has been implicated in the rewarding and the reinforcing properties of cannabinoids (Gardner, 2005; Maldonado et al., 2006). Thus, similarly to most reinforcing drugs (Di Chiara and Imperato, 1988), Δ^9-THC and CB_1R agonists

stimulate the release of DA in the NAcb (Tanda et al., 1997; Lecca et al., 2006). The reciprocal relationship between the DA and cannabinoid system is also illustrated by studies investigating the role of CB_1R in drug relapse (De Vries and Schoffelmeer, 2005). For instance, a CB_1R agonist promotes, whereas a CB_1R antagonist inhibits, cocaine relapse in rats with a history of cocaine self-administration (De Vries et al., 2001). These studies implicate the existence of a functional relationship between the DA system and the cannabinoid system in the control of motivational responses related to drugs of abuse.

Interestingly, the DA release induced by systemic injections of Δ^9-THC is blocked by naloxon, a μ-opioid antagonist (Tanda et al., 1997). Studies using knock-out mice for either the μ-opioid (Ghozland et al., 2002) or the CB1R (Ledent et al., 1999) revealed that the euphoric and addictive properties of Δ^9-THC are regulated by the endogenous opioid system. This interaction is further supported by results obtained with μ-opioid receptor antagonists. Thus, naltrexone reduces self-administration of Δ^9-THC in monkeys (Justinova et al., 2004), and naloxon reduces self-administration of a CB1R agonist in rats (Braida et al., 2001). The opiod-cannabinoid interaction has been recently discussed by Vigano *el al.* (Vigano et al., 2005).

1.4.5 Functional aspects on CB_1 receptors in the basal ganglia

Within the basal ganglia, *in situ* hybridization revealed that the CB_1 gene is expressed in high levels in the striatum and STN, with low expression in GP and the SNpr (Mailleux and Vanderhaeghen, 1992; Matsuda et al., 1993). The localization of the receptor protein shows complementary results. Thus, receptor autoradiography (Herkenham et al., 1990; Herkenham et al., 1991) and immunohistochemistry (Tsou et al., 1998; Egertova and Elphick, 2000; Robbe et al., 2001; Matyas et al., 2006) revealed high CB_1R expression in GP and the SNpr and lower expression in the striatal complex (Herkenham et al., 1991). In addition, it was found that the CB_1Rs are localized both at the level of striatonigral (dynorphin positive) and striatopallidal (enkephalin positive) neurons (Fig 4) (Hohmann and Herkenham, 2000).

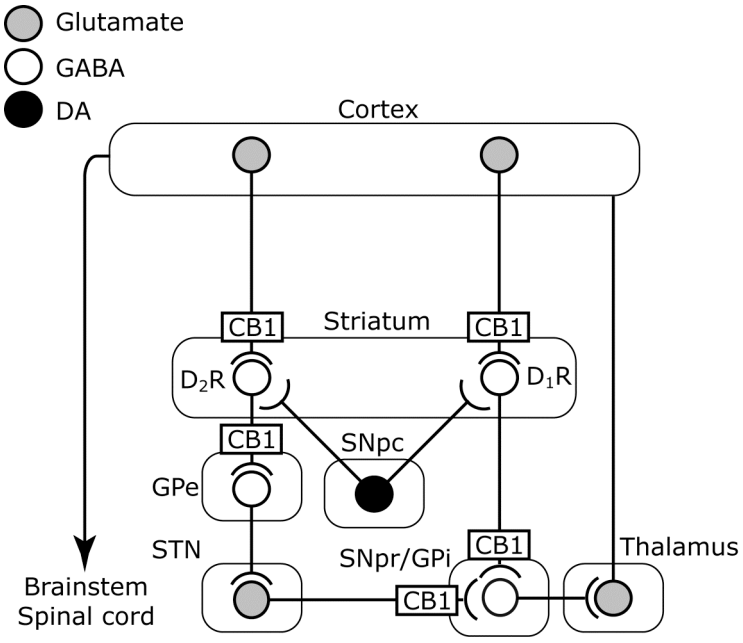

Fig 4. Schematic illustration of CB_1Rs localization in the basal ganglia. CB_1Rs are mainly located presynaptically where they control glutamate and GABA release. In striatum, CB_1Rs are located at the level of striatopallidal and striatonigral MSNs.

Taken together the studies on the expression of CB_1R mRNA and the localization of CB_1R protein suggest that the CB_1Rs are synthesized by the MSNs located in the striatum and then transported to the regions of the basal ganglia where these neurons terminate. Accordingly, CB_1Rs are located presynaptically on the terminals of MSNs in the GP and SNpr, where they inhibit the release of GABA (Miller and Walker, 1995, 1996; Tersigni and Rosenberg, 1996; Chan et al., 1998; Chan and Yung, 1998; Wallmichrath and Szabo, 2002b, a). MSNs give rise to recurrent axon collaterals, which form intrinsic GABAergic synapses (Fujiyama et al., 2000; Tepper and Bolam, 2004). Interestingly, neurotransmitter release from these terminals is regulated by CB_1Rs (Szabo et al., 1998). In addition, considerable electrophysiological evidence supports a role of CB_1Rs in the control of corticostriatal glutamatergic transmission (Gerdeman and Lovinger, 2001; Huang et al., 2001; Brown et al., 2003). An additional anatomical finding is that midbrain DA neurons projecting to the striatal complex do not synthesize or express CB_1Rs (Julian et al., 2003). Thus, the cannabinoid system does not appear to modulate DA transmission via direct effects on DA terminals or cell bodies. In line with this

hypothesis is the observation that CB_1R agonists or antagonists are unable to inhibit electrically evoked release of endogenous DA in the striatum (Szabo et al., 1999).

1.5 OPIOIDS AND OPIOID RECEPTORS

The pharmacology of the opioid system has been thoroughly examined due to its involvement in drug addiction, pain regulation and stress responses [see (Kieffer, 1995)]. The effects of opioids in the central nervous system are mediated by three GPCRs: μ, δ and κ. These receptors bind to three endogenous opioid peptide families, the proopioimelanocortin, proenkephalin and prodynorphin, each with a distinct gene and precursor peptide. Various opioid peptides are produced via enzymatic cleavage of these precursor peptides, generating endorphin, methionine and leucine enkephalin, dynorphin A and B, as well as α- and β-neo-endorphin. The β-endorphins are preferentially agonists at μ-opioid receptors, enkephalin binds to δ receptors, and dynorphin to κ receptors. An additional opioid receptor, N/OFQ, (Mollereau et al., 1994) with the endogenous ligand orphanin FQ (Reinscheid et al., 1995; Mogil et al., 1996) or nociceptin (Meunier et al., 1995) was found recently. The pharmacology and therapeutic potential of this receptor is reviewed in (Chiou et al., 2007).

All opioid receptors are located in brain areas classically associated with processing of emotional and rewarding stimuli. Thus, receptor autoradiography and mRNA expression reveal high levels of opioid receptors throughout limbic areas, including amygdala, hippocampus, prefrontal cortex, striatum and nucleus accumbens (Mansour et al., 1988; Mansour et al., 1995). In the striatum, μ-receptors are markers for the patch-like distribution of MSNs, whereas the δ- and κ-opioid receptors are diffusely located in medial and lateral portions, respectively. At the cellular level, μ-receptors are preferentially expressed together with dynorphin (Guttenberg et al., 1996), whereas δ-receptors are exclusively located on cholinergic interneurons (Le Moine et al., 1994). The predominant opioid receptor in the area of midbrain DA neurons is the μ-receptor, which is expressed in moderate levels in SNpc as well as VTA. On the other hand, DA neurons express high levels of mRNA for the κ-receptor but no apparent receptor protein, suggesting that these receptors are synthesized in cell bodies and transported to terminal areas in the striatum, where they function as autoreceptors (Mansour et al., 1995; Schoffelmeer et al., 1997). Another particular

feature of the μ-receptor is its dense expression in the area of the noradrenergic cells in the locus coeruleus (Mansour et al., 1988; Mansour et al., 1995; Gray et al., 2006).

At the physiological level, all three receptors contribute to pain regulation and have antinociceptive properties. In contrast, whereas exogenous opioid agonists produce reward through activation of either μ- or δ-receptors, stimulation of κ-receptors produce dysphoria and negative effective states (Pfeiffer et al., 1986). Recent neuropharmacological and genetic studies suggest that the μ-receptor, but not the δ-receptor, is critical for morphine and heroin reinforcement and has a central role in addiction (De Vries and Shippenberg, 2002; Kieffer and Gaveriaux-Ruff, 2002; Contet et al., 2004; David et al., 2007). More specifically, it appears that these effects are mediated by μ-receptors located in the VTA and the NAcb (Shippenberg et al., 1992; Shippenberg and Elmer, 1998), and involve both DA-dependent and independent mechanisms (Di Chiara and North, 1992; Self and Nestler, 1998; Nestler, 2001; De Vries and Shippenberg, 2002; Nestler, 2002).

Opioid receptors are prototypical Gi/Go-coupled receptors (Childers, 1991; Law et al., 2000), which reduce neuronal excitability by inhibiting adenylyl cyclase, decreasing the conductance of voltage-gated Ca^{2+} channels or activating inwardly rectifying K^{+} channels. In the striatum, functional studies have shown that activation of μ- and δ-receptors inhibit cAMP formation in membrane homogenates (Noble and Cox, 1995), slices (Schoffelmeer et al., 1987) and primary striatal cultures (Van Vliet et al., 1990). Interestingly, the μ-opioid receptor appears to specifically regulate the activity of adenylyl cyclase in striatonigral neurons, whereas δ-opioid receptors inhibit cAMP formation in the striatopallidal neurons (Noble and Cox, 1995). Further support for this mechanism comes from the observation that μ-receptor stimulation antagonizes DARPP-32 phosphorylation induced by activation of D_1Rs, which are located on striatonigral MSNs, whereas δ-receptor agonists antagonize DARPP-32 phosphorylation induced by activation of adenosine A2A receptors (A_{2A}Rs), which are located on striatonigral MSNs (see below) (Lindskog et al., 1999b). The κ-receptor, on the other hand, does not inhibit adenylyl cyclase in striatal slices (Heijna et al., 1989), and has no effect on D_1R or A_{2A}R mediated DARPP-32 phosphorylation (Lindskog et al., 1999b).

Several *in vivo* studies have found that the cAMP cascade is critically involved in the short- and long-term effects of morphine. The first demonstration was a link between an upregulation of cAMP in the noradrenergic cells of the locus coeruleus

and morphine physical dependence and withdrawal (Duman et al., 1988; Nestler and Tallman, 1988; Nestler et al., 1994). Subsequent studies revealed that increased cAMP is not restricted to morphine dependence or to the locus coeruleus, but appears to be a common mechanism occurring in several brain regions in response to repeated morphine administration (Nestler, 2004). Downstream targets of the cAMP/PKA cascade that followed the same pattern of activation/upregulation are CREB and ΔFosB (Guitart et al., 1992; Nye and Nestler, 1996; Nestler et al., 2001; Han et al., 2006). Thus, the hypothesis from these series of studies is that chronic morphine treatment results in compensatory upregulation of the cAMP/PKA pathway, increased activation of CREB and induction of ΔFosB. The increased transcriptional activity leads to elevated production of dynorphin, which acts retrogradely on κ-receptors expressed by DA terminals and decreases DA release (Steiner and Gerfen, 1998; Nestler, 2004).

1.5.1 Mechanisms involved in morphine induced hyperactivity

Morphine activates VTA DA neurons and enhances DA release in the NAcb via inhibition of GABA interneurons (Di Chiara and Imperato, 1988; Johnson and North, 1992). Although the role of DA in morphine reward remains controversial (Pettit et al., 1984; Bechara et al., 1998; De Vries and Shippenberg, 2002), several lines of evidence suggest that DA is necessary for morphine-induced hyperactivity (Murphy et al., 2001; Hnasko et al., 2005). The locomotor response to morphine is preferentially associated to stimulation of D_1R on MSNs (Jeziorski and White, 1995; Maldonado et al., 1997; Becker et al., 2001) and dependent on activation of cAMP (Kim et al., 2006). Repeated intermittent administration of morphine results in a robust and persistent sensitized response to subsequent morphine injections (Vanderschuren and Kalivas, 2000). This behavioral sensitization is associated with increased DA release in the striatum (Cadoni and Di Chiara, 1999; Vanderschuren et al., 2001). On the other hand, no difference is observed in a receptor-binding assay of DA-receptors (Nestby et al., 1997). This suggests that behavioral sensitization can be caused by supersensitivity of existing DA receptors. In agreement with this hypothesis, the basal levels of cAMP are increased in the striatum of morphine-sensitized rats (Vigano et al., 2003).

1.6 ADENOSINE AND ADENOSINE RECEPTORS

The purine nucleoside adenosine plays an important role in energy transfer as adenosine triphosphate (ATP) and adenosine diphosphate (ADP) and participates in nucleic acid biosynthesis. In addition, adenosine functions as a neuromodulator controlling, for example, motor activity and sleep rhythm (Dunwiddie, 1985; Snyder, 1985; Haas and Selbach, 2000). In the brain, adenosine has primarily inhibitory function on excitatory transmitter release (Fredholm and Dunwiddie, 1988). However, adenosine is not categorized as a neurotransmitter in classical terms, since it is not stored in vesicles, nor is it released by exocytosis, or delivered unidirectionally from presynaptic to postsynaptic sites. Instead, adenosine is accumulated intracellularly as an ATP metabolite, and released into the extracellular space by means of nucleoside transporters (Fredholm et al., 2005).

At present, four different GPCR for adenosine have been identified: A_1, A_{2A}, A_{2B} and A_3 (Fredholm et al., 2001). The predominant isoforms in neuronal tissue are the A_1R and $A_{2A}R$, which are coupled to opposite regulation of adenylyl cylcase. Activation of the A_1R decrease the rate of adenylyl cylcase via Gi/Go (Fredholm et al., 2000), whereas activation of the $A_{2A}R$ leads to accumulation of cAMP via stimulation of Gs/Golf (Fredholm et al., 2000; Kull et al., 2000; Corvol et al., 2001).

The pattern of distribution of A_1R and $A_{2A}R$ in the brain is strikingly different. Whereas A_1R is found in most areas, including cortex, hippocampus, hypothalamus and cerebellum (Mahan et al., 1991; Rivkees et al., 1995), the expression of $A_{2A}R$ is mainly restricted to the basal ganglia (Jarvis and Williams, 1989; Parkinson and Fredholm, 1990). At the subcellular level, the A_1R is preferentially expressed in axons and terminal areas (Rebola et al., 2003) and stimulation of A_1Rs has been shown to regulate the release of glutamate (Marchi et al., 2002), DA (Okada et al., 1996), and acetylcholine (Brown et al., 1990). On the other hand, A_1R receptors are also found postsynaptically in dendrites where they control NMDA receptor function (Fredholm et al., 2005).

The less abundant $A_{2A}R$ is preferentially expressed in the striatum, nucleus accumbens and olfactory tuburcle (Jarvis and Williams, 1989). In the striatum, the $A_{2A}R$ is expressed at the postsynaptic level in asymmetric synapses on enkephalin-containing MSNs (Fink et al., 1992; Schiffmann and Vanderhaeghen, 1993). Thus, it

has been shown that A_{2A}Rs play an important role in the regulation of excitatory corticostriatal and thalamostriatal input to, and inhibitory output from, the striatopallidal pathway of the basal ganglia (Ferre et al., 1993; Norenberg et al., 1997; Norenberg et al., 1998; Gerevich et al., 2002; Wirkner et al., 2004). Because of its anatomical location, the A_{2A}R has been associated to the control of motor activity. Thus, A_{2A}R agonists decrease motor activity (Ferre et al., 1991a; Barraco et al., 1993), whereas A_{2A} antagonists increase motor activity (Svenningsson et al., 1997), through the modulation of the indirect pathway in the basal ganglia.

1.6.1 Adenosine – dopamine interactions in the basal ganglia

The adenosine A_{2A}R has been reported to antagonistically regulate D_2R-mediated behavioral responses (Rimondini et al., 1998), neurotransmitter release (Ferre et al., 1994) and expression of immediate early genes in the striatum (Le Moine et al., 1997). Subcellular analysis demonstrated the localization of A_{2A}R and D_2R in the extrasynaptic compartment at the level of the spines of MSNs (Hersch et al., 1995; Hettinger et al., 2001). On the basis of these results it was hypothesized that A_{2A}R and D_2R may interact physically in the plasma membrane, and that this could have consequences on their individual properties (Ferre et al., 1992). Indeed, stimulation of A_{2A}R reduces the affinity of D_2Rs for dopamine (Ferre et al., 1991b) and the existence of a heteromeric complex between A_{2A}R and D_2R has been demonstrated by co-immunoprecipitation and by fluorescence resonance energy transfer (FRET) in co-transfected neuroblastoma cells and mouse fibroblast cells (Hillion et al., 2002; Canals et al., 2003).

The antagonistic relationship between A_{2A}Rs and D_2Rs at the level of signal transduction has also been extensively documented. As mentioned earlier, adenosine A_{2A}Rs are coupled to Golf and stimulation of adenylyl cyclase, whereas D_2Rs activates Gi proteins, reduces adenylyl cyclase activity and inhibits cAMP formation. Thus, incubation of striatal slices with an A_{2A}R agonist, CGS21680, stimulated PKA catalyzed phosphorylation of DARPP-32 at Thr34 (Svenningsson et al., 1998) and decreased phosphorylation at Thr75 (Lindskog et al., 2002). The regulation of DARPP-32 phoshphorylation by CGS21680 was counteracted by quinpirole, a D_2R-like agonist (Lindskog et al., 1999a). At the systemic level, blockade of D_2Rs with haloperidol or eticlopride increases the PKA-dependent phosphorylation of DARPP-32 and of the GluR1 subunit of the glutamate AMPA receptor (Svenningsson et al.,

2000a; Håkansson et al., 2006). These effects are prevented by genetic inactivation of the $A_{2A}R$ or preadministration of KW6002, a selective $A_{2A}R$ antagonist (Svenningsson et al., 2000b; Håkansson et al., 2006).

These results clearly demonstrate that $A_{2A}Rs$ and D_2Rs oppositely regulate the activity of the cAMP/PKA pathway in striatopallidal neurons *in vitro* and *in vivo*, and that this effect is observed at the level of downstream target proteins. Furthermore, they also suggest that endogenous adenosine acting on $A_{2A}R$ exerts an excitatory tone on striatopallidal neurons by a D_2R independent mechanism (Chen et al., 2001).

2 SPECIFIC AIMS

The main goals of this Ph.D. project have been: 1) to identify specific changes in signaling produced by cannabinoids and morphine in the medium spiny neurons of the striatum, and 2) to examine the contribution of these changes to the short- and long-term actions of these drugs. The Specific Aims were:

PAPER I

- To study the regulation of the state of phosphorylation of DARPP-32 by drugs interacting with the cannabinoid system
- To study the interactions between cannabinoids, dopamine and adenosine at the level of the regulation of DARPP-32 phosphorylation
- To determine the role of DARPP-32 in the action of cannabinoids

PAPER II

- To study the regulation of the state of phosphorylation of DARPP-32 by Δ^9-tetrahydrocannabinol

PAPER III

- To study the regulation of the state of phosphorylation of DARPP-32 by morphine
- To determine the role of DARPP-32 in the action of morphine

PAPER IV

- To study the regulation of ERK signaling in morphine psychomotor sensitization

3 MATERIALS AND METHODS

The methodological approach in this thesis consisted of: 1) determination of the state of phosphorylation of phosphoproteins in the striatum after treatment of mice with drugs interfering with cannabinoid, opioid, DA or adenosine transmission, and 2) behavioral studies using normal or transgenic (Table 1) mice with targeted mutations of proteins involved in striatal signal transduction. Detailed description of the methods can be found in the individual papers.

3.1 ANIMALS

Male C57BL/6 mice (20–30 g) were purchased from Taconic (Denmark), Scanbur (Sweden) or Charles River (Germany) and were used in all papers (Paper I, II, III, IV). All experimentation involving animals presented in this thesis has been approved beforehand by the Swedish Animal Welfare Agency (CFN) and Swedish Ministry for Agriculture.

Table 1 Genetically modified mice used in these studies. * N = number of generations of backcross into recipient strain. More than 10 generations is considered a congenic, genetically homogenous strain.

	Genetic background*	**Paper**	**Reference**
DARPP-32 knock-out	C57Bl/6J ($N_{>20}$)	I, III	(Fienberg et al., 1998)
T34A DARPP-32 mutant	C57Bl/6J 75% 129/Sv 25% (N_2)	I, III	(Svenningsson et al., 2003)
T75A DARPP-32 mutant	C57Bl/6J 75% 129/Sv 25% (N_2)	I, III	(Svenningsson et al., 2003)
D2R knock-out	C57Bl/6J ($N_{>10}$)	I	(Baik et al., 1995)
$A_{2A}R$ knock-out	CD1 ($N_{>10}$)	I	(Ledent et al., 1997)
drd1a-EGFP	C57Bl/6J 75% Swiss Webster 25% (N_2)	IV	(Gong et al., 2003)
drd2-EGFP	C57Bl/6J 75% Swiss Webster 25% (N_2)	IV	(Gong et al., 2003)

3.2 DETERMINATION OF PHOSPHORYLATED PROTEINS

The levels of phosphoproteins in striatal tissue homogenates were determined by immunoblotting (Paper I-IV) as described in (Svenningsson et al., 2000a; Borgkvist et al., 2007) or by immunocytofluorescence according to (Valjent et al., 2005). Phosphorylation state specific antibodies for phosphoThr34-DARPP-32 (Snyder et al., 1992) (monoclonal, used at 1:1'000 dilution) and phosphoThr75-DARPP-32 (Bibb et al., 1999) (polyclonal, 1:20'000) and a DARPP-32 monoclonal antibody that was not phosphorylation state specific (Hemmings and Greengard, 1986) (1:10'000) were kind gifts from Dr. Paul Greengard (The Rockefeller University, New York, NY, USA). Polyclonal antibodies against phospho-Thr202/Tyr204-ERK1/2 (Western blotting: 1:2'000, Immunocytofluorescence: 1:200) and total-(phosphorylated plus unphosphorylated)-ERK1/2 (Western blotting: 1:1'500) were purchased from Cell Signaling Technology (Beverly, MA, USA).

3.3 BEHAVIORAL STUDIES

Table 2. Behavioral studies used in this thesis

	Paper
Catalepsy test	I
Locomotor activity	III, IV
Conditioned Place Preference	III
Hot-plate test	III

4 RESULTS AND DISCUSSION

4.1 REGULATION OF DARPP-32 PHOSPHORYLATION BY CANNABINOIDS (PAPER I AND II)

In Papers I and II we examined the ability of different classes of drugs acting on CB_1Rs to regulate DARPP-32 phosphorylation and we investigated the role of DARPP-32 on CB_1R-induced catalepsy.

Systemic administration of the CB_1R agonist CP 55,490 (0.25-1.0 mg/kg, Paper I) or Δ^9-THC (2.5-10 mg/kg, Paper II) produced a dose dependent increase in DARPP-32 phosphorylation at Thr34, without affecting Thr75 phosphorylation. The effects of both drugs were prevented by co-administration of the selective CB_1R antagonist/inverse agonist SR141716A (3-5 mg/kg) (Rinaldi-Carmona et al., 1994). The stimulation of phosphoThr34-DARPP-32 peaked between 30-60 minutes after injection with 0.5 mg/kg CP 55,940 or 10 mg/kg Δ^9-THC.

The effect of Δ^9-THC was present in both dorsal striatum and NAcb. The dose (10 mg/kg) at which Δ^9-THC was found to increase DARPP-32 phosphorylation is much higher than that previously shown to induce conditioned place preference (e.g. 1 mg/kg) (Valjent and Maldonado, 2000), thereby suggesting a preferential involvement of DARPP-32 in the motor, rather than rewarding, action of cannabinoids. The lack of effect of low doses of Δ^9-THC (and possibly also of other cannabinoids) may be due to the fact that western blotting is currently the only method available to quantify phosphorylated DARPP-32. With this procedure it is possible to quantify phosphoproteins only in relatively large tissue samples, which does not allow changes occurring in small subsets of MSNs to be appreciated.

We also examined the effects of eCBs on DARPP-32 phosphorylation. Blockade of the reuptake of anandamide and 2-AG achieved using 10 mg/kg of AM404 (Beltramo et al., 1997) produced an increase in Thr34 phosphorylation, without affecting Thr75 phosphorylation. Recently, we tested the effect of URB597, an inhibitor of the enzyme fatty acid amide hydrolase (FAAH), which degrades eCB and terminates their activities (Cravatt et al., 2001; Fegley et al., 2005). We found that, similarly to AM404, administration of URB597 (0.3-1.0 mg/kg) increases the levels of phosphoThr34-DARPP-32 in the striatum (A. Borgkvist and G. Fisone,

unpublished). Taken together these experiments indicate that DARPP-32 phosphorylation at Thr34 is stimulated by endogenous cannabinoids. In order to determine the involvement of CB_1Rs in these effects we used the selective CB_1R antagonist/inverse agonist SR141716A (3-5 mg/kg) (Rinaldi-Carmona et al., 1994). In agreement with the experiments performed using CP 55,940, we found that blockade of CB_1Rs prevented the ability of AM404 and URB597 to stimulate DARPP-32 phosphorylation.

4.1.1 Stimulation of DARPP-32 phosphorylation by CB_1R requires intact D_2R transmission (Paper I)

The observation that activation of CB_1Rs increases phosphorylation of DARPP-32 at Thr34, a site regulated by PKA, is inconsistent with the notion that CB_1Rs are coupled to a Gi protein, which inhibits the activity of adenylyl cyclase (Howlett, 1984; Howlett et al., 1988). It should be noted, however, that experiments performed in striatal neurons have shown that the Gi/Go coupling of CB_1Rs can change to Gs coupling following activation of D_2R (Glass and Felder, 1997). The mechanism underlying this phenomenon is not known, but recent evidence has shown that heterodimerization with D_2R is associated with a shift of CB_1R signaling from inhibition to stimulation of adenylyl cyclase (Kearn et al., 2005).

On the basis of the above observations, the potential role of D_2Rs in cannabinoid-induced phosphorylation of Thr34 was evaluated in D_2R knock-out mice. We found that, in these mice, the regulation of Thr34 phosphorylation by CP 55,940 was absent. Whereas these results are in line with the existence of an interaction between CB_1Rs and D_2Rs on striatopallidal MSNs (cf. above), they are in contrast with the view that CB_1Rs are mainly located presynaptically. In fact, in the striatum, CB_1Rs have been shown to inhibit the release of GABA (Szabo et al., 1998; Wallmichrath and Szabo, 2002a) and glutamate (Gerdeman and Lovinger, 2001; Huang et al., 2001; Kofalvi et al., 2005). Previous studies, showed that stimulation of striatal slices with NMDA or AMPA results in activation of calcineurin and dephosphorylation of DARPP-32 at Thr34 (Halpain et al., 1990; Nishi et al., 2002). Thus, CB_1Rs may promote DARPP-32 phosphorylation by decreasing glutamate release, inhibiting calcineurin and suppressing dephosphorylation at Thr34. This effect may be promoted by concomitant activation of D_2Rs. Indeed, activation of

presynaptic D_2Rs located on corticostriatal terminals has been shown to reduce the release of glutamate (Bamford et al., 2004a; Bamford et al., 2004b). Further studies will be necessary to clarify a possible interaction between CB_1Rs and D_2Rs in the regulation of striatal glutamate release and, ultimately, phosphorylation of DARPP-32.

4.1.2 Stimulation of DARPP-32 phosphorylation by CB_1R requires intact A_{2a}R transmission (Papers I and II)

D_2Rs are coexpressed with adenosine A_{2A}Rs in the striatopallidal neurons. In these cells, the activity of adenylyl cylase is regulated by the inhibitory action of D_2Rs and the stimulatory effects exerted by A_{2A}Rs. It has been shown that blockade of D_2Rs by systemic injections of eticlopride unmasks A_{2A}R signaling and results in increased phosphorylation of DARPP-32 on Thr34 (Svenningsson et al., 2000a). Based on the functional interaction between D_2Rs and A_{2A}Rs, CB_1R-mediated stimulation of Thr34 phosphorylation was analyzed in A_{2A}R knock-out mice, or using the selective A_{2A}R antagonist, KW6002 (3 mg/kg). As presented in Paper I, genetic inactivation or pharmacological blockade of A_{2A}R reduced CP 55,940-induced phosphorylation of Thr34. Similarly, administration of KW6002 prevented the ability of Δ^9-THC to increase DARPP-32 phosphorylation (Paper II).

The results described above indicate that CB_1Rs regulate the state of phosphorylation of DARPP-32 at the PKA site, in striatopallidal MSNs. They also raise the question of the existence of a similar regulation exerted by cannabinoids in striatonigral MSNs, which do not expresss D_2Rs and A_{2A}Rs. In Paper II, we examined the effect produced by Δ^9-THC on DARPP-32 phosphorylation, in the presence of SCH23390, a D_1R antagonist that suppresses cAMP signaling specifically in striatonigral MSNs. We found that SCH23390 abolished Δ^9-THC-induced Thr34 phosphorylation. Therefore, it appears that activation of CB_1Rs regulates DARPP-32 in both striatopallidal and striatonigral MSNs

4.1.3 DARPP-32 is required for the action of cannabinoids (Paper I)

The biochemical studies performed in Paper I and II raised the question of the involvement of DARPP-32 in the behavioral effects of cannabinoids. The doses of CP 55,940 and Δ^9-THC able to produce a significant phosphorylation at Thr34 are known to produce a profound depression in motor activity. Therefore we examined the possible role of DARPP-32 phosphorylation in the action of cannabinoids, by evaluating the cataleptic response produced by CP 55,940 in DARPP-32 mutant mice. Genetic inactivation of DARPP-32 resulted in a robust decrease in the cataleptic response to 0.5 mg/kg of CP 55,940. Whereas a targeted mutation of the phosphorylation site Thr75 of DARPP-32 did not modify the response to CP 55,940, mutation of Thr34 resulted in a similar attenuation as observed in the DARPP-32 knock-out mice. Thus, the ability of CP 55,940 to produce catalepsy requires the specific regulation of phosphorylation at Thr34.

In agreement with the biochemical data and further supporting a role of Thr34 on DARPP-32 in the psychomotor effects of cannabinoids, we found that the cataleptic response to CP 55,940 was attenuated in D_2R knock-out mice and in A_{2A}R knock out mice. We were also able to reduce the response to CP 55,940 using the A_{2A}R antagonist, KW6002. These results indicate the importance of increased DARPP-32 phosphorylation in the action of cannabinoids and point to the striatopallidal MSNs as an important neural component involved in motor responses produced by activation of CB_1Rs. Further studies will be necessary to understand the relevance of DARPP-32 phosphorylation in striatonigral MSNs in the action of cannabinoids.

4.2 THE EFFECT OF MORPHINE ON DOPAMINE D1 MEDIATED SIGNALING IN MEDIUM SPINY NEURONS (PAPER III)

In paper III we examined the involvement of DARPP-32 in the acute motor stimulant effect of morphine, and in morphine psychomotor sensitization. Furthermore, we used the place-preference paradigm to address the involvement of DARPP-32 in morphine classical conditioning. In Paper IV we used morphine sensitized mice to: 1) study the ability of a previously morphine-paired environment to activate ERK signaling in the NAcb, and 2) examine the effect produced by a drug-free period of four weeks on the responsiveness to morphine.

4.2.1 Morphine stimulates DARPP-32 phosphorylation in the striatum by activating DA D_1Rs (Paper III)

Systemic administration of morphine (6 and 9 mg/kg) increased DARPP-32 phosphorylation at Thr34 in NAcb and dorsal striatum. The effect on Thr34 phosphorylation was maximal at 30 minutes after injection and was still present after 60 minutes. In the same experiments, we did not observe any regulation of Thr75 phosphorylation by morphine. The phosphorylation on Thr34 was dependent on stimulation of D_1Rs, since it was inhibited by administration of SCH23390 (0.15 mg/kg), a selective D_1R antagonist. These results indicated that morphine promoted Thr34 phosphorylation via activation of D_1Rs and stimulation of the cAMP/PKA pathway. Previous experiments performed in striatal slices showed that activation of μ-opioid receptors reduced, rather than enhanced the state of phosphorylation of DARPP-32 (Lindskog et al., 1999b). This inhibitory regulation of the cAMP/PKA/DARPP-32 cascade reflects the activation of Gi/Go-coupled opioid receptors located on MSNs. In this study, however, we confirm that the preponderant effect observed after systemic administration of morphine depends on the ability of morphine to promote DA release and to activate D_1Rs on MSNs. In this way, morphine produces a similar regulation of the state of DARPP-32 phosphorylation as previously reported for psychostimulant drugs (Svenningsson et al., 2000a; Valjent et al., 2005).

4.2.2 Involvement of DARPP-32 in morphine hyperactivity (Paper III)

We next examined the role of DARPP-32 in the ability of morphine to produce hyperactivity. Morphine, at the doses of 6 and 9 mg/kg, produced a strong activation of locomotion in wild-type mice. This response was attenuated in DARPP-32 knock-out mice and only 9 mg/kg of morphine was able to stimulate locomotor activity in these animals. A similar decrease was found in T34A, but not in T75A mutant mice. Western blotting experiments performed on tissue from DARPP-32 knock-out mice confirmed that the level of μ-receptor expression was unchanged in mutant mice. Furthermore, DARPP-32 knock-out mice responded to morphine as wild-type littermates in the hot-plate test confirming that the attenuated locomotor response was not due to a generalized decreased sensitivity to morphine. Taken together, these

experiments demonstrated that stimulation of PKA-dependent phosphorylation of DARPP-32 at Thr34 is specifically involved in the action of morphine on locomotor activity.

It was previously reported that the hyperlocomotor response to a single acute injection of cocaine was attenuated in DARPP-32 knock-out mice (Fienberg et al., 1998) and T34A DARPP-32 mutant mice (Zachariou et al., 2006). Thus, together with the present study, these reports demonstrate the importance of DARPP-32 in the acute motor stimulant effects produced by addictive substances. The role of DARPP-32 is most likely to enhance the outcome of D_1R stimulation by inhibiting PP1 and amplifying downstream effects of PKA (cf. Introduction). In line with this hypothesis, we did not observe a complete absence of locomotor response to morphine in DARPP-32 mutant mice as observed previously in DA D_1R knock-out mice (Becker et al., 2001).

There is compelling evidence that the locomotor response to morphine is a result of inhibition of GABAergic interneurons located in the VTA and subsequent disinhibition of DA neurons (Joyce and Iversen, 1979; Kalivas et al., 1990; Johnson and North, 1992). Motor activity can, however, also be stimulated by local injections of μ-receptor agonists in the NAcb (Costall et al., 1976; Cunningham and Kelley, 1992). Thus, it is possible that the residual stimulation of motor activity observed in DARPP-32 mutant mice is mediated by morphine acting directly at μ-receptors located on MSNs, without involvement of DAergic transmission (Swerdlow et al., 1986).

4.2.3 Repeated administration of morphine increases DARPP-32 phosphorylation (Paper III)

Repeated treatment with 9 mg/kg of morphine for 9 days, a regimen that leads to psychomotor sensitization (see below), resulted in increased ability of morphine to stimulate Thr34 phosphorylation in NAcb and dorsal striatum. This effect was prevented by SCH23390, confirming that, even after sensitization, the ability of morphine to regulate DARPP-32 phosphorylation depends on D_1Rs. Chronic morphine has been shown to increase the activity of the cAMP/PKA pathway in several brain regions [for review see (Nestler, 2004)]. In the NAcb, this phenomenon may depend on decreased expression of Gi protein, which couples μ-receptors on

MSNs to inhibition of adenylyl cyclase and cAMP synthesis (Terwilliger et al., 1991). Thus, repeated administration of morphine is accompanied by decreased ability of μ-receptors to inhibit forskolin-stimulated cAMP production (Vigano et al., 2003). In Paper III, we showed that repeated treatment with morphine increases the ability of acute morphine to stimulate cAMP/PKA-dependent phosphorylation of DARPP-32 on Thr34. This change in efficacy may depend on the diminished ability of striatal μ-receptors to counteract the accumulation of cAMP produced by morphine via disinhibition of DA release and activation of DA D_1Rs. The enhanced ability of morphine to stimulate DARPP-32 at Thr34 in sensitized animals appears to be a unique feature associated to chronic treatment with morphine, as repeated administration of psychostimulants has been shown to reduce rather than promote Thr34 phosphorylation (Bibb et al., 2001; Chen and Chen, 2005)

4.2.4 Genetic inactivation of DARPP-32 does not prevent morphine psychomotor sensitization or morphine conditioned place preference

In Paper III we showed that the ability of repeated morphine administration (9 mg/kg x 9 days) to induce psychomotor sensitization is preserved in DARPP-32 mutant mice. Interestingly, however, the reduced locomotor response to morphine in DARPP-32 knock-out and T34A mutant mice is maintained also during repeated administration. Morphine psychomotor sensitization has been associated to increased DA transmission in the NAcb and dorsal striatum (Kalivas and Duffy, 1987; Vezina et al., 1987; Spanagel et al., 1993; Cadoni and Di Chiara, 1999; Vanderschuren et al., 2001). On the other hand, studies using D_1R and D_2R antagonists indicate that the development of psychomotor sensitization to opioids is independent of DA (Kalivas, 1985; Vezina and Stewart, 1989; Jeziorski and White, 1995). Thus, it has been suggested that glutamate receptors in the VTA, not DA receptors in the NAcb, are primarily involved in the development of psychomotor sensitization to morphine (Vanderschuren and Kalivas, 2000). Our data, indicating that DARPP-32 mutant mice are able to develop psychomotor sensitization to morphine, are in agreement with this hypothesis.

In Paper III, we also showed that DARPP-32 knock-out mice are fully capable of acquiring preference for a morphine-associated environment in the CPP test. This is surprising considering previous findings of a specific role of D_1Rs in CPP with

opioids [for review see (Shippenberg and Elmer, 1998)]. On the other side, it was found recently that DA-deficient mice are able to establish CPP for morphine (Hnasko et al., 2005) as well as cocaine (Hnasko et al., 2007), which indicates that DA play a complex role in this test. In our experiments we performed 4 conditionings with 3 different doses of morphine: 3, 6 and 9 mg/kg. A recent study showed that DA receptors in the NAcb shell are important for acquisition of morphine CPP (reflecting morphine reward), but not expression (Fenu et al., 2006). Therefore, D_1R-mediated stimulation of DARPP-32 phosphorylation may play a role in single conditioning trials. Further studies will be necessary in order to address the role of DARPP-32 in this type of paradigm or in the effects produced by low doses (<3 mg/kg) of morphine (which have not been tested in our studies).

The ability of cocaine to phosphorylate DARPP-32 at Thr34 is required for psychostimulant-induced sensitization (Valjent et al., 2005) [but see also (Zachariou et al., 2006)]. In addition, DARPP-32 knock-out mice fail to establish cocaine CPP (Zachariou et al., 2002). In contrast, our data show that DARPP-32 phosphorylation at Thr34 is necessary for the acute psychomotor response to morphine, but not for the development of psychomotor sensitization or for morphine CPP. This is in agreement with the role of cAMP/PKA/DARPP-32 pathway in dopaminergic transmission, and consistent with the recent evidence indicating a blunted morphine-induced motor response, but intact morphine reward, in DA-deficient mice (Hnasko et al., 2005).

4.3 CONTEXT AND WITHDRAWAL DEPENDENT ERK SIGNALING IN THE NAcB SHELL OF MORPHINE-SENSITIZED MICE (PAPER IV)

Repeated consumption of addictive drugs results in long-term adaptations in brain reward pathways that persist over prolonged periods of abstinence (Koob et al., 1998; Berke and Hyman, 2000; Nestler, 2001; Everitt and Robbins, 2005; Kalivas and Volkow, 2005). These adaptations may increase the risk for drug relapse, which is provoked by stressful events or by encountering situations that used to be associated with drug intake (O'Brien et al., 1992).

It has been proposed that expression of behavioral sensitization is a form of drug craving that results from hypersensitivity to drugs and drug-associated stimuli in the same neural circuits that mediate incentive salience (wanting) of rewards (Robinson and Berridge, 1993; Berridge, 2007). Recent evidence using experimental

models of drug relapse illustrated that extended withdrawal times increase the ability of drug-associated cues to provoke drug seeking behavior in rats (Lu et al., 2004). This phenomenon has been related to drug-induced molecular adaptations in areas of the brain that process emotional and motivational stimuli (Shaham and Hope, 2005), and is hypothesized to be similar to those that drive a sensitized behavioral response to psychostimulants and opioids (Kalivas and Stewart, 1991; Robinson and Berridge, 1993; Shaham and Hope, 2005).

4.3.1 Withdrawal increases behavioral sensitization to morphine (Paper IV)

In paper IV, we tested the ability of a drug-free period to modulate the sensitivity to morphine in mice that had received 5 daily injections of 9 mg/kg of morphine. The morphine pairings were performed in an environment different from the home cage in order to stimulate drug-context associations. This sensitization protocol was essentially the same as presented in paper III, and resulted in a robust psychomotor sensitization (see below). The degree of psychomotor sensitization was assessed using a dose of morphine that did not produce any effect on locomotor activity in drug-naïve mice. On day 6, following 5 days with 9 mg/kg morphine, we observed a robust increase in locomotor activity to 2 mg/kg morphine. In mice subjected to 4 weeks of withdrawal, the response to 2 mg/kg was increased by almost 80% (from 10991 +/- 1609 cm in 90 min on day 6 to 19656 +/- 2844 cm on day 33). This indicated that the withdrawal period substantially increased the efficacy of morphine in sensitized mice. This is in agreement with previous studies showing increased behavioral responses to amphetamine after extended withdrawal periods (Kolta et al., 1985; Paulson and Robinson, 1991, 1995; Vanderschuren et al., 1999). Interestingly, similar time-dependent increases in cue-induced cocaine or heroin seeking behaviors have also been reported (Grimm et al., 2001; Shaham et al., 2003; Lu et al., 2004).

Using the same protocol of morphine sensitization, we observed that exposure to the morphine-associated cage in the absence of drug produced a robust conditioned locomotor response in mice at day 6 and day 33. Unlike the response observed following morphine challenge, we did not observe any difference in this type of response between withdrawn and non-withdrawn mice. These data, however, indicated that the morphine-associated cage acted as a conditioned stimulus and was

able to elicit a conditioned response, which is in agreement with the involvement of associative learning processes in behavioral sensitization (Anagnostaras and Robinson, 1996; Badiani et al., 2000; Badiani and Robinson, 2004).

After having confirmed that the morphine-associated cage did produce a conditioned response, we investigated the role of this context-association in the response to morphine. To this end, we introduced a single trial of re-exposure to the cage for 30 minutes in the absence of drug (which should disrupt the conditioned response) and then examined the effect of 2 mg/kg of morphine the day after (day 7 or day 34). This manipulation did not change the response to morphine in non-withdrawn mice. In the withdrawn mice, however, the locomotor response was reduced to the level observed in non-withdrawn animals.

The conditional locomotor response is regarded as a component of the general psychomotor activation in sensitized animals (Anagnostaras et al., 2002). Indeed, extinction procedures that eliminate the conditional response, attenuate behavioral sensitization (Stewart and Vezina, 1991; Anagnostaras and Robinson, 1996; Carey and Gui, 1998). The present results indicate that the involvement of the conditional response in the expression of behavioral sensitization to morphine assumes particular importance after a withdrawal period.

4.3.2 Withdrawal confers to the morphine-associated environment the ability to induce D_1R-dependent ERK signaling in the NAcb shell (Paper IV)

Western blotting experiments showed that ERK phosphorylation was increased in the NAcb of withdrawn animals exposed to the morphine-associated cage. However, this increase occurred irrespectively of whether the mice where challenged with morphine (5 mg/kg) or saline. Immunocytofluorescence revealed increased phospho-ERK in the MSNs of NAcb shell but not in the core. Furthermore, the increased activation of ERK occurred only in withdrawn mice exposed to the context, and not in mice injected with saline and returned to the home cage. These data confirms that the withdrawal confers to the morphine-associated cage the ability to activate ERK in the NAcb shell.

We then analyzed if the pattern of ERK activation followed the decreased expression of behavioral sensitization to morphine observed in re-exposed mice (cf above). Indeed, two re-exposures to the context in the absence of morphine

completely abolished activation of ERK. We concluded that ERK is activated in response to the first re-exposure to the context, which correspond the increased behavioral sensitization in withdrawn mice, and that a re-exposure to the cage without morphine devaluates its significance on behavior and prevents ERK activation.

Previously it was found that morphine conditioned stimuli increase DA release in the NAcb shell (Bassareo et al., 2007). Furthermore, it was recently shown that ERK phosphorylation in the NAcb was implicated in cue-induced reward seeking (Shiflett et al., 2008). We therefore hypothesized that the increased ERK phosphorylation could represent a context-dependent stimulation of DA transmission leading to activation of DA receptors on MSNs in the NAcb shell. In order to identify the neuronal population(s) in which re-exposure to the context induced ERK phosphorylation, we used BAC transgenic mice in which EGFP expression is under the control of the promoter for the D_1R (*drd1a*-EGFP) or D_2R (*drd2*-EGFP). Immunofluorescence analysis in withdrawn *drd1a*-EGFP and *drd2*-EGFP mice demonstrated that context-induced ERK phosphorylation occurred almost exclusively in D_1R-expressing neurons. The involvement of D_1R stimulation in context-induced ERK activation in withdrawn mice was confirmed by the lack of ERK phosphorylation in mice treated with SCH23390 prior to exposure to context. This is in line with a recent study showing that blockade of D_1Rs in NAcb shell prevents reinstatement of heroin seeking by contextual cues (Bossert et al., 2007).

Previous work has shown that activation of ERK in the amygdala mediates cue-induced cocaine seeking after a prolonged period of withdrawal (Lu et al., 2005). In addition, ERK phosphorylation in the NAcb has been implicated in cue-induced reward seeking (Shiflett et al., 2008). Together with these evidences, our data highlight the impact of withdrawal on the ability of contextual stimuli to induce dopamine-dependent signaling in the NAcb. Future studies will be necessary to identify mechanisms by which prolonged withdrawal enhances the value of contextual cues that can contribute to drug seeking.

5 FUTURE DEVELOPMENTS

The results presented in this thesis indicate that administration of cannabinoids results in CB_1R-mediated increase in DARPP-32 phosphorylation at the PKA site, Thr34. This effect is exerted at the level of striatal medium spiny neurons and is required for a full behavioral response to the CB_1R agonist, CP 55,940. One important question raised by these findings is related to the mechanism by which activation of CB_1Rs, which are predominantly expressed at the presynaptic level, leads to increased DARPP-32 phosphorylation, which instead occurs at the postsynaptic level. In this regard it will be interesting to examine the possibility that the effect of cannabinoids on DARPP-32 phosphorylation is exerted via regulation of the release of glutamate from corticostriatal terminals. Indeed, cannabinoids are known to reduce glutamatergic transmission in the striatum and this may lead to suppression of DARPP-32 dephosphorylation. One way of testing this possibility would be by using conditional knock-out mouse lines that lack CB_1Rs in different neuronal subpopulations. For instance, CB_1R-induced DARPP-32 phosphorylation could be determined in mice lacking CB_1Rs in cortical glutamatergic neurons [cf (Monory et al., 2007)]. It would also be interesting to examine CB_1R-induced DARPP-32 phosphorylation in mice lacking CB_1Rs in striatal neurons (Monory et al., 2007), in order to unveil a possible regulation of DARPP-32 exerted via activation of CB_1Rs located on MSNs.

The studies performed in Paper II show that the increase in DARPP-32 phosphorylation produced by Δ^9-THC is prevented by blockade of $A_{2A}Rs$ or D_1Rs. This indicates that CB_1R-mediated DARPP-32 phosphorylation occurs in both striatopallidal (i.e. $A_{2A}R$ expressing) and striatonigral (e.g. D_1R expressing) MSNs. What is the relative contribution of changes in DARPP-32 phosphorylation occurring in these two populations of neurons to the action of cannabinoids? This question can be addressed by examining the motor depressant effect of Δ^9-THC and CP 55,940 in mice in which DARPP-32 has been inactivated in striatopallidal or striatonigral MSNs. These mice have been obtained by crossing mice in which the gene coding for DARPP-32 has been flanked by *loxP* sites (DARPP-$32^{flox+/+}$ mice), with bacterial artificial chromosome (BAC) transgenic mice, in which Cre is expressed in D_1R, or D_2R containing MSNs (*drd1a*- and *drd2*-Cre mice).

Another interesting point that will require further investigation is the possible mechanism involved in the withdrawal and context-dependent increase in ERK phosphorylation described in Paper IV. This effect is dependent on activation of D_1Rs, which also increases DARPP-32 phosphorylation. Therefore, it would be interesting to determine the involvement of phosphoThr34-DARPP-32 in this phenomenon. Is the increase in ERK phosphorylation produced by exposure to drug-paired environment still present in T34A mutant mice?

In Paper IV it is shown that ERK is activated in mice that exhibit an increased expression of psychomotor sensitization. What is the involvement of ERK signaling in this behavioral response? This question can be addressed by using drugs that are able to inhibit ERK activation, such as SL327 a selective MEK inhibitor. In addition, DARPP-32 mutant mice could also be tested in the same behavioral paradigm. It would also be interesting to analyze for how long repeated morphine treatment has to be suspended in order to observe context-dependent activation of ERK. This type of analysis could be correlated to the expression of psychomotor sensitization. Stimulation of ERK generally requires concomitant activation of D_1R and glutamate receptors. In a recent study it was shown that context-induced reinstatement of heroin seeking was attenuated by local injections of a metabotropic glutamate receptor 2 agonist (mGluR2) into the NAcb shell (Bossert et al., 2006). This raises the possibility that depressing glutamate release via stimulation of presynaptic mGluR2 may result in decreased activation of ERK in our model. This could be tested by systemic injections of mGluR2 agonists prior to exposing the sensitized mice to the morphine-associated environment.

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