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The Differential Contribution of Striatonigral and Striatopallidal Neurons in Mediating Responses to Therapeutic Agents and Drugs of Abuse: A Dual Role for DARPP-32

Helen S. Bateup

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THE DIFFERENTIAL CONTRIBUTION OF STRIATONIGRAL AND
STRIATOPALLIDAL NEURONS IN MEDIATING RESPONSES TO THERAPEUTIC
AGENTS AND DRUGS OF ABUSE: A DUAL ROLE FOR DARPP-32

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the degree of Doctor of Philosophy

by

Helen S. Bateup

June 2008

THE DIFFERENTIAL CONTRIBUTION OF STRIATONIGRAL AND
STRIATOPALLIDAL NEURONS IN MEDIATING RESPONSES TO THERAPEUTIC
AGENTS AND DRUGS OF ABUSE: A DUAL ROLE FOR DARPP-32

Helen S. Bateup, Ph.D.

The Rockefeller University 2008

The basal ganglia are a set of subcortical structures which integrate information from diverse brain areas to coordinate vital behaviors including movement, reward, and motivational processes. The striatum is the main input center of the basal ganglia which sends projections to the output nuclei via two pathways, the direct striatonigral pathway and the indirect striatopallidal pathway. These two pathways work together to modulate behavior and imbalance of these pathways can have profound physiological consequences.

DARPP-32 is a dual function kinase/phosphatase inhibitor which has been shown to be a key mediator of signaling in both striatonigral and striatopallidal neurons. A variety of therapeutic agents and drugs of abuse can affect the phosphorylation of DARPP-32. Psychostimulants such as cocaine increase DARPP-32 phosphorylation at its main regulatory site, T34. Paradoxically, antipsychotics such as haloperidol also increase T34 phosphorylation to a similar degree. Despite this similar biochemical regulation, psychostimulants and antipsychotics have opposing behavioral and clinical effects. We hypothesized that these drugs act via the same biochemical pathway but in distinct populations of striatal neurons.

To directly test this idea, we generated BAC transgenic mice which express epitope tagged DARPP-32 selectively in striatonigral and striatopallidal neurons using the D1 and D2 receptor promoters. We developed a protocol to immunoprecipitate DARPP-32 from drug treated mice and study phosphorylation in a cell-type specific manner. Using this new methodology we demonstrate that the increases in T34 phosphorylation with acute cocaine and haloperidol are restricted to striatonigral and striatopallidal neurons, respectively. Additionally, we show that the changes in DARPP-32 phosphorylation induced by a variety of drugs targeting the striatum have cell-type specific patterns.

In a complimentary approach, we generated conditional knock-out mice in which DARPP-32 is selectively deleted in striatonigral or striatopallidal neurons. This allowed us to study the behavioral consequences of alteration in the direct and indirect pathways on psychostimulant and antipsychotic mediated locomotor behavior. These studies provided direct evidence for the theory that the direct and indirect pathways exert opposing influences on locomotor behavior. Additionally, we showed that dopamine can differentially modulate activity in these pathways resulting in a synergistic stimulation of locomotor activity.

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TABLE OF CONTENTS

TITLE PAGE	(i)
COPYRIGHT	(ii)
ABSTRACT	
ACKNOWLEDGMENTS	(iii)
TABLE OF CONTENTS	(iv)
LIST OF FIGURES	(vi)
LIST OF TABLES	(viii)
CHAPTER 1: Introduction	1
The Basal Ganglia	2
The Striatum	6
Divisions of the Striatum	7
Striatal Neurotransmitter Systems	10
Dopamine	19
Dopamine Receptors	20
Dopamine Receptors and Synaptic Plasticity	22
Dopamine Receptors and Locomotion	24
Dopamine Receptors in the Actions of Psychostimulants	25
Dopamine Receptors and Schizophrenia	27
Dopamine Receptors and Parkinson's Disease	28
DARPP-32	30
Characteristics of DARPP-32	31
Biochemistry of DARPP-32 Phosphorylation	34
Factors Affecting Phosphorylation of DARPP-32	35
DARPP-32 and Disorders of the Basal Ganglia	40
DARPP-32 Phosphorylation: The Paradox	45
CHAPTER 2: Identification and Characterization of Novel DARPPP-32	
Phosphorylation Sites	47
Introduction	48
Results	49
Discussion	57
CHAPTER 3: Differential Phosphorylation of DARPP-32 in Striatonigral and Striatopallidal Neurons	61
Introduction	62
Results	64
Discussion	85

CHAPTER 4: Behavioral Analysis of Conditional DARPP-32 Knock-out Mice	93
Introduction	94
Results	95
Discussion	107
CHAPTER 5: Conclusions and Future Directions	113
MATERIALS AND METHODS	122
REFERENCES	139

LIST OF FIGURES

Figure 1.1	Basal Ganglia Circuitry	5
Figure 1.2	Signaling Pathways in Striatal Medium Spiny Neurons	11
Figure 1.3	Regulation of Locomotor Behavior by Direct and Indirect Pathway Neurons	25
Figure 1.4	DARPP-32 Phosphorylation Sites	34
Figure 2.1	Immunoprecipitation of DARPP-32 from Mouse Striatum	50
Figure 2.2	Mass Spectrometry Quantification of DARPP-32 Phosphorylation in Response to Dopaminergic Drugs	53
Figure 2.3	<i>In vitro</i> Phosphorylation of DARPP-32 by Brain Kinases	55
Figure 2.4	CKII Phosphorylates DARPP-32 at S45	56
Figure 2.5	Updated Schematic of DARPP-32 Phosphorylation Sites	59
Figure 2.6	Alignment of DARPP-32 Amino Acid Sequence by Species	60
Figure 3.1	Generation of D1R-DARPP-32/Flag and D2R-DARPP-32/Myc mice	66
Figure 3.2	D1R-DARPP-32/Flag and D2R-DARPP-32/Myc Construct Expression in Striatonigral and Striatopallidal Neurons	69
Figure 3.3	Experimental Design of <i>in vivo</i> Biochemical Experiments Using D1/D2-DARPP-32 mice	70
Figure 3.4	Cell-type Specific DARPP-32 Immunoprecipitations	72
Figure 3.5	Effects of D1R and D2R agonists on DARPP-32 Phosphorylation in Striatal Slices	75
Figure 3.6	Effects of D1R and D2R Agonists on DARPP-32 Phosphorylation <i>in vivo</i>	78
Figure 3.7	Differential Regulation of DARPP-32 Phosphorylation by Psychostimulants and Antipsychotics	82
Figure 3.8	Chronic L-DOPA Increases T34 Phosphorylation Selectively in D1R Neurons	84

Figure 4.1	Generation of Conditional DARPP-32 Knock-out Mice	98
Figure 4.2	DARPP-32 Protein Expression in Conditional Knock-out Mice	100
Figure 4.3	Basal Locomotor Activity in D1R-D32-KO and D2R-D32-KO Mice	102
Figure 4.4	Locomotor Responses to Acute Cocaine and Haloperidol in Conditional DARPP-32 KO Mice	106
Figure 5.1	Striatonigral and Striatopallidal Neurons Integrate Afferent Signals and Modulate Behavior via the Direct and Indirect Pathways	119

LIST OF TABLES

Table 1	Factors Affecting DARPP-32 Phosphorylation	39
Table 2	DARPP-32 Phosphopeptide Sequences	51
Table 3	Body Weights of Conditional DARPP-32 Knock-out Mice	101

Chapter 1
INTRODUCTION

The Basal Ganglia

The basal ganglia (BG) are a set of subcortical structures involved in coordinating information from sensorimotor, motivational and cognitive brain areas to control behaviors such as movement and reward learning. Signals from the cortex, mid-brain and limbic regions are processed and integrated through the basal ganglia which in turn send this integrated information back to the cortex and brain stem to drive behavior. Disruption in BG signaling can have profound consequences on an organism's behavior. Parkinson's disease and Huntington's disease are neurodegenerative diseases affecting structures in the BG which result in debilitating disruption of coordinated movement and cognition. The maladaptive reward learning associated with drug addiction has been linked to alterations in cellular processes in particular areas of the BG. Psychiatric disorders such as schizophrenia, obsessive-compulsive disorder, and attention deficit hyperactivity disorder have also been connected to the BG, involving imbalances in neurotransmitter systems within this brain area. A thorough understanding of the normal and disrupted function of the BG is therefore useful to gain insight into a number of diverse and critical brain processes.

The BG consist of several interconnected structures which include the striatum, comprised of the caudate/putamen and nucleus accumbens, the external and internal segments of the globus pallidus, the subthalamic nucleus, and the substantia nigra (Figure 1.1). Together with the cortex and thalamus, the BG participate in a circuit which begins with topographically mapped glutamatergic afferents from the cortex and thalamus to the striatum. Striatal neurons then send GABA-ergic collaterals within the striatum and primary projections to BG output nuclei via two pathways. The direct striatonigral

pathway sends monosynaptic projections to the substantia nigra pars reticulata (SNr) and the globus pallidus internal segment (GPi, entopeduncular nucleus (EPN) in rodents). The indirect striatopallidal pathway projects to the globus pallidus external segment (GPe) which in turn sends GABA-ergic axons to the subthalamic nucleus (STN). The STN sends excitatory glutamatergic projections back to the GPe as well as to the output nuclei, the GPi/SNr. GPi/SNr neurons project to several thalamic nuclei which then complete the circuit by innervating prefrontal, premotor, and motor cortical areas. In addition to the cortex and thalamus, the hippocampus and amygdala also send topographically organized glutamatergic input to the striatum which is most dense in ventral striatal regions.

In addition to transmission via glutamate and GABA, the BG also relies on signaling by modulatory neurotransmitters, among which dopamine plays an essential role. There are several different dopaminergic pathways within the basal ganglia-cortical circuit which are associated with subsets of connections mediating different aspects of BG function. The nigrostriatal pathway sends dopaminergic projections from the SNc to the dorsal striatum and is essential for coordinated voluntary movement. It is the SNc dopaminergic neurons which degenerate in Parkinson's disease resulting in disruption of motor activity. The mesolimbic pathway is comprised of dopaminergic projections from the ventral tegmental area to the nucleus accumbens and this system is largely involved in motivation, emotion, and reward processes. Another set of dopaminergic projections from the VTA to the limbic cortex known as the mesocortical pathway, is associated with working memory, learning and reward.

In addition to glutamate and dopamine, striatal neurons respond to a variety of other neurotransmitters including serotonin, adenosine, acetylcholine and opiates, discussed in more detail below. Taken together, the combination of interconnections between BG structures, the inputs from a wide-range of brain regions, and the ability to respond to a varied array of signals, endows the BG with the ability to integrate diverse information in a selective and complex way.

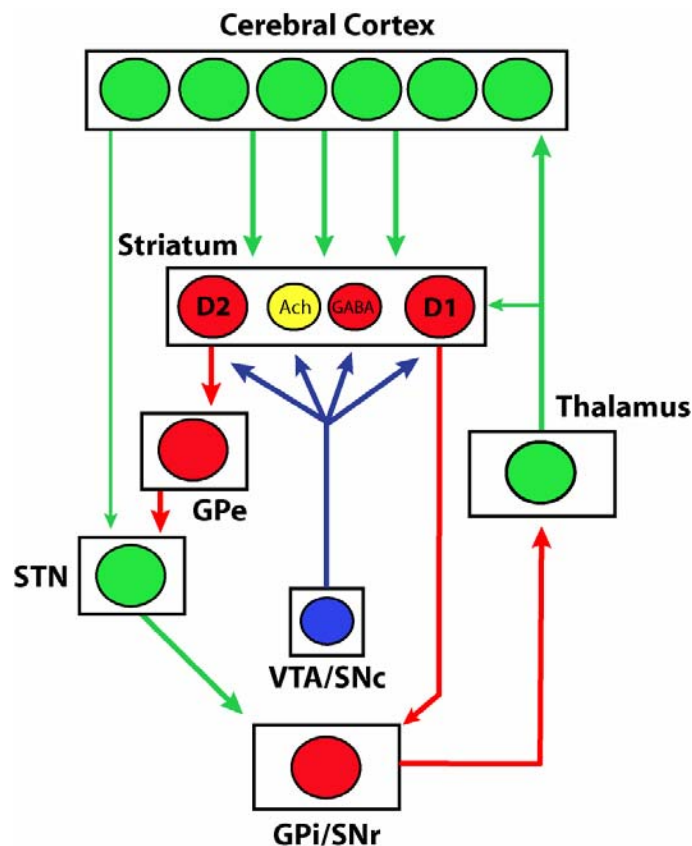


Figure 1.1 Basal Ganglia Circuitry

Schematic drawing of the connections between structures of the basal ganglia. Green arrows indicate excitatory glutamatergic projections, red arrows indicate GABA-ergic inhibitory projections, and blue arrows represent dopaminergic projections. Abbreviations: D1, dopamine receptor type 1; D2, dopamine receptor type 2; Ach, acetylcholinergic interneuron; GABA, GABA-ergic interneuron; GPe, globus pallidus external segment; GPi, globus pallidus internal segment; STN, subthalamic nucleus; SNr, substantia nigra pars reticulata; SNc, substantia nigra pars compacta; VTA, ventral tegmental area. Adapted from (Svenningsson et al., 2004).

The Striatum

The striatum is the main input center for the BG which responds to and integrates signals from a variety of brain areas and neurotransmitter systems. The striatum is therefore an excellent model system for studying neuronal signal transduction and integration. The striatum is mainly comprised of medium spiny neurons (MSNs) which represent 90-95% of all cells in this structure (Kemp and Powell, 1971). These neurons contain a large number of dendritic spines which are the primary sites receiving dopaminergic and glutamatergic input. MSN's are GABA-ergic and send axons collaterally within the striatum and project via the direct and indirect pathways to the output structures of the BG. The division of MSN's into direct and indirect sub-populations is 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 (Gerfen et al., 1990). Indirect pathway MSN's express enkephalin, type 2 dopamine receptors, and adenosine type 2 receptors (Gerfen et al., 1990).

In addition to MSNs, the striatum contains several classes of interneurons defined by their physiology, morphology, and expression of specific markers. Striatal interneurons fall into four main categories, one class of large cholinergic neurons which represent the main source of acetylcholine (ACh) in the striatum, and three types of GABA-ergic interneurons which express either parvalbumin, calretinin, or nitric oxide synthase (NOS) and 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 MSN's where they can strongly regulate action potential firing (Tepper et al.,

2004). The striatum, therefore, can be viewed as a site of integration not only at the level of varied inputs converging on individual cells but also at the level of signal integration across networks of striatal neurons.

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.

Dorsal versus Ventral Striatum. The dorsal-ventral division of the striatum was originally based on differences in afferent connections, whereby the dorsal region receives somatotopically organized sensorimotor-related information from the cortex and dopaminergic innervation primarily from the SNc (Donoghue and Herkenham, 1986; Kitai et al., 1976). The ventral striatum, on the other hand, receives visceral and limbic related afferents with a higher density of inputs from the amygdala, hippocampus, and VTA (Voorn et al., 2004). The intermediate zone receives polymodal information from the thalamus as well as input from higher associational cortical centers (Voorn et al., 2004). These divisions are loosely based, however, as there are no strict anatomical boundaries between these regions and innervation by all afferent brain regions is found throughout the striatum. It has also recently been suggested that a more accurate

dorsal/ventral division of the striatum may be along a dorsolateral to ventromedial axis (Voorn et al., 2004).

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. Within the ventral striatum, the nucleus accumbens can be further subdivided into core and shell regions which differ in their efferent and afferents connections. There has been significant research in the field of drug abuse to examine the contributions of the core versus shell 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).

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 acetylcholinesterase staining (AChE) (Graybiel and Ragsdale, 1978). The AChE poor striosomes or patches were later shown to correspond to small striatal regions rich in μ -opioid 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).

Although the patch/matrix distinction is not completely rigid, it 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.

Direct and Indirect Pathway Projections. As mentioned previously, 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 sub-class of GABA-ergic MSNs which express 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. 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, which allow selective identification, purification, and/or targeting of these

cells (Gong et al., 2003; Heintz, 2001). Studies utilizing these mice have revealed important differences between striatonigral and striatopallidal neurons which were not appreciated with conventional techniques. Chapters three and four of this thesis will present further data revealing important differences in intracellular signaling processes between these two cell populations.

Striatal neurotransmitter systems

As stated previously, the striatum is innervated by an abundance of neurotransmitters and neuromodulators which are integrated at the level of the single cell and in the context of the entire BG. Striatal cells are therefore an excellent system to study signal transduction, specifically the interactions between fast and slow signaling pathways (Figure 1.2). Fast synaptic responses occur within milliseconds and are due to the rapid influx of charged ions into the cell which depolarize or hyperpolarize the cell membrane, ultimately making a cell more or less likely to fire an action potential (Greengard, 2001). Slow transmission, on the other hand, requires seconds to minutes, and occurs largely through G-protein coupled receptors which are linked to a vast array of second messengers and signaling molecules including protein kinases and phosphatases (Greengard, 2001). Signaling via these second messengers allows multiple points of convergence or inhibition between the numerous signaling molecules that act on the striatum and provides an opportunity to study the complex interactions between these pathways.

In order to respond to a vast array of neurotransmitters and neuromodulators, 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 which will be discussed in more detail in the next section. Here I will present a brief overview of the non-dopaminergic signaling pathways with regard to their role in striatal function.

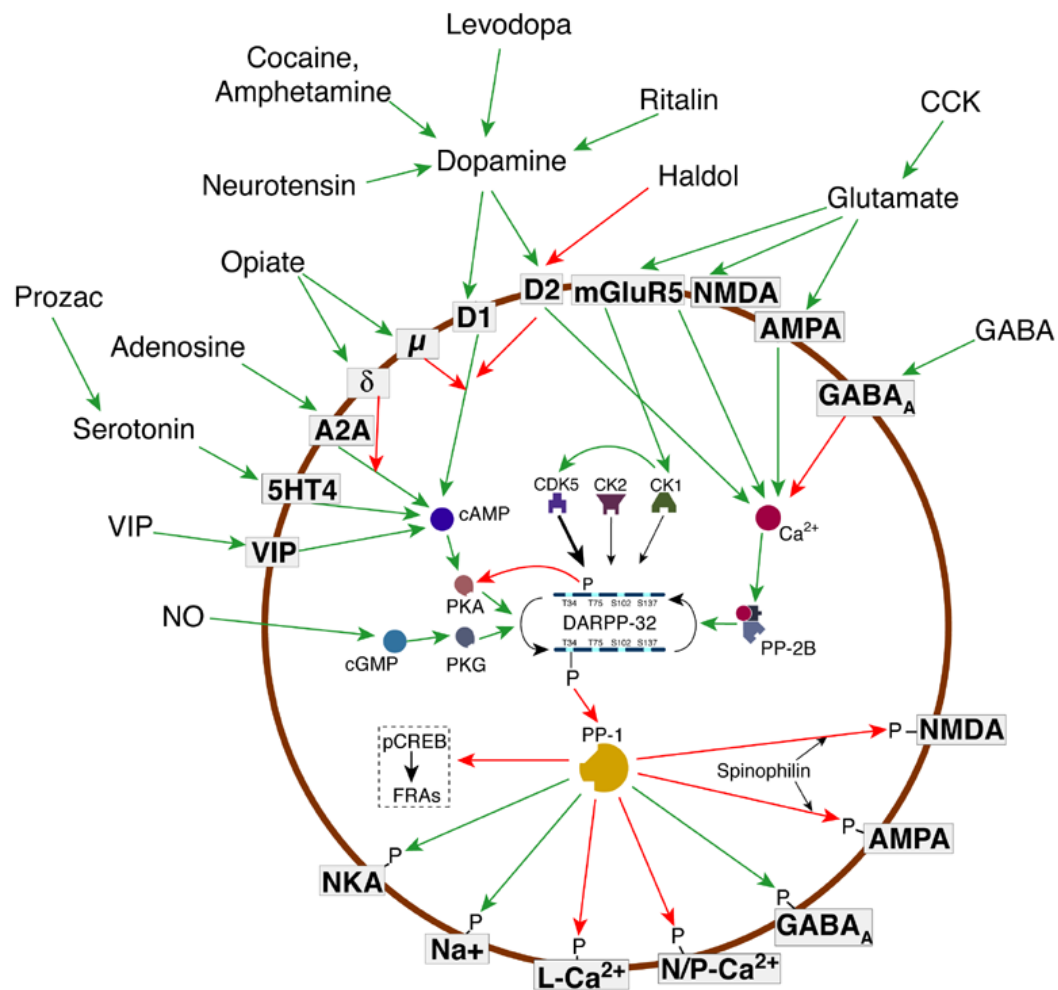


Figure 1.2 Signaling Pathways in Striatal Medium Spiny Neurons

Striatal medium spiny neurons respond to a variety of neurotransmitters, therapeutic drugs, and drugs of abuse which are integrated at the level of direct receptor interactions and second messenger cascades.

Green arrows represent excitatory actions, red arrows represent inhibitory actions. See text for abbreviations. Adapted from (Greengard, 2001)

Glutamate. Glutamatergic input from the cortex and thalamus provides the primary excitatory drive to striatal neurons. Glutamate acts on AMPA and NMDA ionotropic receptors expressed on striatal cells which control the influx of sodium and calcium into these neurons. AMPA and NMDA receptors are the primary players mediating cellular learning and memory processes which are known as long-term potentiation (LTP) and long-term depression (LTD). LTP and LTD are processes whereby coordinated periods of high frequency (LTP) or low frequency (LTD) glutamate input combined with a permissive post-synaptic environment result in long-term changes in synaptic strength and responses to future stimulation. The induction phase of these processes relies largely on local changes in the phosphorylation of AMPA and NMDA receptors which changes their functional properties and/or trafficking to the membrane (Malinow and Malenka, 2002; Raymond, 2007). Late-phase LTP and LTD, however, require gene transcription and protein synthesis (Mameli et al., 2007; Raymond, 2007).

LTP and LTD are important forms of synaptic plasticity expressed at corticostriatal synapses and may underlie primary striatal functions such as motor and reward learning, and cognitive performance (Calabresi TINS 2007). Disruptions in normal LTP and LTD processes are seen in response to drugs of abuse and in maladaptive responses to drugs used to treat Parkinson's disease (Kauer and Malenka, 2007; Picconi et al., 2003; Ungless et al., 2001). Although striatal LTP and LTD are primarily mediated by AMPA and NMDA receptor activation, these forms of plasticity also critically depend on input from striatal neuromodulators such as dopamine, acetylcholine, endocannabinoids, and nitric oxide as discussed below (Surmeier et al., 2007).

In addition to ionotropic transmission, glutamate also acts via metabotropic glutamate receptors (mGluRs) which are expressed in the striatum. mGluRs are divided into three groups based on their sequence homology and coupling to G-proteins. Group I mGluR1 receptors are expressed post-synaptically on MSNs and interneurons of the striatum as well as pre-synaptically on dopaminergic neurons of the SNc. mGluR1 receptors are coupled to G_q proteins which can activate the phospholipase C (PLC)/diacylglycerol (DAG)/inositol triphosphate (IP_3) cascade which increases intracellular calcium levels and protein kinase C (PKC) activity. In dopaminergic neurons of the SNc, activation of mGluR1 receptors on axon terminals suppresses dopamine release to the striatum, likely due to the activity of calcium-activated potassium channels (Zhang and Sulzer, 2003). mGluR5 receptors are expressed in indirect striatopallidal MSNs where they couple to G_q and can amplify NMDA signaling (Conn et al., 2005). mGluR5 receptors have recently been shown to physically interact with A2A receptors acting synergistically to promote downstream signaling pathways including the activation of mitogen-activated protein kinase (MAPK) which in turn activate gene expression in these cells (Ferre et al., 2002; Nishi et al., 2003).

Group II mGluRs (mGluR2 and mGluR3) receptors are also involved in striatal signaling. They are expressed presynaptically on corticostriatal nerve terminals where they can negatively regulate glutamate release onto striatal cells (Lovinger and McCool, 1995). In addition to expression within the striatum, ionotropic and metabotropic glutamate receptors are expressed either pre- or post-synaptically throughout the basal ganglia which contributes to a complex network of signaling by glutamate in this brain region.

GABA. Ionotropic GABA_A and metabotropic GABA_B receptors are expressed in the striatum and respond to GABA released from MSN axon collaterals and striatal interneurons. GABA_A receptors are ligand-gated ion channels that allow hyperpolarizing chloride ions to enter the cell which generates inhibitory post synaptic potentials and counter-balances glutamatergic transmission. GABA_A receptors are expressed postsynaptically on MSNs. Recent studies have suggested that GABA_A receptors may be upregulated in the drug addicted state serving as a homeostatic mechanism to counterbalance increases in dopaminergic transmission (Heiman et al., 2007; Volkow et al., 1998). Metabotropic G-protein coupled GABA_B receptors are expressed in striatal interneurons, MSNs, and in GP, SN and VTA neurons (Waldvogel et al., 2004). Recent studies suggest that GABA_B receptors expressed in dopaminergic cells play an important role in regulating dopamine release in the striatum and therefore mediating the rewarding properties of drugs of abuse (Vacher et al., 2006).

Serotonin. The serotonergic system plays an important role in the regulation of emotion, mood, reward and cognition. Serotonin imbalance is implicated in several common psychiatric disorders including depression, schizophrenia, and drug addiction and the serotonergic system is the target for many of the current medications used to treat these types of disorders. The prefrontal cortex and hippocampus are well studied sites of serotonin action. However, the important role of serotonin signaling in the striatum is becoming increasingly clear (Svenningsson et al., 2002a; Svenningsson et al., 2002b; Zhang et al., 2007). Medium spiny neurons express several sub-types of serotonin receptors including 5-HT_{1B}, 5-HT_{1F}, 5-HT_{2A}, 5-HT_{2C}, 5-HT₃, 5-HT₄, and 5-HT₆ (Svenningsson et al., 2004). All of these receptors are metabotropic, with the exception

of 5-HT₃ which is ionotropic, and activate a variety of intracellular signaling pathways which converge with second messenger systems initiated by dopamine and other neurotransmitters. Striatal serotonin receptors act primarily via the following second messenger systems: 5-HT_{1B/E} receptors decrease cyclic AMP (cAMP) formation, 5-HT_{2A/C} receptors increase DAG and IP₃ production, 5-HT₃ receptors increase Na⁺ and Ca⁺ influx, and 5-HT₄ and 5-HT₆ receptors increase cAMP production (Svenningsson et al., 2004). Two recent studies showed that Prozac, a commonly prescribed anti-depressant drug, acts via 5-HT₄ and 5-HT₆ receptors in the striatum to increase cAMP and protein kinase A (PKA) signaling, a pathway which is also important in dopamine signaling (Svenningsson et al., 2002a; Svenningsson et al., 2002b).

Adenosine. Like dopamine and serotonin, adenosine has an important modulatory role in the striatum. Adenosine is primarily formed via the breakdown of intracellular ATP which is transported to the extracellular space via transporter molecules. Released adenosine acts via two types of G-protein coupled metabotropic receptors, A₁ and A_{2A}, which are highly expressed in the brain (Fredholm et al., 2005). The A₁ receptor subtype is widely expressed throughout the brain, in both types of striatal projection neurons, and on pre-synaptic dopaminergic nerve terminals (Ferre et al., 1997). A₁ receptors are coupled to G_i proteins which inhibit cAMP and PKA signaling. A₁ receptors have recently been shown to modulate dopamine signaling in both direct and indirect MSN's via their influence on pre-synaptic dopamine release as well as through G-protein mediated second messenger signaling (Yabuuchi et al., 2006).

A_{2A} receptors, on the other hand, are highly expressed in the striatum where they are selectively localized to indirect striatopallidal MSN's (Schiffmann et al., 1991). A_{2A}

receptors are coupled to G_{olf} proteins which stimulate the adenylyl cyclase (AC)/cAMP/PKA cascade (Kull et al., 2000). A_{2A} receptors form heteromeric complexes with D2 dopamine receptors in striatopallidal neurons where they can oppose D2 R signaling via modulation of ligand binding affinity and through opposing action on the AC/cAMP/PKA cascade (Ferre et al., 1991; Yabuuchi et al., 2006). It was recently discovered that the mechanism of action of the widely used psychostimulant, caffeine, is via antagonism of A_{2A} receptors (Fredholm et al., 1999). Inhibition of A_{2A} Rs by caffeine is “pro-dopaminergic” since it relieves the A_{2A} R-mediated inhibition of D2 receptor signaling (Lindskog et al., 2002). A role for A_{2A} receptor signaling in Parkinson’s disease is also becoming increasingly clear and A_{2A} receptor antagonists are being touted as potential non-dopaminergic therapies for PD due to their lower risk for debilitating side-effects and potential neuroprotective properties (Schwarzschild et al., 2006).

Acetylcholine. As mentioned previously, acetylcholine is released in the striatum by large cholinergic interneurons and a proper balance between dopamine and acetylcholine is necessary to govern coordinated movement. Several classes of nicotinic and muscarinic acetylcholine receptors (AChRs) are expressed in specific pre- and post-synaptic striatal compartments where they regulate striatal signaling. $\alpha 4\beta 2$ and $\alpha 7$ nicotinic AChRs are found at pre-synaptic dopaminergic and glutamatergic nerve terminals respectively and stimulation of these receptors induces either DA or glutamate release (Hamada et al., 2005). It is thought that the rewarding and addictive properties of nicotine are mediated by direct action of this drug at nAChRs resulting in increased dopamine release in the striatum.

In addition to nicotinic receptors, muscarinic G-protein coupled acetylcholine receptors also play an important role in BG signaling. Pre-synaptic muscarinic M5 receptors are expressed in SNc dopaminergic cells and can facilitate the release of dopamine (Zhang et al., 2002). M4 receptors are expressed abundantly on MSNs and facilitate dopamine release likely by inhibiting GABA-ergic negative feedback to dopaminergic neurons (Zhang et al., 2002). M3 receptors, on the other hand, are expressed at low levels in MSNs but can also inhibit dopamine release (Zhang et al., 2002). M1 ACh receptors located post-synaptically on MSNs were recently shown to be involved in corticostriatal LTD whereby lowering M1 receptor tone promoted this type of plasticity via disinhibition of L-type calcium channels (Wang et al., 2006).

Opiates, nitric oxide, and endocannabinoids. The roles of the neurotransmitters described above, either in the striatum or in other brain systems, have been well studied. In addition to these factors, signaling molecules outside of the classical categories have also been shown to exert important modulatory roles in the striatum. Among these are the neuropeptide opiates, the gaseous signaling molecule nitric oxide, and the membrane lipid-derived endocannabinoids which were recently found to play a role in striatal function.

Endogenous opiates regulate striatal function by indirect action on dopaminergic neurons and by direct activity at opioid receptors located post-synaptically on MSNs. Opioid receptors are highly expressed in the striatum where κ -opioid receptors are expressed primarily on dopaminergic nerve terminals, and μ - and δ -receptors are expressed on MSNs (Schoffelmeer et al., 1986). μ -receptors are selectively enriched in direct pathway striatonigral neurons where they are co-localized with D1 receptors and

negatively regulate D1 receptor signaling (Georges et al., 1999). δ -receptors are expressed in interneurons as well as in indirect striatopallidal MSNs where they can counteract signaling via A_{2A} receptors (Lindskog et al., 1999).

Striatal nitric oxide (NO) is generated by neuronal nitric oxide synthase-(nNOS)-expressing interneurons upon stimulation of dopamine type 5 receptors expressed in these interneurons (Centonze et al., 2003). NO diffuses across the cell membrane where it activates soluble guanylyl cyclase, thereby increasing cGMP levels, and protein kinase G (PKG) activity. Via this pathway, NO has been shown to be involved in striatal synaptic plasticity via regulation of the induction phase of LTD in MSNs (Centonze et al., 2003).

In addition to the modulation of synaptic plasticity by opiates and NO, endogenous cannabinoids (ECBs) have emerged as additional regulators of LTD in the striatum as well as in other brain areas (Calabresi et al., 2007). ECBs are synthesized from membrane derived lipid precursors released from post-synaptic MSNs upon D2 R activation. These signaling molecules then bind to CB-1 receptors located in glutamatergic axon terminals which serve to suppress glutamate release and induce LTD in corticostriatal synapses (Yin and Lovinger, 2006).

Taken together these descriptions provide a brief overview into the variety and complexity of non-dopaminergic signaling pathways in the striatum. All of these pathways are linked to each other either via direct receptor interactions, modulation of pre-synaptic neurotransmitter release, or interactions at the level of second messengers. A common theme in these signaling pathways is that they are all somehow connected back to dopamine which appears to be either modulating or modulated by these signaling molecules. In the next section I will discuss in more detail what is known about

dopamine in the striatum and the signaling properties of dopamine receptors which are key regulators of basal ganglia function.

III. Dopamine

Dopamine (DA) belongs to the class of catecholamine neurotransmitters which also includes epinephrine (E) and norepinephrine (NE, also known as noradrenaline). These three neurotransmitters are synthesized from the amino acid tyrosine via a series of enzymatic steps. The first enzyme in the pathway is tyrosine hydroxylase (TH) which converts tyrosine to L-dihydroxy-phenylalanine (L-DOPA) which is the rate limiting step in DA and NE synthesis (Holtz, 1959). TH is present in all catecholamine-producing neurons and is commonly used as a histological marker for these cell types. L-DOPA is then converted to dopamine by a carboxylase. In NE and E expressing neurons, two additional enzymes are required to convert DA to NE and NE to E. L-DOPA is the “gold-standard” drug used to rescue the effects of dopamine loss associated with Parkinson’s disease (PD) and will be discussed in more detail below.

Dopamine is released by several populations of midbrain and forebrain neurons. The A8-10 mid-brain dopaminergic cell groups include the SNc and VTA which send ascending projections to the striatum, cortex and limbic system. The A11-14 cell populations arise from the hypothalamus and wall of the third ventricle and send descending projections to the autonomic areas of the brain stem and spinal cord, as well as regulate neuroendocrine function. There are additional dopaminergic cell groups (A15-17) located in olfactory structures and in the retina.

This work will focus on the SNc and VTA dopaminergic projection neurons which innervate the BG. These ascending dopaminergic inputs are linked to several important brain processes including control of voluntary movement, reward learning, and motivational processes. A large body of behavioral, biochemical, and electrophysiological work has been devoted to understanding the precise aspects of these processes which are controlled by dopamine. Dopamine's role in motor function has been long recognized and there are several current hypotheses regarding DA's function in reward. Prevailing views suggest that DA is critical for reinforcement processes which are the strengthening of stimulus-response and stimulus-reward associations (Wise, 2004). DA has also been shown to be involved in priming, motivational arousal, and reward prediction (Wise, 2004). Many of these functions require learning and memory processes and DA plays an essential role in modulating LTP and LTD, the cellular correlates of learning, which will be further discussed below (Calabresi et al., 2007).

Dopamine Receptors

Dopamine's actions are carried out by five sub-types of DA receptors selectively expressed in discrete brain regions. Dopamine receptors are seven transmembrane domain metabotropic receptors which are coupled to G-proteins. The D1-class of receptors includes the D1 and D5 sub-types which couple to G_s proteins and activate adenylyl cyclase (AC), the enzyme that converts ATP to cAMP (Stoof and Kebabian, 1981). cAMP activates a major effector enzyme, protein kinase A (PKA). Activated PKA phosphorylates a variety of intracellular targets such as neurotransmitter receptors, ion channels, transcription factors, and signaling molecules which changes their signaling

properties and can influence cellular excitability. Protein phosphorylation has been shown to be a key mechanism underlying synaptic signal transduction as it allows relatively fast modification of protein targets without the need for further transcriptional or translational processes (Greengard 2001 Science). The D2 class of receptors includes D2 R, D3 R and D4 R which are coupled to G_i proteins and inhibit AC, thereby decreasing cAMP and PKA activity (Stoof and Kebabian, 1981). D2 receptors can also elevate intracellular calcium levels and activate a phospholipase C (PLC)/protein phosphatase-2b (PP-2B) cascade (Nishi et al., 1997). D2 receptors have been shown to have a higher affinity for dopamine than D1 R which may impact the relative contribution of signaling via these receptors in low and high dopamine states (Hamada et al., 2004; Missale et al., 1998).

D1 receptors show widespread neocortical expression including the prefrontal cortex (PFC) and striatum. D5 receptors are concentrated in the hippocampus and entorhinal cortex (EC) with very low expression in the striatum. D2 receptors are highly expressed in the striatum where they are found both post-synaptically on MSNs and pre-synaptically as autoreceptors on dopaminergic nerve terminals. D2 receptors are also expressed at low levels in the hippocampus, EC, amygdala, and weakly in the PFC. D2 receptors exist as two different splice variants termed $D2_{\text{short}}$ and $D2_{\text{long}}$ which are differentially expressed pre-synaptically ($D2_{\text{short}}$) or post-synaptically ($D2_{\text{long}}$) (Centonze et al., 2004). D3 Rs 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 (Guillin et al., 2007; Sealfon and Olanow, 2000).

Within the striatum, post-synaptic D1 and D2 receptors are largely segregated to the two populations of MSNs comprising the direct striatonigral, and indirect striatopallidal pathways (Gerfen et al., 1990). The early evidence for this segregation was based on retrograde labeling from BG output structures and histochemical staining with cell specific markers (Deng et al., 2006; Gerfen and Young, 1988). Current techniques relying on genetically targeted constructs using the D1R and D2R promoters have also verified this segregation (Gong et al., 2003). Studies utilizing both BAC transgenic mice as well as traditional knock-outs and knock-ins have shown differential responses to various agents in D1R and D2R-expressing cell populations (Day et al., 2006; Gantois et al., 2007; Lee et al., 2006; Moratalla et al., 1996).

Despite strong evidence for segregation of D1R and D2Rs in the striatum, some studies have suggested varying degrees of co-localization (Aizman et al., 2000; Surmeier et al., 1996). These studies relied on techniques such as immunohistochemistry and RT-PCR which are sensitive and can have problems with background contamination. Additionally, these studies were performed in cultured striatal neurons which may not accurately represent the *in vivo* situation. The work presented here in subsequent chapters provides additional evidence that D1 and D2 receptors are segregated to different striatal cell populations.

Dopamine Receptors and Synaptic Plasticity

It is widely accepted that one of the major functions of dopamine is to modulate glutamatergic signaling. In the case of the striatum this involves modulation of excitatory corticostriatal input. Glutamatergic and dopaminergic axons terminate of the head and

neck, respectively, of dendritic spines on medium spiny neurons (Chase and Oh, 2000).

As mentioned previously, the striatum is involved in several types of learning and memory processes and balanced striatal synaptic plasticity including LTP and LTD is essential for its function. Electrophysiological studies have examined the relative contributions of D1 and D2 receptors to these processes.

D1 receptors have been shown to interact with NMDA-type glutamate receptors which impacts the trafficking and function of each receptor in a bi-directional manner (Lee et al., 2002; Scott et al., 2002). Via several different pathways, dopamine can either enhance or inhibit NMDA signaling based on the depolarization state of the neuron (Surmeier et al., 2007). Acting through the cAMP/PKA pathway, D1R signaling has been shown to increase phosphorylation of NMDA receptors and calcium channels which can enhance glutamate-induced currents (Flores-Hernandez et al., 2002). It is thought that this mechanism may underlie the D1 receptor activation of LTP, a process which is lost by treatment with D1R antagonists, or in D1 receptor knock-out mice (Calabresi et al., 2007).

D2 receptors, conversely, have an inhibitory effect on LTP and are known to be vital in the induction of LTD. D2R activation leads to dephosphorylation of the GluR1 subunit of the AMPA receptor which reduces glutamate-mediated currents and can promote trafficking of the receptor out of the membrane (Hakansson et al., 2006). Stimulation of D2 receptors can also lead to diminished pre-synaptic release of glutamate (Yin and Lovinger, 2006). As mentioned previously, D2 receptors play an important role in striatal LTD. D2 receptors expressed in cholinergic interneurons regulate acetylcholine release, while D2Rs expressed post-synaptically on MSNs are important in

facilitating endocannabinoid release, both essential mediators of LTD (Calabresi et al., 2007).

Dopamine Receptors and Locomotion

In the case of the D1 receptor, the classical model of basal ganglia 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 GABA-ergic 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 (Figure 1.3). 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 by the following mechanism. Stimulation of D2Rs is inhibitory in indirect pathway cells, resulting in decreased GABA-ergic 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 (Figure 1.3). As a result, agents which block D2 receptors such as antipsychotics, decrease locomotion and in some cases result in catalepsy, characterized by loss of voluntary movement and muscular rigidity.

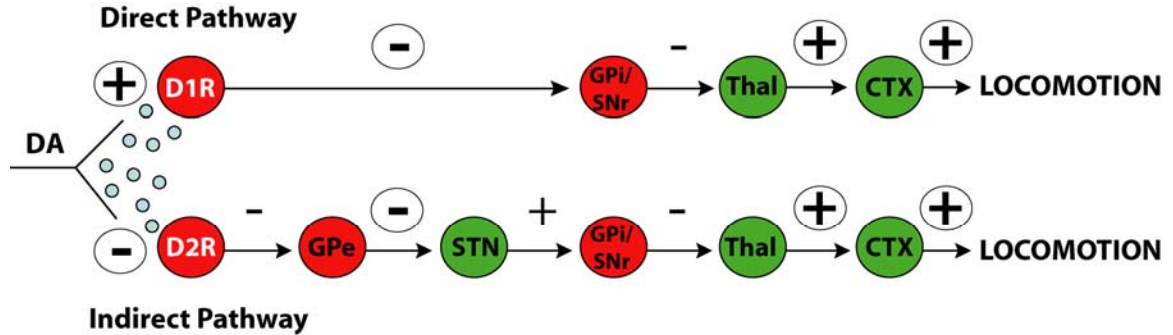


Figure 1.3 Regulation of Locomotor Behavior by Direct and Indirect Pathway Neurons

Schematic of the excitatory and inhibitory connections of the basal ganglia output pathways controlling locomotion. Minus signs indicate GABA-ergic inhibitory connections, plus signs indicate glutamatergic excitatory connections. Bolded and circled plus and minus signs indicate increased excitation or inhibition in response to dopamine. Abbreviations: DA, dopamine; D1R, type 1 dopamine receptor; D2R, type 2 dopamine receptor; SNr, substantia nigra pars reticulata; TH, thalamus; Ctx, cortex; GPe, globus pallidus external segment; STN, subthalamic nucleus.

Dopamine Receptors in the Action of Psychostimulants

Nearly all drugs of abuse have been shown to be dependent on or alter dopaminergic signaling (Nestler, 2001). Psychostimulant drugs have direct actions on the dopamine system by enhancing levels of synaptic dopamine. This enhancement occurs either by blocking the re-uptake of dopamine by the pre-synaptic dopamine transporter (DAT) or by facilitating the release of dopamine from synaptic vesicles. Dopamine receptor signaling is essential for mediating both the locomotor stimulant and addictive properties of these drugs.

Studies with D1 receptor knock-out (D1R KO) mice have shown that these mice have altered sensitization and locomotor responses to cocaine (Xu et al., 2000). D1R KO mice have also been shown to have reduced or absent biochemical responses to

psychostimulants including blunted dynorphin and cfos responses (Moratalla et al., 1996). Activation of the cAMP/PKA pathway by D1 receptors stimulates CREB and Δ FosB expression. These molecules are increased in response to chronic cocaine and can produce long lasting changes in striatal neurons associated with the drug addicted state (Ron and Jurd, 2005). Further evidence implicating D1R signaling in mediating the long term changes associated with psychostimulant use is the finding that increases in spine density occurring with chronic cocaine use are selectively stable in D1R but not in D2R-expressing nucleus accumbens neurons (Lee et al., 2006).

In addition to D1 receptors, D2Rs are also important in mediating the locomotor-activating and rewarding effects of psychostimulant drugs of abuse. Aspects of the induction phase of cocaine-induced locomotor activity are selectively blocked by D2R antagonists but not by non-specific motor depressant drugs (Chausmer and Katz, 2001). Additionally, cocaine-seeking behavior is enhanced by stimulation of the nucleus accumbens with D2 R but not D1 R agonists (Self et al., 1996). Recent studies have shown that a regulator of G protein signaling, RGS9-2, plays an essential role in cocaine-induced hyperlocomotion and conditioned place preference. The primary target of RGS9-2 was shown to be the D2 receptor (Rahman et al., 2003). Another study showed that D2 receptors can interact with a subunit of the NMDA receptor, NR2B, and that this interaction is induced by cocaine administration and required for the full induction of the locomotor response to cocaine. Functionally, this interaction resulted in decreased NMDA activity and inhibition of indirect pathway neurons which is associated with increased locomotor activity (Liu et al., 2006). Together these results suggest that both

D1 and D2 receptors are important for different aspects of the biochemical and behavioral responses to psychostimulant drugs of abuse.

Dopamine Receptors and Schizophrenia

Abnormalities in dopamine signaling were classically linked to schizophrenia based on the discovery that compounds with high D2 receptor antagonism were successful in reducing the positive or psychotic symptoms of schizophrenia (Creese et al., 1976; Seeman and Lee, 1975). Newer generation “atypical” antipsychotic drugs were developed to target the negative symptoms and cognitive disruptions in schizophrenia which were not resolved by classical drugs. Atypical antipsychotics are active at different classes of serotonin and adrenergic receptors. Clinically effective doses of these drugs, however, still retain the ability to bind striatal D2 receptors (Abi-Dargham and Laruelle, 2005). In addition to a D2 receptor role in antipsychotic drug action, imaging studies have demonstrated enhanced striatal D2R binding in untreated schizophrenic patients suggesting an elevation of D2R activity in this disorder (Laruelle and Weinberger, 2001).

Balanced D1 receptor activation has been shown to be important for optimal performance in cognitive tasks mediated by the prefrontal cortex (PFC) (Goldman-Rakic et al., 2000). It is thought that deficiency in PFC function is associated with the negative and cognitive symptoms of schizophrenia. Therefore a link between D1 receptor activity in the PFC and schizophrenia has been made. The current dopamine hypothesis of schizophrenia states that hyperactivity of striatal D2 receptors contributes to the positive, psychotic symptoms of schizophrenia, while hypoactivity of PFC D1 receptors underlies

the negative and cognitive disturbances seen in this disease (Guillin et al., 2007). Along with a strong dopaminergic component, schizophrenia has also been associated with perturbations in other neurotransmitter systems including serotonin, glutamate, GABA and acetylcholine. Given the high level of overlap of these pathways in the BG and limbic system, it is very likely that there are complex interactions and imbalances among these systems which contribute to the psychopathology of schizophrenia.

Dopamine Receptor Signaling in Parkinson's Disease

Parkinson's disease (PD) is a neurodegenerative disorder characterized by the progressive loss of dopamine due to the death of dopaminergic neurons in the SNc. Clinically, PD is manifested by an inability to translate thought into coordinated movement resulting in bradykinesia (slowed movement), resting tremor, and muscular rigidity. In addition to disruptions in movement, PD is also associated with cognitive dysfunctions which often manifest in the later stages of PD. The loss of dopaminergic innervation of the striatum results in profound secondary adaptations in MSNs which underlie the manifestation of PD symptoms. It is likely that imbalance in direct and indirect pathway signaling is an important contributor to PD symptomology (Albin et al., 1989). Due to the difficulty in studying biochemical processes selectively in direct and indirect pathway neurons, progress has been limited in understanding the precise pattern of dysfunction in these pathways. Recent studies, however, using BAC transgenic mouse technology to label striatonigral and striatopallidal neurons, have shown differences in these cell populations in response to dopamine loss. A study using transgenic mice which express EGFP under the control of the D1R and D2R promoters, showed that

striatal dopamine depletion induced a selective loss of glutamatergic synapses and spines on D2R striatopallidal neurons with no change in spine structure or physiology in D1R striatonigral neurons (Day et al., 2006). The authors suggest that the loss of D2 receptor signaling after DA depletion results in disinhibition of synaptic calcium channels causing destabilization of spines and synaptic contacts. This loss of glutamatergic input results in further adaptations in striatopallidal neurons which alters their pattern of firing to GPi neurons. Disrupted striatopallidal input results in expression of synchronous bursting rhythms in the GPi/STN network which has been thought to underlie the expression of PD motor symptoms, as lesions or deep-brain stimulation of these structures alleviates these symptoms in PD patients (Chan et al., 2005).

Abnormal D1 receptor signaling has also been implicated in Parkinson's disease, specifically in the emergence of motor complications associated with the pharmacological treatment of PD. L-DOPA has been the leading drug prescribed for PD for the past 40 years owing to its ability to successfully alleviate the manifestation of PD motor symptoms. L-DOPA is highly effective during the early stages of PD, but its efficiency declines with sustained use and over time it can induce debilitating motor fluctuations and complications, known as L-DOPA induced dyskinesias (Mercuri and Bernardi, 2005). Specific alterations in D1 receptor signaling have been linked to the expression of L-DOPA induced dyskinesias (LIDs). Although there is no change in striatal D1 receptor expression, downstream signaling pathways of the D1 receptor become hypersensitized during chronic L-DOPA treatment resulting in robust acute locomotor induction in response to L-DOPA. One substrate of this sensitization is the $G_{\alpha/olf}$ protein which is coupled to striatal D1 receptors and mediates D1R stimulation of

cAMP production. A recent study found that membrane levels of $G_{\alpha/olf}$ were increased in the putamen of PD patients and in 6-OHDA lesioned rats, a rodent model of PD. This increase in $G_{\alpha/olf}$ was associated with an increase in DA-dependent adenylyl cyclase activity (Corvol et al., 2004). Downstream signaling molecules such as ERK1/2 and the immediate early gene c-fos also exhibit sensitized responses to L-DOPA which have been shown to be D1 receptor mediated (Gerfen, 2003; Westin et al., 2007).

It is clear from these studies that balanced D1 and D2 receptor signaling is vital for normal brain function. Although studies have uncovered some differential functions of D1 and D2 receptors, studies have been limited by the lack of ability to fully distinguish signaling selectively in D1R and D2R-expressing cell populations. Behavioral and biochemical responses to receptor agonists and antagonists have produced valuable information. However, the effects measured in response to these drugs reflect an average of responses from all striatal cell types. Better tools are needed to understand more precisely the biochemical signaling cascades mediated by different classes of dopamine receptors in the different striatal cell types.

IV. DARPP-32

Striatal signal transduction is dependent upon the coordination of fast and slow signaling processes. As mentioned previously, slow synaptic transmission involves a variety of second messenger molecules, among which protein kinases and phosphatases play a vital role. Twenty-five years ago, a phospho-protein named Dopamine- and cyclic AMP-regulated Phosphoprotein of 32kD (DARPP-32) was discovered to be enriched in the dopaminergic 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). DARPP-32 has the unique ability to act as a protein kinase inhibitor or a protein phosphatase inhibitor depending on its state of phosphorylation at four well-characterized sites (see below). Every striatal signaling pathway mentioned in the previous sections can affect the phosphorylation of DARPP-32 at one or more sites. Studies with the DARPP-32 knock-out mouse have shown that DARPP-32 mediates the actions of many of these molecules (Fienberg and Greengard, 2000).

Characteristics of DARPP-32

DARPP-32 is an ~200 amino acid protein which exists as a soluble elongated monomer and has an N-terminal domain which binds protein phosphatase 1 (PP-1) (Hemmings et al., 1984c; Hemmings et al., 1984d) (Figure 1.4). This N-terminal domain contains two regions which are involved in the binding of DARPP-32 to PP-1. A small domain consisting of amino acids 7-11 (KKIQF) interacts with PP-1 at a site removed from the active site and this KKIQF docking motif is shared with other proteins that bind PP-1, including inhibitor-1, spinophilin, and neurabin (Greengard et al., 1999). The region surrounding the threonine 34 residue (T34) binds PP-1 at its active site preventing access to phosphorylated substrates (Kwon et al., 1997). When DARPP-32 is phosphorylated at T34, it is converted into a potent high-affinity inhibitor of PP-1 with an IC_{50} of approximately 10^{-9} M (Hemmings et al., 1984a). DARPP-32 is expressed at very

high concentrations (~50 μ M) in both striatonigral and striatopallidal neurons and therefore a substantial portion of PP-1 activity is inhibited *in vivo* in response to dopaminergic stimulation (Ouimet et al., 1998). PP-1 is a major multifunctional serine/threonine phosphatase in the brain which regulates the phosphorylation of many downstream physiological effectors including neurotransmitter receptors and voltage-gated ion channels (Greengard et al., 1999). Therefore, by regulating PP-1 function, DARPP-32 can regulate the physiological properties of a neuron.

One of the first notable features of DARPP-32 was its high level of expression in dopaminergic neurons in the brain. Robust expression of DARPP-32 is seen in the striatum, olfactory tubercle, bed nucleus of the stria terminalis and regions of the amygdaloid complex, all of which receive strong dopaminergic input. Moderate to weak expression of DARPP-32 is also found in the neocortex, primarily layers II, III, and VI, in the Purkinje cells of the cerebellum, in several subregions of the hypothalamus, and in specific cell types in the retina (Ouimet et al., 1984; Witkovsky et al., 2007). Within these cells DARPP-32 immunoreactivity has been detected in cell bodies, dendrites and axon terminals (Ouimet et al., 1984).

A hypothesis concerning the overall function of DARPP-32 in striatal signaling was elucidated by studies of the DARPP-32 knock-out mouse, generated in 1998. 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 to supraphysiological levels. 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 PP-1, high levels of phosphorylation and activation are sustained. When DARPP-32 is absent, inhibition of PP-1 is relieved allowing it to dephosphorylate substrates, thereby shutting off or reducing the effects of the drug.

As mentioned above, DARPP-32 can also serve as an inhibitor of PKA activity when it is phosphorylated at the threonine 75 site (see below) (Bibb et al., 1999). Taken together, these findings suggest that DARPP-32 is a dual-function amplifier of signaling in MSNs. During periods of high cAMP/PKA activity, DARPP-32 inhibits PP-1 allowing heightened phosphorylation of PKA targets. In periods of low cAMP/PKA activity, DARPP-32 further dampens PKA activity and allows dephosphorylation of targets by PP-1.

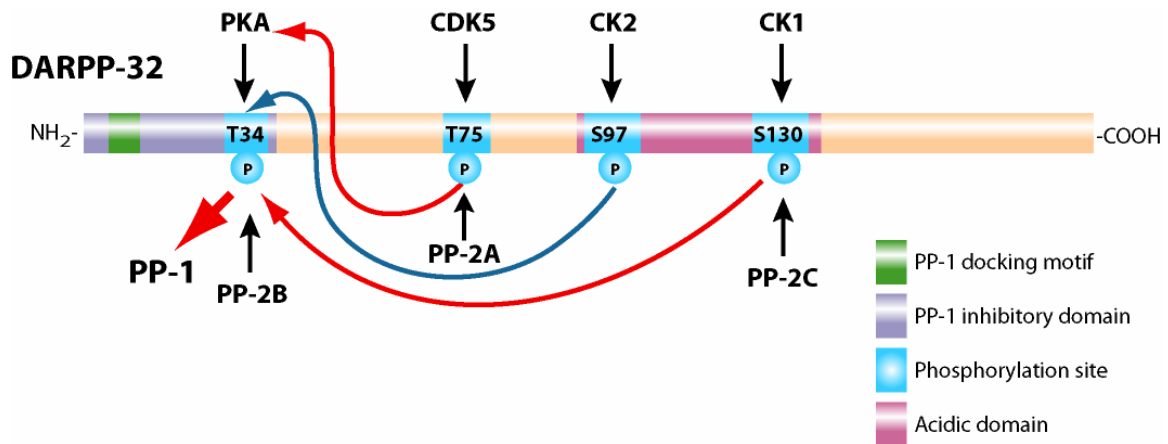


Figure 1.4 DARPP-32 Phosphorylation Sites

Schematic of DARPP-32 protein showing the location of the 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. Adapted from (Svenningsson et al., 2004).

Biochemistry of DARPP-32 Phosphorylation

The function of DARPP-32 depends on the relative phosphorylation level of four well characterized residues (Figure 1.4). DARPP-32 is phosphorylated by PKA at T34 in response to dopamine acting via D1 receptors and in response to other cAMP-activating molecules (Nishi et al., 1997). As mentioned previously, T34 phosphorylation turns DARPP-32 into a potent inhibitor of PP-1 activity. Phospho-T34 levels are maintained at a low basal state but can be strongly increased in response to activation of the cAMP/PKA pathway (Nishi et al., 1999). In addition to phosphorylation by PKA, T34 is also a good substrate for protein kinase G (PKG) (Hemmings et al., 1984d). In striatal slices, Thr 34 is primarily dephosphorylated by the calcium activated protein phosphatase calcineurin (also known as PP-2B) but can also be dephosphorylated by the phosphatase PP-2A (Nishi et al., 1999).

Phosphorylation at serine 97 (S102 in the rat) or serine 130 in the rat (S137 in the rat) modulates the likelihood of phosphorylation at T34. S97 is phosphorylated by casein kinase 2 (CK2) and *in vitro*, S97 phosphorylation increases the efficiency of T34 phosphorylation by PKA but not PKG (Girault et al., 1989). *In vitro* studies have shown that S97 is most efficiently dephosphorylated by PP-2A although PP-1 also shows activity at this site (Girault et al., 1989). S137 is phosphorylated by casein kinase 1 (CK1) and increases T34 phosphorylation by decreasing the rate of dephosphorylation of this site by PP-2B (Desdouits et al., 1995). *In vitro* and in intact cells, S130 is preferentially dephosphorylated by PP-2C (Desdouits et al., 1998).

In contrast to T34 phosphorylation, phosphorylation 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 and activated by the noncyclin co-factor p35. T75 is dephosphorylated by PP-2A and distinct isoforms of this enzyme are activated in response to both calcium signaling via AMPA and NMDA receptors, and PKA signaling stimulated by D1 receptor activation (Ahn et al., 2007a; Ahn et al., 2007b).

Factors affecting phosphorylation of DARPP-32

As mentioned previously, the striatum receives rich innervation from a variety of neurotransmitters and neuromodulators. All of these factors can affect the phosphorylation of DARPP-32 at one or more sites (Table 1). Here I will discuss the changes in DARPP-32 phosphorylation that occur in response to the striatal signaling molecules dopamine, serotonin, adenosine, GABA and glutamate.

Dopamine and DARPP-32. Studies have shown that dopamine acts in a bidirectional manner whereby it exerts stimulatory biochemical effects via D1 receptors and inhibitory biochemical effects via D2 receptors (Gerfen et al., 1990; West and Grace, 2002). This opposing action of D1 and D2 receptor stimulation is also true for the regulation of DARPP-32 phosphorylation. Studies *in vivo* and in striatal slices have shown that dopamine action via D1 R stimulates the cAMP/PKA cascade and increases phosphorylation at T34. This pathway also dephosphorylates T75 via a PKA stimulated increase in PP-2A activity (Ahn et al., 2007a). D2 receptor activation, conversely, 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_i mediated inhibition of AC/cAMP signaling which decreases PKA and PP-2A activity and the other is via a D2 R mediated increase in intracellular calcium which activates PP-2B, resulting in 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 D1 R-mediated potentiation of PKA activity and the D2 R-mediated inhibition of PKA signaling.

Serotonin and DARPP-32. Together with dopamine, serotonin is an important modulator of striatal function. With regard to DARPP-32, the phosphorylation pattern induced by serotonin in the striatum is similar to that produced by dopamine stimulation of D1 receptors, although these pathways are largely independent (Svenningsson et al., 2002a). Serotonin acting on 5-HT₄ and 5-HT₆ receptors increases the cAMP/PKA cascade resulting in an increase in T34 phosphorylation and a decrease in T75 phosphorylation. An additional serotonergic pathway increases S130 phosphorylation

due to a 5-HT₂ receptor mediated increase in the PLC/CK1 pathway (Svenningsson et al., 2002a).

Adenosine and DARPP-32. Adenosine has been shown to be an important modulator of dopamine signaling. A_{2A} receptors are expressed selectively in striatopallidal neurons and stimulation of A_{2A}Rs increases the cAMP/PKA cascade resulting in an increase in T34 phosphorylation (Svenningsson et al., 1998b). The mechanism of action of the psychostimulant caffeine is via antagonism of A_{2A} receptors and this results in an increase in T75 phosphorylation due to inhibition of PP-2A catalyzed dephosphorylation at this site (Lindskog et al., 2002).

Recently, signaling via adenosine type 1 receptors (A₁R) was also shown to affect DARPP-32 phosphorylation. In contrast to A_{2A} receptors, A₁ receptors are expressed in both direct and indirect MSNs and are coupled to G_i proteins which inhibit cAMP/PKA signaling. A₁ receptor activation in direct pathway neurons antagonizes D1R signaling and decreases T34 phosphorylation (Yabuuchi et al., 2006). In indirect pathway neurons, A₁R stimulation is thought to have a bi-phasic response whereby T34 phosphorylation is initially increased and then subsequently decreased. The early increase in T34 phosphorylation was attributed to pre-synaptic A₁R effects on dopamine release while the late phase was likely mediated by post-synaptic A₁R inhibition of cAMP and PKA (Yabuuchi et al., 2006).

GABA and DARPP-32. MSNs receive GABA projections from recurrent collaterals or from striatal interneurons which can have a strong effect on membrane potentials in these cells. In striatal slices, GABA stimulation produced a rapid increase in the state of phosphorylation of DARPP-32 at T34. This effect was potentiated by

forskolin, a cAMP activator, which suggests that the GABA mediated increase in T34 is due to inhibition of PP-2B (Snyder et al., 1994). This effect is most likely mediated via stimulation of GABA_A ionotropic receptors which increase Cl⁻ influx, decrease neuronal excitability, and decrease calcium influx thereby inactivating PP-2B.

Glutamate and DARPP-32. Both ionotropic and metabotropic glutamate receptors are expressed in MSNs and these receptors initiate different signaling pathways with different temporal patterns. As a result, the regulation of DARPP-32 phosphorylation by glutamate is complex and involves at least five different signaling cascades which operate on distinct time scales (Nishi et al., 2005).

Upon stimulation of striatal slices by glutamate, T34 phosphorylation was rapidly increased (at 1 minute), then decreased (at 1-5 minutes), and subsequently increased at later time points (5-10 minutes) (Nishi et al., 2005). The early increase in T34 was shown to be dependent upon AMPA, NMDA, and GluR5 activation of intracellular calcium which induced an increase in nitric oxide release. Nitric oxide stimulated the cGMP and PKG pathway resulting in an increase in T34 phosphorylation. The intermediate decrease in T34 phosphorylation was due to calcium activation of PP-2B and dephosphorylation of this site. The increase in T34 seen at later time points was attributed to mGluR5 signaling activating both a cAMP/PKA and a PLC/CK1 cascade. These signaling pathways had the net effect of inhibiting dephosphorylation of T34 and also increasing phosphorylation of S130.

The regulation of T75 phosphorylation by glutamate was also found to be complex (Nishi et al., 2005). At intermediate (1-5 minutes) time points, T75 phosphorylation was decreased by glutamate. This decrease was due to an AMPA and

NMDA receptor mediated increase in calcium which has been shown to activate the B''/PR72 subunit of PP-2A resulting in dephosphorylation of T75 (Ahn et al., 2007b). At later time points (5-10 minutes), T75 phosphorylation was increased by activation of cdk5 which occurred via a PLC/Ca⁺⁺/PP-2B/CK1 cascade (Nishi et al., 2005). This signaling cascade has also been shown to increase phosphorylation at S130 by CK1 and is dependent upon activation of mGluR1 receptors (Liu et al., 2001).

Table 1 Factors Affecting DARPP-32 Phosphorylation

All signaling pathways are increased in response to the factors listed, except for pathways in red which are decreased. For details, see text. Adapted from (Svenningsson et al., 2004).

Factor	Receptor	Signaling Pathway	T34	T75
Dopamine	D1R	cAMP/PKA/PP-2A	↑	↓
	D2R	cAMP/PKA	↓	↑
Serotonin	5-HT4/6	cAMP/PKA	↑	↓
Adenosine	A1R	cAMP/PKA	↓	
	A2AR	cAMP/PKA	↑	↓
GABA	GABA-A	Ca ⁺⁺ /PP-2B	↑	
Glutamate	NMDA	Ca ⁺⁺ /PP-2B	↑↓	↓
	AMPA	Ca ⁺⁺ /PP-2B	↑↓	↓
	mGluR1/5	PLC/CK1/cdk5		↑
		A2A/cAMP/PKA	↑	
Opioids	mu/delta R	cAMP/PKA	↓	↑
Nitric Oxide		cGMP/PKG	↑	
Cannabinoids	CB-1	inhibition of D2R	↑	
Nicotine	nAchRs	increase in DA	↑	↓

DARPP-32 and Disorders of the Basal Ganglia

DARPP-32 and Drugs of Abuse. DARPP-32 is involved in mediating the actions of many categories of drugs of abuse including opioids, ethanol, caffeine, cannabinoids, and psychostimulants (Svenningsson et al., 2004). It is well established that the dopaminergic system plays an important role in reward-related behaviors and drugs with reinforcing properties share the ability to modulate dopamine signaling (Nestler, 2001). I will focus here on the data implicating DARPP-32 in the acute and chronic effects of the psychostimulant drugs, cocaine and amphetamine, which act directly on dopaminergic neurons.

Both cocaine and amphetamine cause a robust increase in dopamine availability at the synapse by blocking the reuptake of dopamine by pre-synaptic transporter molecules and promoting release of dopamine from nerve terminals, respectively. This increased dopamine binds to both D1 and D2 receptors and alters signaling in direct and indirect pathway striatal neurons. To date there has been no method to study DARPP-32 phosphorylation independently in these cell populations. Therefore studies which are reviewed here are based on an average of striatal signaling responses in both cell types.

Acute treatment with either cocaine or amphetamine increases phosphorylation of T34 and decreases phosphorylation of T75 (Nishi et al., 2000). Both of these drugs cause a robust acute induction of locomotion and this response is attenuated in the DARPP-32 knockout mouse (Fienberg et al., 1998). Psychostimulant-induced locomotion is also reduced in mice bearing a point mutation in which T34 is replaced by the non-phosphorylatable amino acid alanine (T34A), suggesting that DARPP-32 phosphorylation at T34 is necessary for this behavioral effect (Zachariou et al., 2006).

Chronic treatment with psychostimulants causes behavioral sensitization which is characterized by an increased response to subsequent exposure to a drug and reflects adaptive changes in cells responsive to these drugs. Immediate early genes such as Δ FosB, which are activated by CREB and c-fos dependent pathways, are upregulated following chronic administration of psychostimulants (Nestler, 2001). Δ FosB regulates the transcription of cdk5 and chronic cocaine upregulates p35, the activator of cdk5 in the ventral striatum (Bibb et al., 2001). Likely via these pathways, chronic treatment with cocaine is associated with an increase in DARPP-32 T75 phosphorylation and a decrease in T34 phosphorylation (Bibb et al., 1999). It has been suggested that this may represent a homeostatic mechanisms to counteract enhanced cAMP/PKA signaling.

In the DARPP-32 knock-out mouse, there is attenuated expression of c-fos and Δ FosB in response to chronic cocaine (Fienberg et al., 1998). DARPP-32 knock-out mice also show reduced acquisition of cocaine self-administration and place preference, which are indices of drugs addiction (Zachariou et al., 2006). Interestingly, T34A mutant mice were also shown to exhibit reduced cocaine self-administration, cocaine-induced place conditioning, locomotor sensitization, and c-fos induction (Zachariou et al., 2006; Zhang et al., 2006). There was no change observed in these outcomes in the T75A mutant mice suggesting that the early increase in T34 is important in setting the stage for these latent addictive changes. In addition, S130A mutant mice shared similar phenotypes to the T34A mice implicating S130 phosphorylation in the reinforcing effects of cocaine (Zhang et al., 2006). These results show that psychostimulants differentially affect DARPP-32 phosphorylation at multiple sites at different time points and that

changes in phosphorylation at these sites are responsible for mediating many of the long-term biochemical and behavioral effects associated with chronic drug use.

DARPP-32 and Schizophrenia. Schizophrenia is a complex neurological disorder which involves imbalances in dopamine, glutamate, serotonin and acetylcholine signaling. The etiology and pathophysiology of schizophrenia are still unclear, however, pharmacological interventions have proven successful in the treatment of schizophrenic symptoms. The typical antipsychotic haloperidol has been widely used in the treatment of schizophrenia. Haloperidol has relatively high antagonistic activity at the D2 receptor and acute injection of haloperidol increases DARPP-32 phosphorylation at T34 (Pozzi et al., 2003). An increase in T34 phosphorylation is also seen by administration of eticlopride or raclopride which are selective D2 receptor antagonists (Pozzi et al., 2003). A_{2A} receptors are co-expressed with D2 receptors and activation of A_{2A}Rs increases cAMP/PKA signaling while D2 receptors decrease cAMP/PKA signaling. The A_{2A} pathway is tonically active; therefore removal of D2 receptor inhibitory tone by antipsychotic treatment likely shifts the balance towards increased PKA activity and T34 phosphorylation.

Stimulation of the indirect striatopallidal pathway results in an inhibition of locomotion, an effect seen after treatment with antipsychotic drugs. High doses of antipsychotics can also induce catalepsy which is extreme hypolocomotion characterized by rigid immobility. In the DARPP-32 knock-out mouse, higher doses of raclopride were required to induce catalepsy suggesting that DARPP-32 plays a role in the locomotor effects of antipsychotics (Fienberg et al., 1998).

D2 receptor antagonists have been shown to increase the phosphorylation of the GluR1 subunit of the AMPA receptor at the PKA S845 site. Phosphorylation at this site promotes glutamate transmission by increasing AMPA receptor currents and enhancing cell surface expression of this receptor (Roche et al., 1996). This change may increase excitability in striatopallidal neurons and contribute to the inhibitory effects of antipsychotics on locomotor activity. This mechanism may also help counteract overactive D2 receptor signaling which has been suggested to underlie some symptoms of schizophrenia. The haloperidol-induced increase in GluR1 S845 phosphorylation is abolished in both DARPP-32 knock-out mice and in T34A mutant mice (Hakansson et al., 2006).

DARPP-32 and Parkinson's Disease. As mentioned previously, Parkinson's disease is a debilitating neurodegenerative disorder caused by the death of SNc dopaminergic neurons. This loss of dopamine causes profound imbalances and maladaptations in both direct and indirect pathway striatal neurons. As DARPP-32 plays an essential role in mediating dopamine signaling, there are changes in DARPP-32 phosphorylation both in the dopamine depleted and the drug treated state.

In rodents, the most common model of Parkinson's disease is the 6-hydroxydopamine (6-OHDA) lesion. 6-OHDA is a toxin which is taken up by axon terminals expressing the dopamine transporter. 6-OHDA first causes loss of dopaminergic axons and then subsequent loss of dopaminergic cell bodies in the SNc. A recent study using a 6-OHDA rat model of PD showed that phosphorylation of DARPP-32 at the T75 site was elevated in the dopamine depleted state while T34 phosphorylation

was unchanged. This increase in T75 phosphorylation was maintained for up to 20 months post-lesion and was reversed with L-DOPA treatment (Brown et al., 2005).

L-DOPA is a commonly prescribed treatment for Parkinson's disease which is efficacious during the early stages of treatment, but is associated with debilitating motor side-effects at later stages. L-DOPA induced dyskinesias (LIDs) are characterized by uncontrollable abnormal movements which likely represent supersensitivity of stimulatory locomotor pathways in the BG. One recent study showed that 6-OHDA lesioned rats expressing high levels of LIDs did not show synaptic depotentiation to low-frequency stimulation following induction of LTP at cortico-striatal synapses (Picconi et al., 2003). L-DOPA treated lesioned rats which did not become dyskinetic did show synaptic depotentiation similar to control rats suggesting that abnormal synaptic plasticity may play a role in the dyskinesic response. In the same study it was shown that the dyskinetic rats also had abnormally high levels of T34 phosphorylation although it was not known which striatal cell type(s) this effect occurred in (Picconi et al., 2003). Additionally, a recent study showed that in 6-OHDA lesioned mice treated chronically with L-DOPA, the severity of dyskinesia was correlated with the level of T34 phosphorylation (Santini et al., 2007). Highly dyskinetic mice showed the highest levels of T34 phosphorylation. Furthermore, DARPP-32 knock-out mice showed a significant reduction in their dyskinetic response following chronic L-DOPA treatment (Santini et al., 2007). As mentioned above, supersensitivity of signaling molecules downstream from the D1 receptor have been implicated in LIDs. Since activation of D1 receptors results in an increase in the cAMP/PKA/T34 cascade, one possibility is that the increase in DARPP-32 T34 phosphorylation occurs mainly in D1 R striatonigral neurons.

DARPP-32 phosphorylation: The paradox

Although much of the biochemistry of DARPP-32 phosphorylation and the signaling cascades of many striatal neurotransmitters have been elucidated in detail, vital questions remain in the field concerning how these various signals are processed in the context of striatonigral and striatopallidal neurons, specifically how DARPP-32 is regulated differently in these two cell populations. This problem is exemplified by the major paradox that drugs such as L-DOPA and haloperidol can both increase DARPP-32 phosphorylation at T34, although these drugs have opposing behavioral and clinical effects. Thus, L-DOPA can cause schizophrenic-like symptoms while haloperidol can produce locomotor inhibition that resembles a Parkinson's disease state. How then can these drugs work through the same biochemical pathway? The straightforward solution is that these drugs are acting via the same protein but in different neuronal cell populations. The classical model of basal ganglia circuitry predicts that dopamine acting on D1 receptors will increase DARPP-32 phosphorylation at T34, stimulate direct pathway neurons, and cause an increase in locomotion. Conversely, if D2 receptor activity is blocked, there will also be an increase in T34 phosphorylation, a stimulation of indirect pathway neurons, and the net effect of a decrease in locomotion. If the increase in T34 phosphorylation seen with L-DOPA is restricted to D1 R striatonigral cells and the T34 activation seen with haloperidol treatment occurs in D2 R striatopallidal cells, then the opposing behavioral effects of these drugs are reconciled.

Direct evidence for this cell-type specific regulation is lacking due to an inability to distinguish phosphorylation responses in D1R-striatonigral and D2R-striatopallidal neurons. This is due to the fact that these cells are anatomically intermixed and

morphologically indistinguishable. In order to overcome this limitation, we have generated novel mouse models which can distinguish DARPP-32 responses in D1 R and D2 R-expressing neurons. These tools allow us to selectively study DARPP-32 phosphorylation in striatonigral and striatopallidal neurons *in vivo* as well as understand the effects of cell-type specific deletion of DARPP-32 on behavior. These studies will be presented in chapters three and four.

In addition to understanding the role of DARPP-32 in D1 and D2 receptor expressing neurons, we also asked whether there are any additional residues on DARPP-32 which are phosphorylated *in vivo* that may be relevant to DARPP-32 signaling and function. To do this we utilized a novel mass spectrometry method developed by Dr. Brain Chait at the Rockefeller University in combination with *in vitro* studies of DARPP-32 phosphorylation. These studies are presented in the following chapter.

Chapter 2

IDENTIFICATION AND CHARACTERIZATION OF NOVEL

DARPP-32 PHOSPHORYLATION SITES

Introduction

DARPP-32 is a dual function protein which has the ability to act as a kinase inhibitor or a phosphatase inhibitor depending on its state of phosphorylation at four well-characterized sites, T34, T75, S97, and S130. When DARPP-32 is phosphorylated at T34, it becomes a potent inhibitor of PP-1. When phosphorylated at T75, DARPP-32 becomes an inhibitor of PKA. The S97 and S130 sites play an important modulatory role by facilitating phosphorylation at T34.

Analysis of the amino acid sequence of mouse DARPP-32 protein reveals that there are 24 potential serine/threonine phosphorylation sites. To date, only four of these are confirmed phosphorylation sites *in vivo*. We sought to examine whether there are additional residues on DARPP-32 which are phosphorylated *in vivo*, which could play a role in DARPP-32 signaling. To do this, we collaborated with Dr. Brian Chait and Dr. Mi Jin of the Laboratory of Mass Spectrometry and Gaseous Ion Chemistry at The Rockefeller University. Dr. Chait and colleagues recently established a mass spectrometric approach which allows identification of all phosphorylated residues on a given protein (Chang et al., 2004). We used this protocol to identify residues on DARPP-32 purified from mouse striatal tissue which were basally phosphorylated *in vivo*.

Results

Purification of DARPP-32 from mouse striatum

In order to look for new *in vivo* phosphorylation sites on DARPP-32 by mass spectrometry, we needed picomolar amounts of highly purified DARPP-32 protein. To do this, we devised a two step immunoprecipitation protocol which resulted in a pure sample of DARPP-32 protein isolated from mouse striatum. We first prepared mouse striatal tissue by focused microwave irradiation (FMI) of wild-type and DARPP-32 knock-out mice. FMI has been shown to be a superior method for preserving the phosphorylation state of *in vivo* proteins as it induces rapid heat inactivation of protein kinases, phosphatases, and proteases (O'Callaghan and Sriram, 2004). Following FMI, dissected striata were homogenized in an acidic lysis buffer. DARPP-32 protein contains a high number of acidic residues, which make it soluble at low pH (Hemmings et al., 1984b). The large majority of striatal proteins are insoluble at this pH, therefore this step resulted in removal of approximately 90% of contaminating proteins. Following the acid extraction, DARPP-32 was immunoprecipitated using a DARPP-32 antibody which is not phosphorylation state specific (Hemmings and Greengard, 1986).

By western blot we were able to detect a DARPP-32 positive band in immunoprecipitated samples from wild-type but not DARPP-32 knock-out mice (Fig. 2.1, left panel). Coomassie staining revealed the presence of protein bands at 32kD from wild-type but not from DARPP-32 knock-out mice (Fig. 2.1, right panel). There were no major contaminating proteins in this region. The bands seen in both panels at approximately 50 and 25kD represent the heavy and light chains of the DARPP-32 antibody used in the immunoprecipitation.

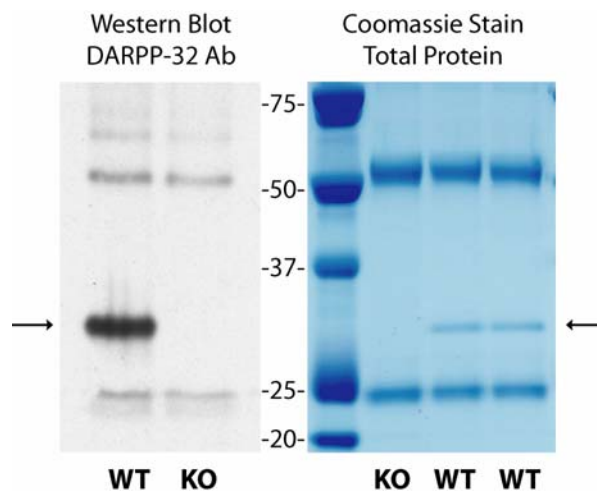


Figure 2.1 Immunoprecipitation of DARPP-32 from Mouse Striatum

The left panel shows detection of a DARPP-32 positive band in the immunoprecipitated sample from wild-type mouse striatum by western blot with a DARPP-32 antibody. The right panel shows a coomassie stained gel of immunoprecipitated samples from knock-out and wild-type mice. Arrows indicates DARPP-32 protein bands at 32kD. Numbers correspond to molecular weight markers, in kD. Abbreviations: WT, wild-type; KO, DARPP-32 knock-out.

Mass Spectrometry Phosphorylation Analysis of DARPP-32

Following purification of striatal DARPP-32 protein, gel bands were digested with trypsin and peptide fragments were analyzed for phosphorylation using the hypothesis-driven multi-stage mass spectrometry approach (HMS-MS) (Chang et al., 2004; Jin et al., 2005). This approach assumes that all serine and threonine residues in a given protein can be potential phosphorylation sites. Because the level of protein phosphorylation *in vivo* can be very low, phosphopeptides are often not discernable in single stage MS. According to the HMS-MS protocol, all hypothesized phosphopeptides are targeted for isolation and subjected to a second round of MS where phosphorylated residues are identified by the loss of 80 or 98 Daltons (Da) in the mass spectrum, corresponding to the mass of the H_3PO_4 or HPO_3 molecules. Using this approach, in 6

independent samples, seven basally phosphorylated residues were found on mouse DARPP-32 protein. Table 2 shows a list of the phosphorylated tryptic peptides with the phosphorylated residues indicated in blue. In addition to T34, T75, S97, and S130, three additional sites were found to be basally phosphorylated *in vivo*. These were S45/46, S52, and S192. The phosphopeptide spanning amino acids 41-56 was found as two separate entities. Based on mass analysis, one phosphopeptide entity was shown to be phosphorylated at one residue, either S45 or S46. Since these serines are next to each other it could not be unambiguously determined which one was phosphorylated. The other 41-56 phosphopeptide entity contained two phosphorylated residues which were determined to be S52 and another site, most likely S45/S56.

Table 2 DARPP-32 Phosphopeptide Sequences

Phosphorylated residues are indicated in blue in the phosphopeptide sequence. Amino acid ranges correspond to the amino acid numbers in the mouse DARPP-32 protein sequence. MH+P is the mass of the peptide plus one phosphate group indicating that the peptide was phosphorylated at one residue, MH+2P is the mass of the peptide plus two phosphates indicating that the peptide was phosphorylated at two residues.

Phosphopeptide Sequence	Amino Acids	Phosphorylation Site	MH + P	MH + 2P
RRPT P AMLFR	32-40	T34	1324.7	
VSEH SS PEEEASPHQR	41-56	S45 or S46	1885.8	
VSEHSSPEEEA S PHQR	41-56	S52 and another		1965.7
RPNPCAY T PPSLK	68-80	T75	1580.7	
AVQHLQTISNLSENQAS EE DELGELR	81-107	S97	3119.4	
ELGYPQEDDEEDEDEE ES QA E VLK	108-136	S130	3522.3	
ATLSEPGEEPQH PS PP	179-194	S192	1752.7	

Quantification of phosphorylation by HMS-MS

In order to quantify changes in the phosphorylation level of the new sites in response to drug treatments, we utilized a differential labeling technique combined with the HMS-MS approach developed in Dr. Chait's lab. DARPP-32 protein was purified from mice treated with either saline or drug. The purified samples were subjected to tryptic digestion and the resulting peptides were differentially labeled with isotopically distinct mass tags. The tags differ in weight by 5-10Da allowing identification of the peaks corresponding to saline or drug treated samples in the later stages of MS analysis. The height of the phosphorylated peak in a given sample is divided by the quantity of an unphosphorylated control peptide to normalize to total protein levels. This phospho/dephospho ratio can be compared between saline and drug treated samples to give a value corresponding to the amount of phosphorylation induced by the drug treatment relative to control (Fig. 2.2a).

We used this methodology to quantify changes in phosphorylation in response to acute injection of drugs which target the dopamine system and are known to increase the cAMP/PKA pathway. In response to cocaine, amphetamine, or a D1 receptor agonist (SKF81297), DARPP-32 phosphorylation at T34 was increased to 143-204% of control levels (Fig. 2.2b). These values are consistent with previous findings (Nishi et al., 2000; Svenningsson et al., 2003) and are also consistent with results using traditional western blotting with a phospho-specific T34 antibody (Fig. 2.2c). When we examined the phosphorylation pattern of two of the new sites, S45/46 and S192, we saw no significant changes in phosphorylation with any of the drug treatments suggesting that these sites are not regulated by the cAMP/PKA pathway. Only the S45/46 and S192 sites were

examined in this experiment because these sites yielded phosphorylation signals high enough for quantitative analysis.

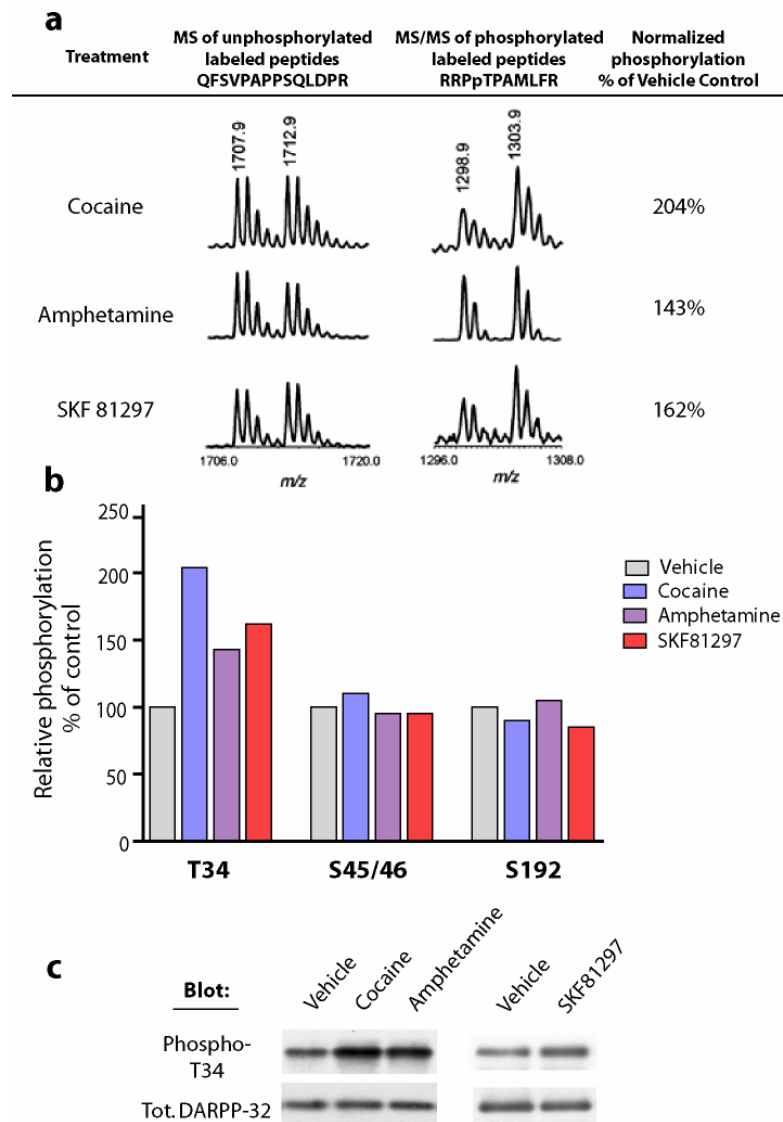


Figure 2.2 MS Quantification of DARPP-32 Phosphorylation in Response to Dopaminergic Drugs

(a) Peaks obtained from HMS-MS analysis used to quantify T34 phosphorylation in drug treated samples. Left peaks correspond to a dephospho-peptide used to quantify total protein levels in the sample. Right peaks correspond to phospho-T34 peptides. Lower mass peaks (1707.9 & 1298.9) correspond to saline treated samples, 5Da higher mass peaks correspond to drug treated samples. (b) MS quantitation of changes in phosphorylation at T34, S45/46, and S192 in response to the drugs indicated. (c) T34 phospho-antibody western blots corresponding to an aliquot of the samples used in (b). a & b are adapted from Fig. 6 in (Jin et al., 2005).

Identification of the kinases phosphorylating DARPP-32 at S45/46, S52 and S192

Since S45/46 and S192 were not regulated by PKA signaling pathways, we sought to find the kinase(s) which can phosphorylate these sites. To do this we performed *in vitro* assays whereby active kinases were added to solutions containing recombinant DARPP-32 protein and radioactive ³²P labeled ATP. Kinase activity was measured by quantifying the incorporation of radioactive phosphate at various time points.

We first determined the kinases which were able to phosphorylate DARPP-32 *in vitro* by testing a panel of commonly expressed brain kinases (Fig. 2.3). We found that the kinases previously known to phosphorylate DARPP-32, i.e. PKA, PKG, CKI, CKII and cdk5, all showed moderate to high activity towards recombinant mouse DARPP-32 protein *in vitro*. Additionally, CamKIV and CamKII kinases also showed high activity towards DARPP-32 *in vitro*. GSK-3a, MAPK, and PKC had low activity while Trk-B, CamKI, and ROK had very little or no activity towards DARPP-32 *in vitro* (Fig. 2.3). Data in Figure 2.3 provide an estimate of the relative activity of each kinase towards DARPP-32. Because we cannot control for the overall enzymatic activity of each kinase *in vitro* it is possible that a kinase with low activity in this assay, can still phosphorylate DARPP-32 *in vivo*.

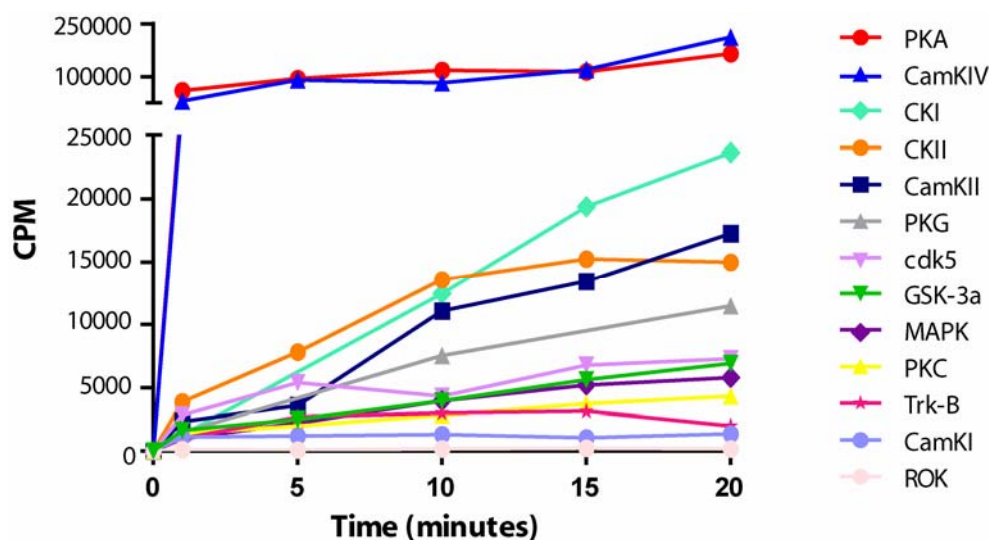


Figure 2.3 *In vitro* Phosphorylation of DARPP-32 by Brain Kinases

Graph shows the time course of ^{32}P labeled phosphate incorporation to recombinant DARPP-32 protein. The Y axis is split as PKA and CamKIV had very high levels of activity compared with the other kinases. CPM = counts per minute.

To determine whether these kinases could specifically phosphorylate the S45/46, S52 or S192 sites, we made point mutant proteins for each of these sites in which the serine was replaced by a non-phosphorylatable alanine residue. We then compared the incorporation of radioactive phosphate between wild-type and mutant DARPP-32 protein in response to the panel of active kinases. If the kinase was active at one of the new sites, then the incorporation of ^{32}P should be reduced in the point mutant protein. We chose to mutate serine 45 as opposed to 46 since it had been previously reported to be phosphorylated *in vitro* (Girault et al., 1989).

Out of all the kinases tested with the point mutant proteins, a significant reduction in radioactive phosphate incorporation was seen only for CKII phosphorylation of S45A protein (Figure 2.4a-c). At the 60 minute time point, there was a 60.7% reduction in

phosphate incorporation in the S45A mutant protein compared to wild-type protein (Fig. 2.4b). The residual CKII phosphorylation seen in the S45A samples likely reflects CKII phosphorylation at an additional site on DARPP-32.

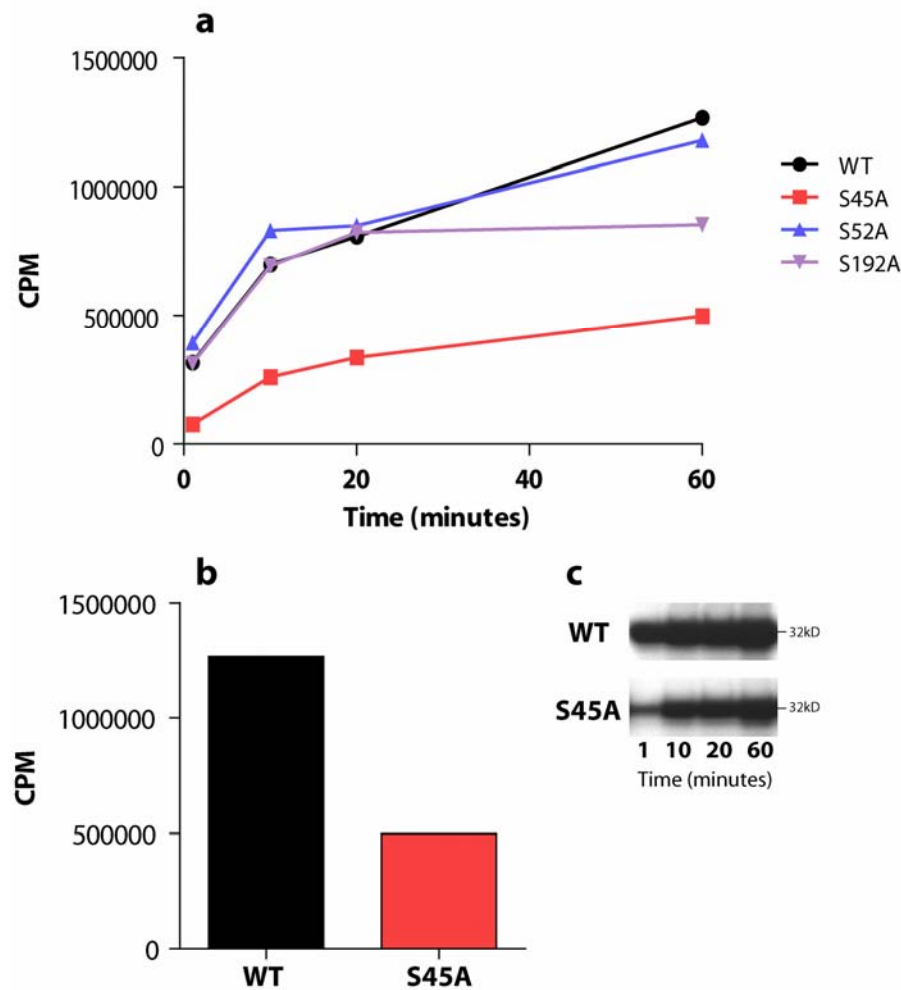


Figure 2.4 CKII Phosphorylates DARPP-32 at S45

(a) Time course of ^{32}P labeled phosphate incorporation to recombinant DARPP-32 proteins after incubation with CKII. (b) Total radioactivity measured after 60 minutes incubation with CKII, wild-type versus S45A mutant protein. (c) Autoradiograms comparing the intensity of ^{32}P incorporation over-time between wild-type and S45A mutant proteins. Abbreviations: WT, wild-type recombinant mouse DARPP-32 protein; S45A, S52A, S192A, serine-to-alanine point mutant DARPP-32 protein at amino acids 45, 52, & 192; CPM, counts per minute.

To confirm the ability of CKII to phosphorylate S45, we submitted CKII phosphorylated wild-type recombinant DARPP-32 to the Proteomics Resource Center at The Rockefeller University for mass spectrometry analysis. Results confirmed that CKII phosphorylated the S45 site as well as the S97 site. S97 was previously shown to be phosphorylated by CKII (Girault et al., 1989). Phosphorylation at S97 likely represents the remaining phosphorylation by CKII in the S45A mutant protein sample (Fig. 2.4). To confirm the specificity of CKII activity towards S45, we also submitted CKI phosphorylated DARPP-32 for MS analysis. In this case, only S130 was found to be phosphorylated which was expected based on previous reports (Desdouits et al., 1995). Additionally, MS analysis of PKA phosphorylated DARPP-32 revealed that only the T34 site was phosphorylated *in vitro* by PKA. This finding is in agreement with the MS quantification data presented in Figure 2.3 showing that drugs known to stimulate PKA signaling have a selective effect on T34 phosphorylation with no regulation of S45 or S192 phosphorylation.

Discussion

The data presented here show that in addition to the four known phosphorylation sites on DARPP-32, there are three additional sites which are basally phosphorylated in mouse striatum *in vivo* (Fig. 2.5). These sites were found repeatedly in independent samples using an unbiased mass spectrometry approach. *In vitro* data show that the S45 site is a good substrate for CKII. A previous study examining *in vitro* phosphorylation of bovine and rat DARPP-32 also identified the S45 site as a CKII target, in addition to S102 (S102 in the rat sequence corresponds to S97 in the mouse) (Girault et al., 1989).

In that study, it was found that the k_{cat} for CKII stimulated phosphorylation of S102 was 5-6 times higher than for S45. Additionally, S102 was determined to be the primary site phosphorylated in rat striatal slices based on Edman degradation analysis. Based on those results it was concluded that S45 was not a physiologically relevant phosphorylation site. Here we show that this site is phosphorylated in mouse striatum *in vivo* and we provide additional evidence that it can be phosphorylated by CKII *in vitro*. Comparison of the amino acid sequences of human, mouse, rat, and bovine DARPP-32 (Fig. 2.6) shows that S45 is conserved across all of these species and may therefore serve an important role in DARPP-32 signaling.

The factors regulating CKII activity in the brain are largely unknown. However, CKII has been implicated in a variety of neuronal processes including protein degradation, neuronal survival and differentiation, ion channel function, and synaptic plasticity ((Ulery et al., 2006). In addition to DARPP-32, CKII has been shown to phosphorylate other proteins important in striatal signaling such as Δ FosB, PTEN, and NMDA receptors (Lieberman and Mody, 1999; Torres and Pulido, 2001; Ulery et al., 2006). Further studies are needed to fully understand the signaling pathways that stimulate CKII activity, and how phosphorylation at S45 and S97 by CKII regulates DARPP-32 function.

In contrast to S45, we did not find any effect of mutation at S52 or S192 on the ability of the kinases tested to phosphorylate DARPP-32 *in vitro*. Both of these serines are followed by prolines and therefore they could be substrates for proline-directed kinases such as cdk5 or MAPK. cdk5/p35 has previously been shown to phosphorylate DARPP-32 at T75 *in vivo* (Bibb et al., 1999). *In vitro*, T75 is also good substrate for other proline-directed kinases such as cdc2 and cdk1 (Bibb et al., 1999; Svenningsson et

al., 2004). Although we did not see any major effects of S52A or S192A mutations on the phosphorylation of DARPP-32 by cdk5, it may have been because *in vitro* cdk5 activity was weak compared to the other kinases known to phosphorylate DARPP-32 (Fig. 2.3). This may be due to the necessity of additional co-activators of cdk5 which were not present in our *in vitro* system. It is also possible that another kinase not tested here is responsible for phosphorylating these sites. Comparison of the DARPP-32 amino acid sequences revealed that the S52 site is conserved across human, mouse, rat, and bovine species while the S192 site is present only in the mouse and human sequence (Fig. 2.6). Further studies are needed to determine the kinases and phosphatases regulating the new sites in order to understand their potential role in DARPP-32 signaling.

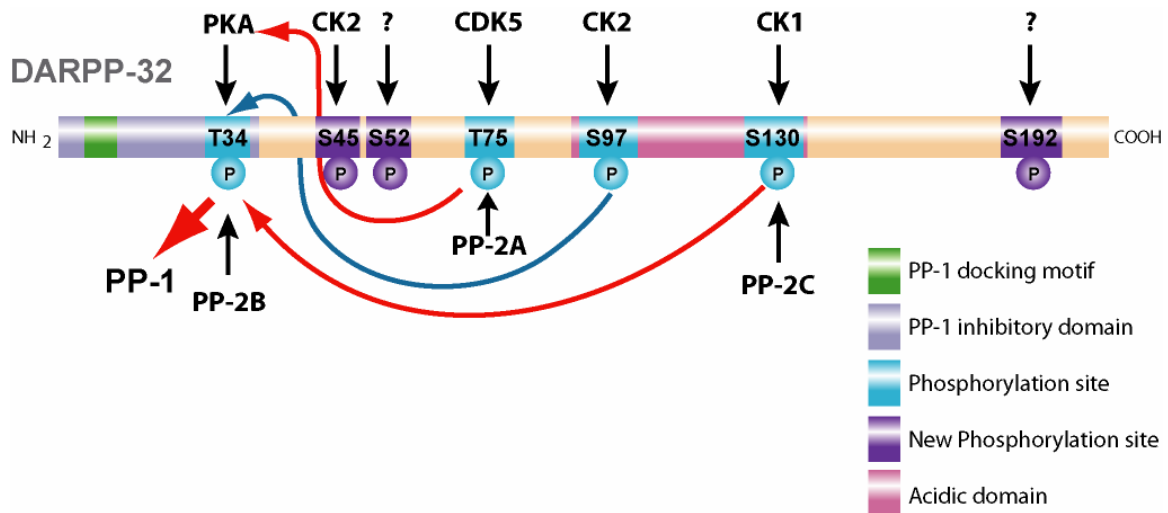


Figure 2.5 Updated Schematic of DARPP-32 Phosphorylation Sites

Schematic of DARPP-32 protein showing the location of the three new phosphorylation sites in relation to the four known 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. Adapted from (Svenningsson et al., 2004).

		M	D	P	K	D	R	K	K	I	Q	F	S	V	P	A	P	P	S	Q	L	D	P	R	Q	V	E	M	I	R	R	Consensus	
											10									20											30		
1		M	D	P	K	D	R	K	K	I	Q	F	S	V	P	A	P	P	S	Q	L	D	P	R	Q	V	E	M	I	R	R	Homo sapiens	
1		M	D	P	K	D	R	K	K	I	Q	F	S	V	P	A	P	P	S	Q	L	D	P	R	Q	V	E	M	I	R	R	Mus musculus	
1		M	D	P	K	D	R	K	K	I	Q	F	S	V	P	A	P	P	S	Q	L	D	P	R	Q	V	E	M	I	R	R	Rattus norvegicus	
1		M	D	P	K	D	R	K	K	I	Q	F	S	V	P	A	P	P	S	Q	L	D	P	R	Q	V	E	M	I	R	R	Bos taurus	
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31		R	R	P	T	P	A	M	L	F	R	L	S	E	H	S	S	P	E	E	E	A	S	P	H	Q	R	A	S	G	E	Homo sapiens	
31		R	R	P	T	P	A	M	L	F	R	V	S	E	H	S	S	P	E	E	E	S	S	P	H	Q	R	T	S	G	E	Mus musculus	
31		R	R	P	T	P	A	L	F	R	V	S	E	H	S	S	S	P	E	E	E	S	S	P	H	Q	R	T	S	G	E	Rattus norvegicus	
31		R	R	P	T	P	A	M	L	F	R	L	S	E	H	S	S	P	E	E	E	A	S	P	H	Q	R	A	S	G	E	Bos taurus	
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61		G	H	H	L	K	S	K	R	P	N	P	C	A	Y	T	P	P	S	L	K	A	V	Q	R	I	A	E	S	H	L	Homo sapiens	
61		G	H	H	P	K	S	K	R	P	N	P	C	A	Y	T	P	P	S	L	K	A	V	Q	-	-	-	-	-	-	-	Mus musculus	
61		G	H	H	P	K	S	K	R	P	N	P	C	A	Y	T	P	P	S	L	K	A	V	Q	R	I	A	E	S	H	L	Rattus norvegicus	
61		G	H	H	L	K	S	K	R	S	N	P	C	A	Y	T	P	P	S	L	K	A	V	Q	R	I	A	E	S	H	L	Bos taurus	
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86		Q	T	I	S	N	L	S	E	N	Q	A	S	E	E	E	D	E	L	G	E	L	R	E	L	G	Y	P	Q	E	D	Mus musculus	
91		Q	T	I	S	N	L	S	E	N	Q	A	S	E	E	E	D	E	L	G	E	L	R	E	L	G	Y	P	Q	E	D	Rattus norvegicus	
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116		D	E	E	D	E	D	-	-	E	E	D	E	E	E	D	S	Q	A	E	V	L	K	G	S	R	G	T	V	G	Mus musculus		
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											160									170											180		
151		Q	K	T	T	R	G	L	R	G	L	E	G	P	W	E	R	P	P	P	L	D	E	S	E	R	D	G	G	S	E	D	Homo sapiens
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151		Q	K	L	T	S	G	Q	G	L	E	G	P	W	E	R	P	P	P	P	L	D	E	P	Q	R	D	G	N	S	E	D	Rattus norvegicus
149		Q	K	T	T	Y	G	Q	G	L	E	G	P	W	E	R	P	P	P	P	L	D	G	P	Q	R	D	G	S	S	E	D	Bos taurus
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174		Q	V	E	G	R	A	T	L	S	E	P	G	E	E	P	Q	H	P	S	P	P	Mus musculus										
181		Q	G	E	G	R	A	T	Q	N	E	P	G	E	E	P	R	H	P	T	A	P	P	E	S	G	T	Rattus norvegicus					
179		Q	V	E	D	-	P	A	L	N	E	P	G	E	E	P	Q	R	P	A	H	P	E	P	G	T	Bos taurus						

Figure 2.6 Alignment of DARPP-32 Amino Acid Sequence by Species

Comparison of human, mouse, rat and bovine DARPP-32 amino acid sequences. Blue residues are conserved across all species. Regions where the sequence differs between species are shown in red. Grey highlighted regions show the location of the known phosphorylation sites, T34, T75, S97, and S130. Yellow highlighted regions indicate the location of the new phospho-sites, S45, S52, and S192.

Chapter 3

DIFFERENTIAL PHOSPHORYLATION OF DARPP-32 IN STRIATONIGRAL AND STRIATOPALLIDAL NEURONS

Introduction

The striatum is the main input center of the basal ganglia and receives innervation from various sources including corticostriatal glutamatergic neurons (Kemp and Powell, 1971) and mid-brain dopaminergic neurons (Dahlstroem and Fuxe, 1964). Medium-sized spiny neurons (MSNs) process and integrate these varied inputs and send projections to the output nuclei of the basal ganglia via two pathways. The striatonigral pathway projects directly to the substantia nigra pars reticulata (SNr)/entopeduncular nucleus while the indirect striatopallidal pathway sends projections via two intermediate structures, the globus pallidus and subthalamic nucleus (Albin et al., 1989). These two pathways work together to modulate behavior. Imbalance or dysregulation of these pathways can have profound physiological consequences. MSNs express high levels of dopamine receptors, particularly type 1 and 2 receptors (D1R and D2R). Many studies have shown that MSNs of the direct pathway preferentially express D1 receptors which are coupled to $G_{s/olf}$ proteins and positively regulate cAMP and PKA activity (Gerfen et al., 1990). In contrast, MSNs of the indirect pathway express D2 receptors coupled to G_i proteins which inhibit cAMP signaling (Gerfen et al., 1990).

DARPP-32 is a key regulator of signaling in both types of MSNs (Fienberg and Greengard, 2000; Greengard et al., 1999). DARPP-32 function depends on its state of phosphorylation at two main regulatory sites, T34 and T75 (Greengard, 2001). When DARPP-32 is phosphorylated at T34 by PKA it becomes a potent inhibitor of PP-1, which in turn regulates the phosphorylation state of several classes of effector proteins including transcription factors, ionotropic receptors, and ion channels (Greengard, 2001). PKA also phosphorylates and activates the protein phosphatase PP-2A which

dephosphorylates DARPP-32 at T75 (Ahn et al., 2007a; Nishi et al., 2000). When phosphorylated at T75 by cdk5, DARPP-32 becomes an inhibitor of PKA signaling and inhibition of PP-1 is relieved (Bibb et al., 1999).

Biochemical studies have shown that a variety of therapeutic agents and drugs of abuse can regulate DARPP-32 phosphorylation at T34 and T75 and studies utilizing mice with point mutations of these residues show that they are necessary for mediating many of the actions of these drugs (Hakansson et al., 2006; Santini et al., 2007; Svenningsson et al., 2003; Zachariou et al., 2006; Zhang et al., 2006). Administration of dopamine-enhancing drugs such as cocaine and the Parkinson's disease therapeutic, L-DOPA, has been shown to increase T34 phosphorylation. Antipsychotics such as haloperidol antagonize dopamine signaling via D2 receptors but can also increase T34 phosphorylation to a similar degree. Despite this similar biochemical effect, dopamine-enhancing drugs and antipsychotics have opposing behavioral and clinical outcomes. Furthermore, treatment with L-DOPA can result in "schizophrenic-like" symptoms, whereas treatment with haloperidol can mimic the locomotor suppression seen in Parkinson's disease and produce the movement disorder known as tardive dyskinesia.

One possible explanation for these seemingly contradictory findings is that these drugs may selectively activate different sub-populations of striatal neurons. Cell-type specific analysis of signal transduction in MSNs has been difficult to address due to the anatomical and morphological homogeneity of these cells. Traditional biochemical studies have the limitation that they examine a mixed population of cells and results represent only an average of the signaling events in this brain region.

To overcome these limitations and to provide a more detailed view of signaling in striatal cells, we genetically targeted epitope-tagged DARPP-32 protein selectively to D1R-enriched striatonigral and D2R-enriched striatopallidal neurons. Using a selective immunoprecipitation protocol we were able to purify phosphorylated DARPP-32 protein specifically from D1R or D2R neurons from mouse striatal tissue. We applied this technique to analyze phosphorylation responses to several classes of drugs which target the striatum, namely dopamine receptor agonists, psychostimulants, antipsychotics, and L-DOPA. Our findings show that all of these drugs exhibit cell-type specific effects which could not be revealed with conventional techniques. These results uncover important biochemical differences between striatonigral and striatopallidal neurons and demonstrate the advantages of using a cell-type targeted approach.

Results

Targeting striatonigral and striatopallidal MSNs

In order to study DARPP-32 phosphorylation selectively in striatonigral and striatopallidal neurons, we generated BAC transgenic mice that express c-terminal Flag tagged DARPP-32 and Venus fluorescent protein under the control of the *drd1a* (D1R) promoter and mice which express c-terminal Myc tagged DARPP-32 and ECFP under the control of the *drd2* (D2R) promoter (Fig. 3.1a). The Internal Ribosome Entry Site (IRES) in these constructs allows separate translation of tagged DARPP-32 and fluorescent marker proteins from the same mRNA transcript. We chose the D1R and D2R promoters to target striatonigral and striatopallidal cells since the levels of these receptors have been shown to be differentially expressed in the two neuronal populations.

In order to confirm expression of these constructs in the correct brain regions, we performed in situ hybridization with a 35S-labeled antisense probe against GFP that recognized both Venus and ECFP mRNA (Fig. 3.1b). Sagittal sections from the D1R-DARPP-32/Flag mice showed high expression in the striatum and olfactory tubercle. Moderate expression was seen in the deep layers of the cortex with weak expression in the hippocampus. Sections from the D2R-DARPP-32/Myc mice showed high levels of expression in striatum and the SNpc/ventral tegmental area (VTA) with moderate expression in the hippocampus. The expression patterns of our constructs recapitulate the known patterns of *drd1a* and *drd2* expression in mouse brain confirming accurate expression of the transgenes. At the cellular level, we found that only a subset of striatal nuclei labeled with Nissl stain were positive for GFP mRNA in the D1R-DARPP-32/Flag (36.7% - 188/512) and D2R-DARPP-32/Myc (41.4% - 147/355) mouse lines respectively (Fig. 3.1b inset).

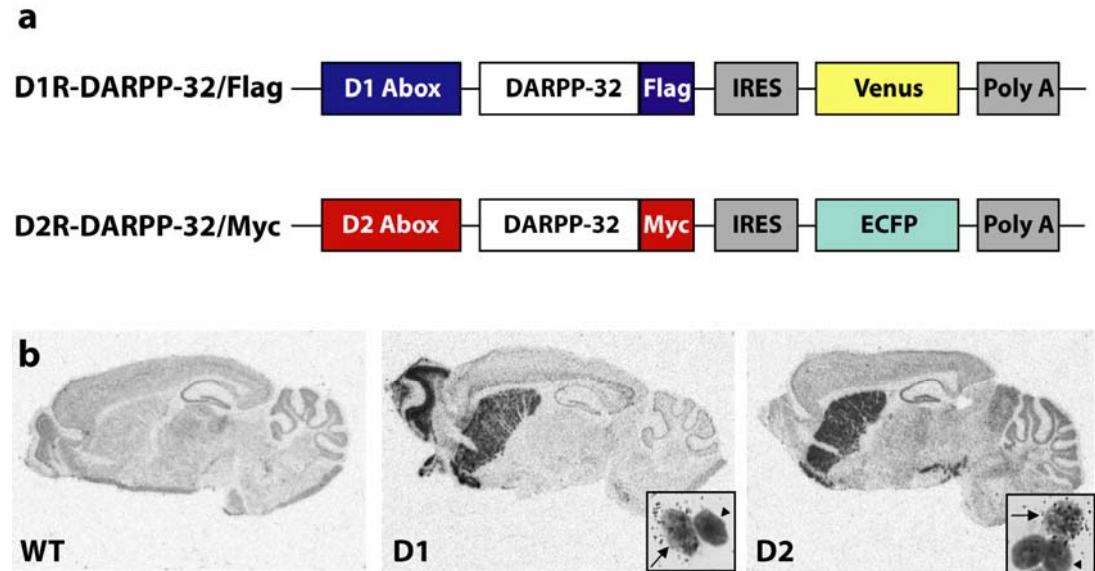


Figure 3.1 Generation of D1R-DARPP-32/Flag and D2R-DARPP-32/Myc mice

(a) Schematic of the modified shuttle vectors used to generate D1R-DARPP-32/Flag and D2R-DARPP-32/Myc mice. IRES, internal ribosome entry site; Poly A, polyadenylation sequence; Venus, Venus fluorescent protein; ECFP, enhanced cyan fluorescent protein. (b) In situ hybridization using a ³⁵S labeled antisense probe against GFP which recognizes both Venus and ECFP mRNA shows the expression of the constructs in sagittal brain sections from wild-type (WT), D1R-DARPP-32/Flag (D1), and D2R-DARPP-32/Myc (D2) mice. Insets show magnification of GFP positive striatal cells labeled with silver grains (arrows) and unlabeled cells (arrowheads) after dipping sections from (b) in emulsion.

After demonstrating targeting of the constructs to the correct brain regions, we confirmed that the labeled striatal cells were indeed striatonigral or striatopallidal neurons. To do this we took advantage of the fact that striatonigral and striatopallidal cells have different projection patterns. Striatonigral neurons send projections to the SNpr while striatopallidal neurons project more proximally to the globus pallidus. We injected Fluoro-Gold (FG) retrograde tracer into the SNpr which is taken up by axon terminals of striatonigral neurons and sent via retrograde transport to cell bodies in the striatum. Only striatonigral neurons are labeled with this method since FG is unable to traverse synapses. Correct targeting of the FG injection is shown by the overlap in immunofluorescence

between reactive astrocytes accumulated at the injection site (red), and the SN shown by tyrosine hydroxylase staining (green) (Fig. 3.2a).

In the striatum we examined whether the D1R-DARPP-32/Flag and D2R-DARPP-32/Myc constructs were co-expressed with the FG tracer. To identify cells which express the D1R and D2R constructs, we used a GFP antibody that recognized Venus protein for the D1R-DARPP-32/Flag mice and a Myc antibody for the D2R-DARPP-32/Myc mice. We used the Myc antibody in the D2R line because ECFP protein was not detectable with our GFP antibody. Although mRNA for both Venus and ECFP was present, there were very low levels of protein expression due to the weak activity of the IRES sequence *in vivo*. The fact that Venus protein was detectable by indirect fluorescence in the D1R-DARPP-32/Flag line was likely due to the higher expression level of the D1R-targeted transcript compared to the D2R-targeted transcript (see below). This differential expression could be due to differences in the strength of the D1R and D2R promoters, differences in BAC copy number, or differences in insertion location between the lines. It is also possible that the antibodies used preferentially recognized Venus protein over ECFP.

Double immunofluorescence revealed near 100% co-localization of FG and the GFP marker in the D1R-DARPP-32/Flag line (Fig. 3.2b) and no co-localization of FG and Myc in the D2R-DARPP-32/Myc line (Fig. 3.2c). These results confirm that the D1R construct was expressed in striatonigral neurons whereas the D2R construct was excluded from these cells.

After confirming proper targeting of the D1R and D2R constructs to striatonigral and striatopallidal cells, the D1R-DARPP-32/Flag and D2R-DARPP-32/Myc mice were

crossed to generate double transgenic mice. Co-staining with Flag and Myc antibodies in striatal sections from D1R/D2R-DARPP-32 mice showed that expression of the epitope tags was restricted to different striatal cell populations (Fig. 3.2d). This further confirmed that we targeted distinct populations of striatal neurons with these transgenic mouse lines.

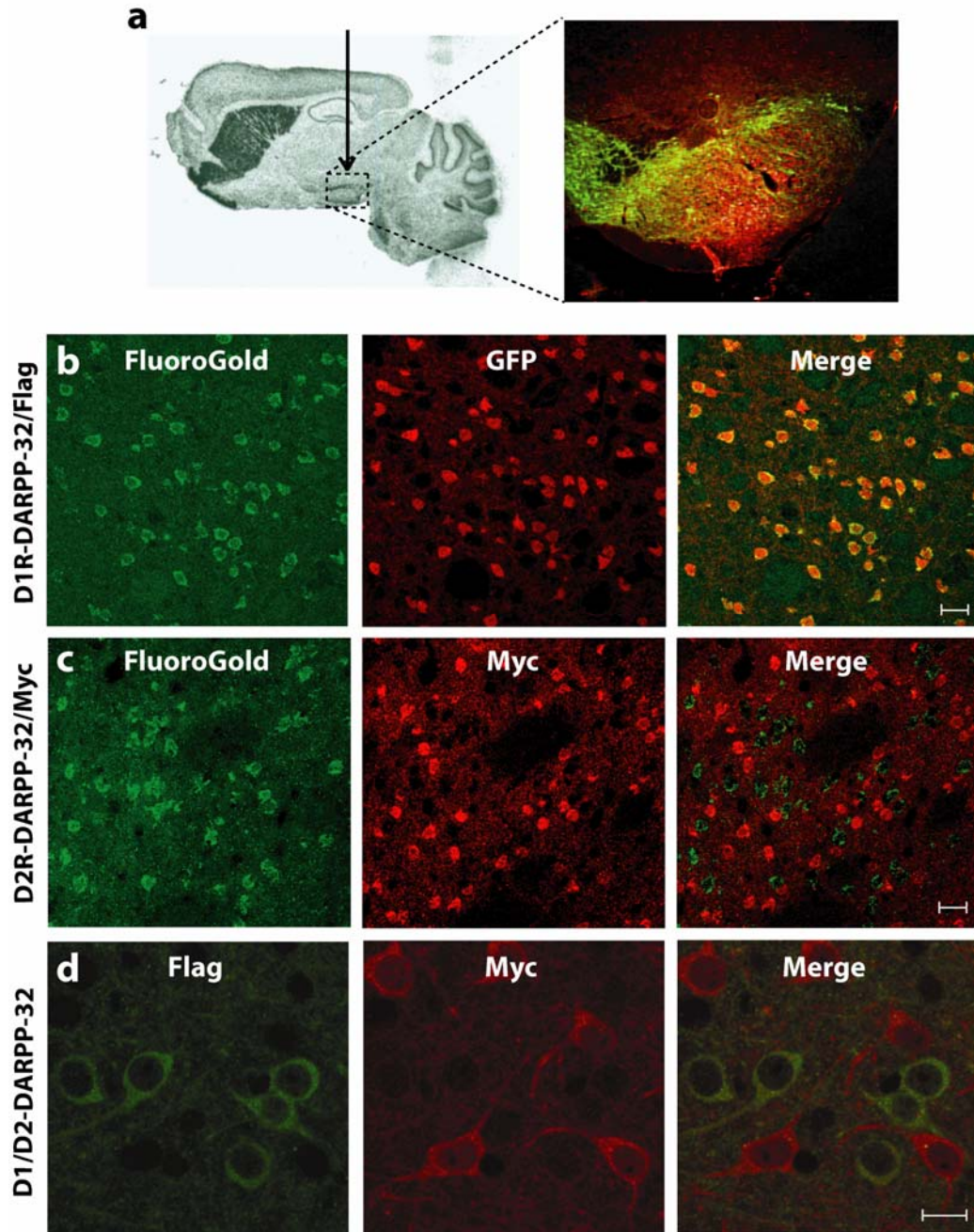


Figure 3.2 D1R-DARPP-32/Flag and D2R-DARPP-32/Myc Construct Expression in Striatonigral and Striatopallidal Neurons

(a) FluoroGold retrograde tracer was injected into the substantia nigra pars reticulata (SNpr) shown by the dashed box. Double immunofluorescence (IF) shows the overlap of tyrosine hydroxylase positive SN neurons (green) and the injection site identified by the presence of reactive astrocytes stained with GFAP antibody (red). (b) IF staining in striatal sections shows the co-localization of Fluoro-Gold (green) and GFP (red) in the D1R-DARPP-32/Flag mice, scale bar = 20µm. (c) There is no co-localization of Fluoro-Gold (green) and Myc (red) in striata from the D2R-DARPP-32/Myc mice, scale bar = 20µm (d) Double immunofluorescence staining using Flag (green) and Myc (red) antibodies in striatal sections from D1R/D2R-DARPP-32 double transgenic mice showing absence of co-localization, scale bar = 10µm.

Cell type-specific immunoprecipitations

Double transgenic D1R/D2R-DARPP-32 mice were used for all biochemical experiments because they allow direct comparison of DARPP-32 phosphorylation in both D1R and D2R-expressing neurons in response to drug treatments within the same mouse. A schematic outline of the *in vivo* biochemical experiments is shown in Figure 3.3. The success of this method relies on accurate expression of tagged DARPP-32 protein as well as high specificity of the Flag and Myc immunoprecipitations (IP's).

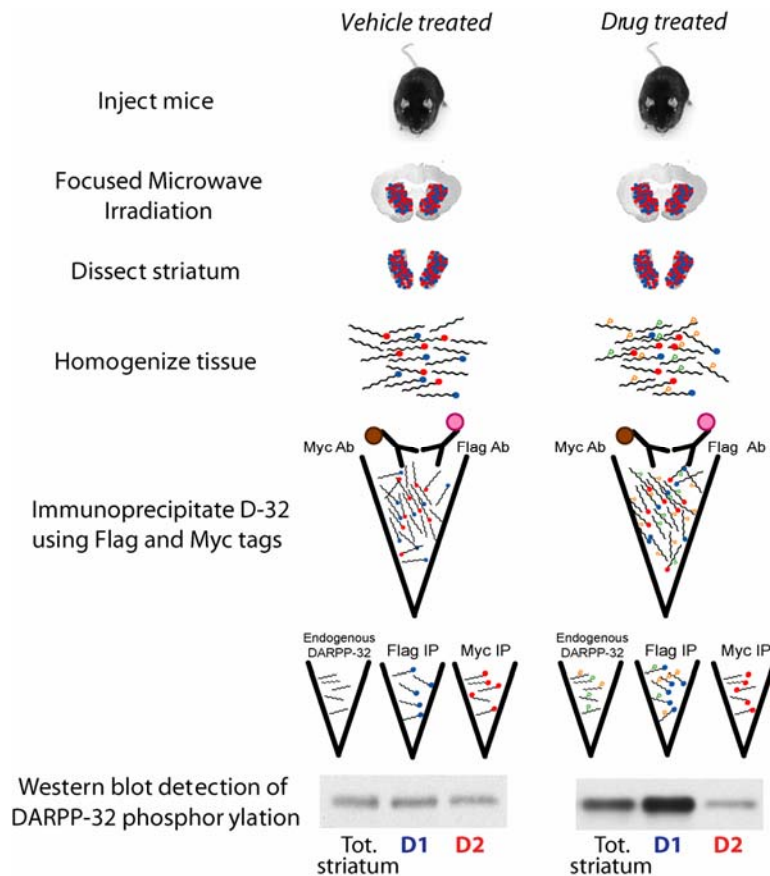


Figure 3.3 Experimental Design of *in vivo* Biochemical Experiments Using D1/D2-DARPP-32 Mice

One feature of this method is the ability to incubate striatal homogenates simultaneously with Myc and Flag antibodies by differentially coupling the antibodies to magnetic beads (brown circles) and affinity gel (pink circles), respectively. Blue circles represent Flag tags, red circles represent Myc tags. “P” indicates phosphorylated protein.

We first checked protein levels of tagged DARPP-32 in single transgenic mice. Western blotting with Flag and Myc antibodies revealed positive bands in D1R-DARPP-32/Flag and D2R-DARPP-32/Myc mice respectively which ran at approximately the same molecular weight as native DARPP-32 (i.e. 32kD). There was no significant change in total DARPP-32 levels between wild-type, D1R-DARPP-32/Flag and D2R-DARPP-32/Myc mice (Fig. 3.4a). There were also no significant differences in T34 or T75 phosphorylation between the lines (Fig. 3.4a). These results suggest that the tagged DARPP-32 represents only a very small fraction of total DARPP-32 expressed in these cells which is likely due to differences in the strength of the DARPP-32 promoter compared to the D1R and D2R promoters. This difference in expression is advantageous because by tagging a very small percentage of DARPP-32 protein in these cells, we can study cell-specific changes in phosphorylation without perturbing the endogenous system.

After confirming expression of the tagged DARPP-32 proteins, we optimized the IP protocols to ensure high selectivity and specificity. Figure 3.4b shows that in the single transgenic lines, IP using the Flag antibody selectively pulled down striatal DARPP-32 from D1R-DARPP-32/Flag mice while the Myc IP specifically pulled down DARPP-32 from D2R-DARPP-32/Myc mice. Further IP controls revealed that in the double D1R/D2R-DARPP-32 mice, there was no contaminating Flag tagged protein present in the Myc IP material and there was no Myc contamination in the Flag IP sample (Figure 3.4c). Additionally, the IP's were highly efficient as over 90% of the Flag and Myc tagged proteins are pulled down with this method (Fig. 3.4d).

We also compared the amount of endogenous DARPP-32 which was not pulled down (Fig. 3.4e, lane 4) versus the tagged DARPP-32 IP'd by the Flag and Myc

antibodies (Fig. 3.4e, lanes 2 and 3). There was 2-3 fold higher expression of Flag-tagged versus Myc-tagged DARPP-32 and approximately 30-fold higher expression of endogenous DARPP-32 compared with Flag-tagged DARPP-32. Importantly, we were able to detect basal levels of T34 and T75 phosphorylation in both the Flag and Myc IP samples showing that tagged DARPP-32 protein was basally phosphorylated *in vivo* (Fig. 3.4f).

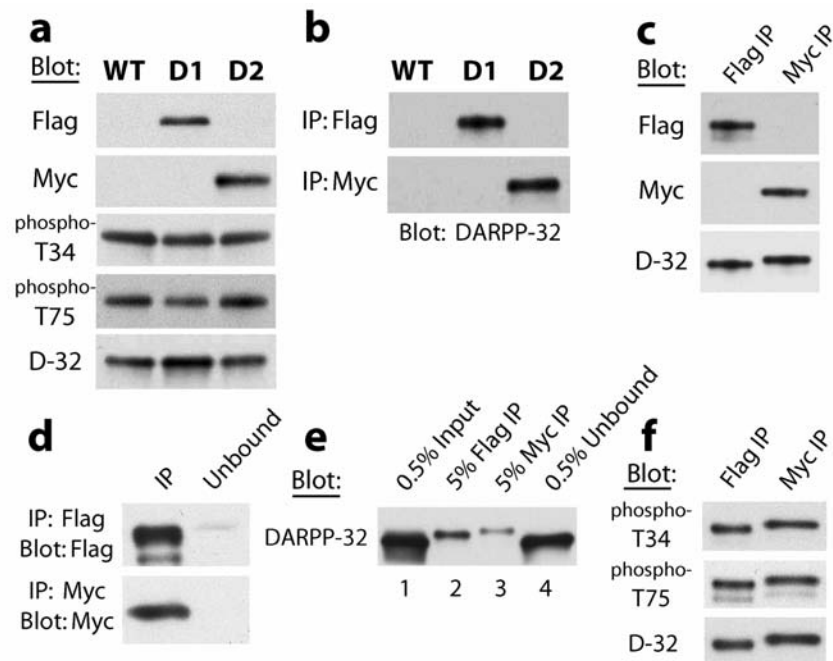


Figure 3.4 Cell-type Specific DARPP-32 Immunoprecipitations

(a) Equal amounts of striatal homogenate from wild-type (WT), D1R-DARPP-32/Flag (D1), and D2R-DARPP-32/Myc (D2) single transgenic mice were blotted with Flag, Myc, phospho-T34, phospho-T75 and total DARPP-32 antibodies. (b) Striatal homogenates from wild-type (WT), D1R-DARPP-32/Flag (D1), and D2R-DARPP-32/Myc (D2) mice were incubated with Flag and Myc antibodies. Equal amounts of IP'd sample was loaded in each lane and blotted with an antibody against total DARPP-32. (c) Striatal homogenate prepared from D1R/D2R-DARPP-32 mice was incubated with Flag and Myc antibodies and IP eluates were blotted with either Flag or Myc antibody. Stripped membranes were re-probed with total DARPP-32 antibody, bottom panel, showing equal loading of IP's. (d) Striatal homogenate from D1R/D2R-DARPP-32 mice was incubated with Flag and Myc antibodies. 5% of each IP was loaded next to 5% of the unbound material post-IP and blotted with Flag and Myc antibodies as shown. (e) 5% of the Flag and Myc IP'd samples from D1R/D2R-DARPP-32 mice were loaded next to 0.5% of the homogenate before (Input) and after (Unbound) the IPs and blotted with total DARPP-32 antibody. (f) Representative blot of the basal level of T34 and T75 phosphorylation in Flag and Myc IP'd samples from D1R/D2R-DARPP-32 mice detected by phospho-specific antibodies. pT34 blot was stripped and re-probed with total DARPP-32 antibody (bottom panel) showing equal loading.

Biochemistry of selective D1 and D2 receptor agonists

After establishing the specificity of the IP's and the ability to detect DARPP-32 phosphorylation in the IP'd samples, we tested whether we could observe cell-type specific D1R and D2R agonist mediated effects on DARPP-32 phosphorylation. Drugs were tested in acutely prepared striatal slices and in whole animals. These complementary approaches allowed us to compare the effects of direct stimulation of post-synaptic receptors in striatal slices versus stimulation of these receptors throughout the brain with *in vivo* injections.

We first tested the effects of the D1R agonist SKF 81297 on DARPP-32 phosphorylation in striatal slices (Fig. 3.5a,b). For the slice experiments, a method was used analogous to that described in Fig. 3.3 except slices were prepared from fresh brain sections. Stimulation of D1 receptors has been shown to cause an increase in the cAMP/PKA cascade which increases phosphorylation of T34 and decreases phosphorylation of T75 (Nishi et al., 2000). In agreement with previous studies, the D1R agonist SKF 81297 caused a significant increase in T34 phosphorylation ($441.3\% \pm 64.6\%$ of control, $p < .01$) and a small and variable decrease in T75 ($87.4\% \pm 8.3\%$ of control) in total striatal homogenates (Fig. 3.5a, left panel). In D1R neurons, SKF 81297 induced a robust 12-fold increase ($1220\% \pm 90.3\%$ of control, $p < .001$) in T34 phosphorylation with a concomitant decrease in T75 phosphorylation ($70.3\% \pm 2\%$ of control, $p < .001$) (Fig. 3.5a, middle panel). There were no significant changes in phosphorylation at either site in D2R neurons (Fig. 3.5a, right panel).

Application of the D2R agonist quinpirole also produced effects on DARPP-32 phosphorylation which were largely cell-type specific (Fig. 3.5c,d). Activation of D2

receptors inhibits the cAMP/PKA signaling cascade which results in a decrease in T34 phosphorylation and an increase in T75 phosphorylation (Nishi et al., 2000). As expected, quinpirole caused a significant decrease in T34 phosphorylation ($63\% \pm 3.7\%$ of control, $p < .01$) and an increase in T75 phosphorylation ($151.6\% \pm 19.3\%$ of control, $p < .05$) in total striatal homogenates (Fig. 3.5c, left panel). In D2R neurons, application of quinpirole also caused a large decrease ($38.4\% \pm 5\%$ of control, $p < .01$) in T34 phosphorylation and an increase in T75 phosphorylation ($151.7\% \pm 14.9\%$ of control, $p < .01$) (Fig. 3.5c, right panel). In D1R neurons, there was no change in T34 phosphorylation. There was a small increase in T75 phosphorylation in D1R neurons ($122.2\% \pm 6\%$ of control, $p < .05$) (Fig. 3.5c, middle panel) which may reflect stimulation of some intact pre-synaptic D2 autoreceptors by quinpirole, and a consequent decrease in dopamine release.

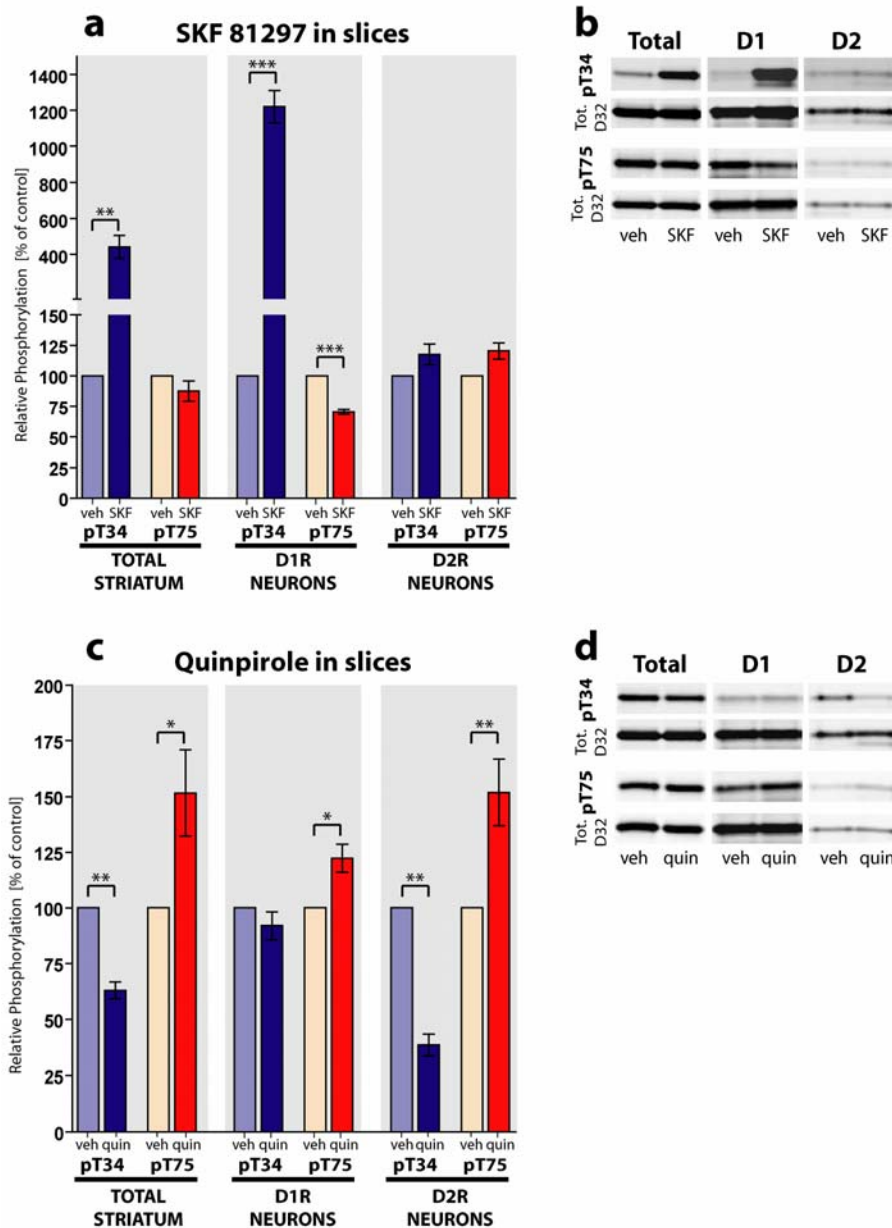


Figure 3.5 Effects of D1R and D2R agonists on DARPP-32 Phosphorylation in Striatal Slices

(a,c) Bar graphs represent group averages of DARPP-32 phosphorylation from striatal slices incubated with vehicle, SKF 81297 (10 μ M, 5 min.), or quinpirole (1 μ M, 10 min.). Phospho-T34 and T75 data were normalized to total DARPP-32 levels and expressed as percent of vehicle control. The left panels represent data from non-tagged endogenous DARPP-32 (Total Striatum), the center panels represent data from Flag IP'd DARPP-32 (D1R Neurons), and the right panels represent data from Myc IP'd DARPP-32 (D2R Neurons). In each experiment 6 striatal slices from 1 mouse were divided into vehicle, SKF 81297, and quinpirole treatment groups and slices from 3 mice were pooled by treatment for the analysis of DARPP-32 phosphorylation. Data represent averages from 4-8 independent experiments. Error bars represent SEM. *, $p < .05$, **, $p < .01$, ***, $p < .001$; unpaired, two-tailed student's t-test (b,d) Representative western blots from treated slices. T34 and T75 phospho-specific blots (top panels) were stripped and re-probed with total DARPP-32 antibody (bottom panels). This data was generated in collaboration with Dr. Akinori Nishi and Mahomi Kuroiwa at Kurume University, Japan.

Based on the findings in striatal slices showing that we could identify selective DARPP-32 phosphorylation responses in D1R and D2R neurons, we then examined cell-type specific responses to drugs injected *in vivo* (Fig. 3.6a,b). Similar to slices, injection of the D1R agonist SKF 81297 *in vivo* resulted in a significant increase in T34 phosphorylation ($140\% \pm 11.2\%$ of control, $p < .01$) with little change in T75 phosphorylation in total striatal homogenates (Fig. 3.6a, left panel). In D1R neurons, there was a large increase in T34 phosphorylation ($219\% \pm 19.3\%$ of control, $p < .001$) but no change in T75 phosphorylation (Fig. 3.6a, middle panel). Additionally, in contrast to slices, there were moderate but significant increases in both T34 and T75 phosphorylation in D2R neurons *in vivo* ($144\% \pm 10.7\%$ of control, $p < .05$ for T34, and $148\% \pm 13.4\%$ of control, $p < .01$ for T75, Fig. 3.6a, right panel). Because these effects were seen *in vivo* and not in striatal slices, it is likely that the changes in D2R neurons were due to stimulation of extrastriatal D1 receptors which indirectly influence the striatum. This finding highlights the complex changes that can occur *in vivo* with injection of a D1R agonist.

Injection of the D2R agonist quinpirole *in vivo* also resulted in changes in DARPP-32 phosphorylation in both cell types (Fig. 3.6c,d). As seen in slices, quinpirole significantly decreased T34 phosphorylation ($67.9\% \pm 4.9\%$ of control, $p < .001$) and increased T75 phosphorylation ($112.2\% \pm 4.2\%$ of control, $p < .05$) in total striatal homogenates (Fig. 3.6c, left panel). In contrast to slices, quinpirole caused a significant decrease in T34 phosphorylation in both D1R and D2R neurons ($79.4\% \pm 7.7\%$ of control, $p < .05$ in D1R neurons and $62.4\% \pm 5.6\%$ of control, $p < .01$ in D2R neurons, Fig. 3.6c). T75 phosphorylation was also increased significantly in both cell types ($137.3\% \pm 5.4\%$

of control, $p < .001$ in D1R neurons, and $123.9\% \pm 8.2\%$ of control, $p < .05$ in D2R neurons). This D2R agonist effect on D1R neurons *in vivo* can be explained by the fact that there are D2 autoreceptors expressed on dopaminergic nerve terminals which negatively regulate the release of dopamine. Decreased dopamine binding at D1 receptors would result in decreased T34 phosphorylation and increased T75 phosphorylation as found here.

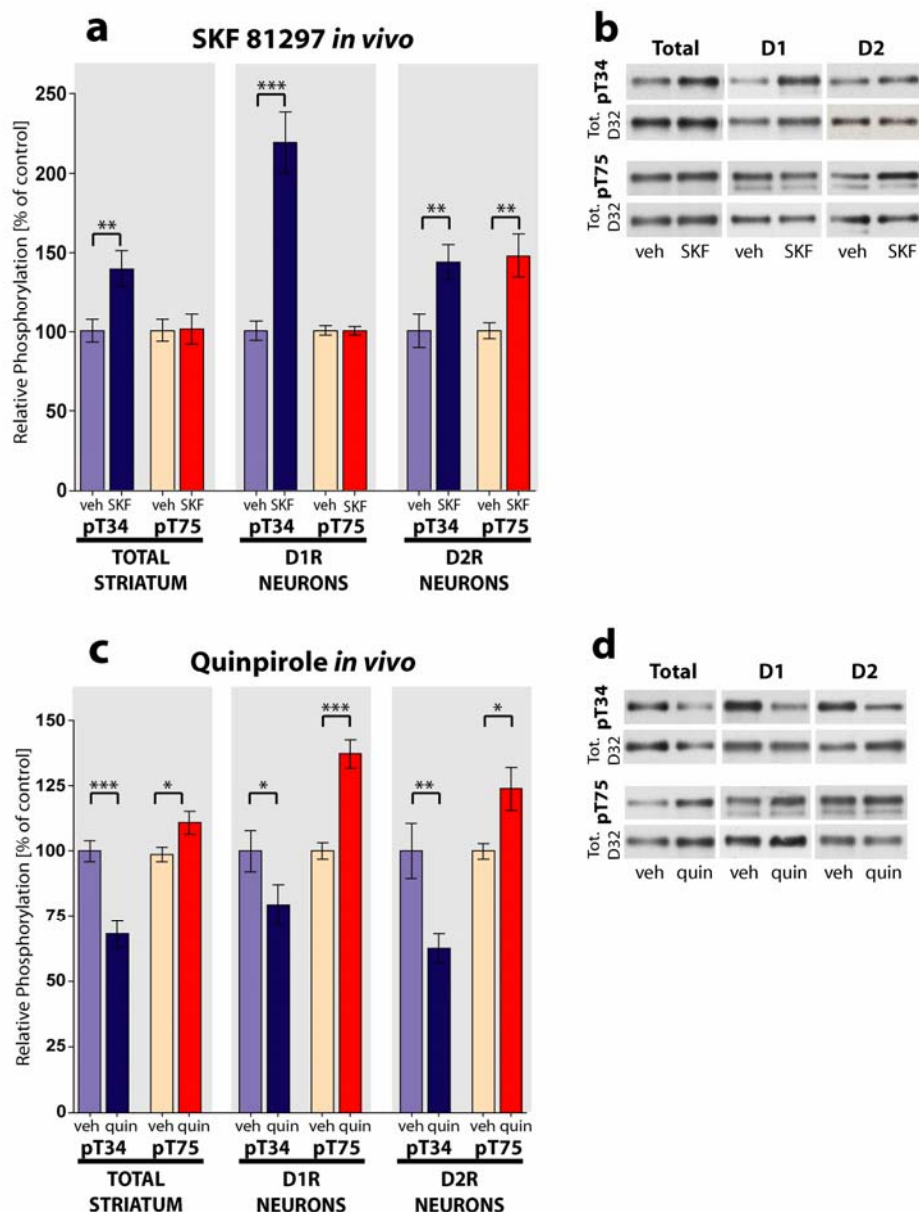


Figure 3.6 Effects of D1R and D2R Agonists on DARPP-32 Phosphorylation *in vivo*

(a,c) Bar graphs represent group averages of DARPP-32 phosphorylation from D1R/D2R-DARPP-32 mice injected i.p. with vehicle, SKF 81297 (5mg/kg), or quinpirole (0.2 mg/kg) and sacrificed 15 minutes later.

Phospho-T34 and T75 data were normalized to total DARPP-32 levels expressed as percent of vehicle control. The left panels represent data from non-tagged endogenous DARPP-32 (Total Striatum), the center panels represent data from Flag IP'd DARPP-32 (D1R Neurons), and the right panels represent data from Myc IP'd DARPP-32 (D2R Neurons). Each bar represents the average of 7-12 mice from 2-3 independent experiments. Error bars represent SEM. *, $p < .05$, **, $p < .01$, ***, $p < .001$; unpaired, two-tailed student's t-test. (b,d) Representative western blots from drug treated D1R/D2R-DARPP-32 mice.

T34 and T75 phospho-specific blots (top panels) were stripped and re-probed with total DARPP-32 antibody (bottom panels).

Inhibition of A_{2A} receptors affects DARPP-32 phosphorylation in D2R neurons

A_{2A} receptors are highly expressed in the striatum and selectively localized to striatopallidal neurons where they are co-expressed with D2 receptors (Fredholm et al., 2005). A_{2A} receptors positively regulate the cAMP/PKA cascade, thereby opposing D2R signaling in these cells (Fredholm and Svenningsson, 2003). A_{2A}R antagonists such as caffeine have been shown to increase DARPP-32 T75 phosphorylation due to decreased activity of the phosphatase PP-2A (Lindskog et al., 2002). In agreement with this study, caffeine increased T75 phosphorylation in total striatal homogenates (130.1% ± 8% of control, $p < .01$) with little effect on T34 phosphorylation (Fig. 3.7a, left panel). In accordance with its selective role in striatopallidal neurons, we found that the increase in T75 phosphorylation seen with caffeine was restricted to D2R neurons (203.5% ± 21.3% of control, $p < .001$) (Fig. 3.7a, right panel).

Cocaine and haloperidol increase T34 phosphorylation in D1R and D2R neurons respectively

To address the paradox of how psychostimulants and antipsychotics can produce similar biochemical effects on T34 phosphorylation in samples from whole striatum, we injected D1R/D2R-DARPP-32 mice acutely with cocaine or haloperidol. Cocaine blocks the dopamine transporter increasing the availability of dopamine at the synapse. It is expected that increased dopamine acting via D1 receptors would increase T34 phosphorylation and decrease T75 phosphorylation while stimulation of D2 receptors would decrease T34 phosphorylation and increase T75 phosphorylation. All of these effects were found in our *in vivo* experiment. Cocaine increased T34 phosphorylation to

192% \pm 10.5% of control, ($p < .001$) in D1R neurons and decreased T34 phosphorylation to 77.5% \pm 3.3% of control, ($p < .01$) in D2R neurons (Fig. 3.7b). Conversely, T75 phosphorylation was decreased to 65% \pm 4.4% of control, ($p < .001$) in D1R neurons and increased to 123.4% \pm 9.3% of control, ($p < .05$) in D2 R neurons (Fig. 3.7b).

The typical antipsychotic drug haloperidol showed a different pattern of phosphorylation (Fig. 3.7c). Although haloperidol induced an increase in T34 phosphorylation in the total striatal sample similar to that found with cocaine (153.2% \pm 5.2% of control, $p < .001$, Fig. 3.7c, left panel), this increase was highly specific to D2R cells as opposed to D1R cells (207.8% \pm 22.9% of control, $p < .001$ in D2R cells, 106.2 \pm 4.2% in D1R cells). Additionally, there was no significant regulation of T75 phosphorylation in either cell type. Typical antipsychotics such as haloperidol are known to be strong D2R antagonists which explains the selective increase in T34 phosphorylation in D2R cells. These results clearly show that although both cocaine and haloperidol stimulate T34 phosphorylation, these biochemical effects are restricted to different cell populations.

“Typical” versus “atypical” antipsychotics differ in their phosphorylation profiles

Schizophrenia is a psychiatric disorder proposed to involve dysfunction in dopaminergic, serotonergic and/or glutamatergic signaling in the cortex and basal ganglia (Meltzer, 1999; Svenningsson et al., 2003). Classical therapeutic drugs used to treat this disorder, termed “typical” antipsychotics, targeted the dopamine system, specifically D2R neurons. Typical antipsychotics such as haloperidol show strong and selective antagonism of D2 receptors. Newer compounds known as “atypical” antipsychotics

exhibit less activity at D2 receptors and greater binding of several classes of adrenergic and/or serotonergic receptors. Clozapine is an example of an “atypical” antipsychotic. Despite differences in their receptor profiles, haloperidol and clozapine both increased T34 phosphorylation in total striatal homogenates ($153.2\% \pm 5.2\%$ of control, $p < .001$ for haloperidol and $140.7\% \pm 7.7\%$ of control, $p < .001$ for clozapine, Fig. 3,7c,d left panels).

To better understand the effects of haloperidol and clozapine on DARPP-32 phosphorylation in D1R and D2R neurons, we compared the cell-type specific phosphorylation profiles of these drugs after acute *in vivo* injection. Similar to haloperidol treatment, clozapine treated mice showed an increase in T34 phosphorylation in D2R neurons, although this increase was smaller than with haloperidol ($158.8\% \pm 16.4\%$ of control, $p < .001$ for clozapine, versus $207.8\% \pm 22.9\%$ of control, $p < .01$ for haloperidol, Fig. 3.7c,d). There were also moderate but significant increases in T75 phosphorylation in both D1R and D2R neurons with acute clozapine treatment which were not seen with haloperidol ($140\% \pm 3.5\%$ of control, $p < .001$ in D1R neurons, $130.2\% \pm 5.2\%$ of control, $p < .001$ in D2R neurons, Fig. 3.7d). Because this change in T75 phosphorylation was seen in both D1R and D2R neurons, it is possible that this effect was mediated by a signaling pathway present in both cell types (e.g. 5-HT_{4/6} receptors).

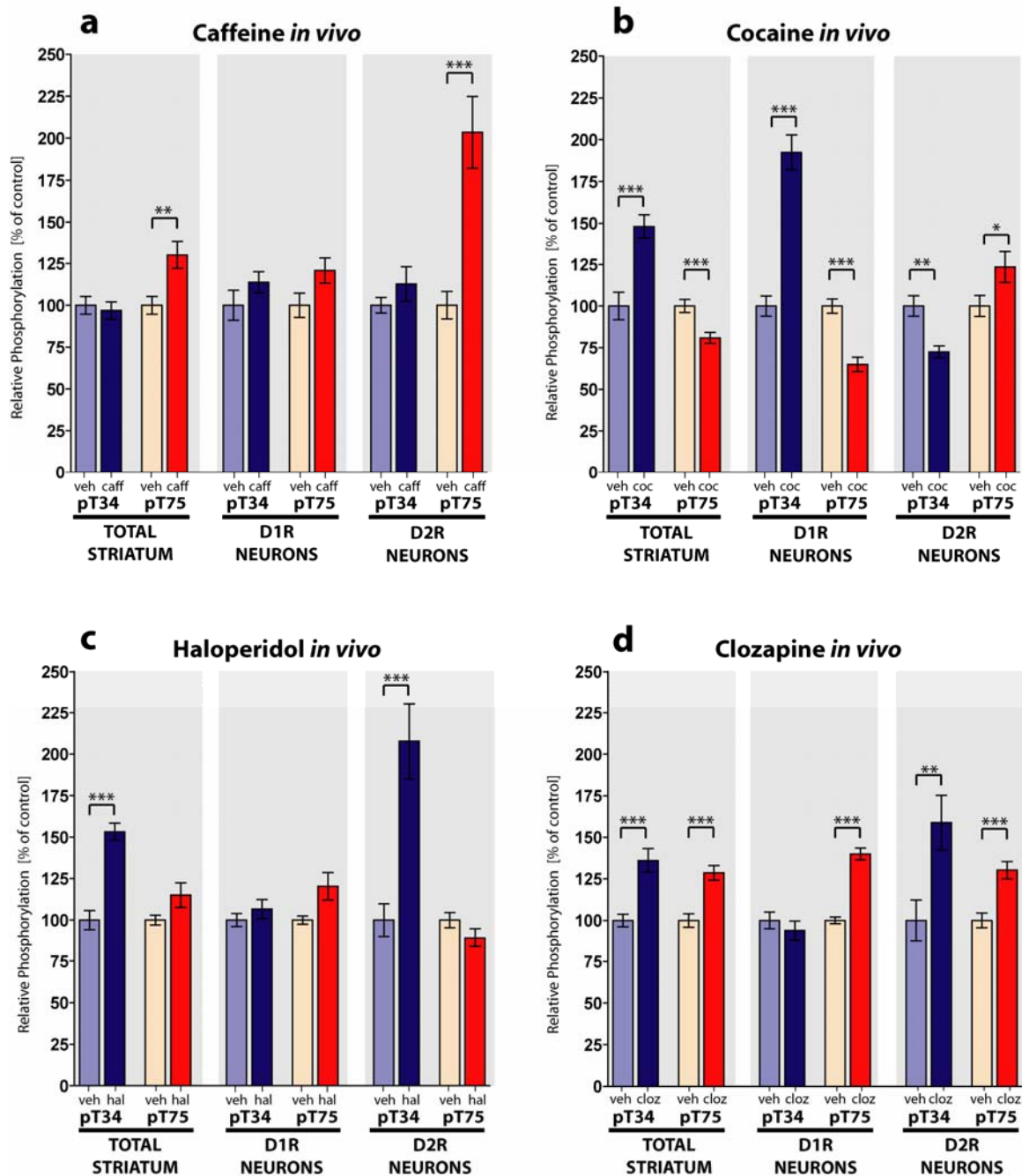


Figure 3.7 Differential Regulation of DARPP-32 Phosphorylation by Psychostimulants and Antipsychotics

D1R/D2R-DARPP-32 mice were injected i.p. with either (a) caffeine (7.5 mg/kg), (b) cocaine (20 mg/kg), (c) haloperidol (1 mg/kg), (d) clozapine (5 mg/kg), or vehicle and sacrificed 15 minutes later. Phospho-T34 and T75 data were normalized to total DARPP-32 levels and expressed as percent of control. The left panels represent data from non-tagged endogenous DARPP-32 (Total Striatum), the center panels represent data from Flag IP'd DARPP-32 (D1R Neurons), and the right panels represent data from Myc IP'd DARPP-32 (D2R Neurons). Each bar represents the average of 7-14 mice from 2-4 experiments. Error bars represent SEM. *, $p < .05$, **, $p < .01$, ***, $p < .001$; unpaired, two-tailed student's t-test.

Chronic L-DOPA increases DARPP-32 phosphorylation selectively in D1R neurons

Parkinson's disease (PD) is a debilitating neurodegenerative disorder caused by the death of dopamine neurons in the SNpc. This loss of dopaminergic input to the striatum results in maladaptations in a variety of signaling pathways. PD is commonly treated with the dopamine precursor L-DOPA which is effective at alleviating the motor symptoms of PD in the early stages of the disorder. Long-term L-DOPA treatment, however, results in debilitating dyskinesias and abnormal movements both in PD patients and in animal models of PD. Several studies have shown that T34 phosphorylation is selectively increased in the dyskinetic state (Picconi et al., 2003; Santini et al., 2007). However, it is unknown which cell type(s) this effect occurs in.

In accordance with previous reports, we found that T34 phosphorylation was increased in striatal homogenates from 6-OHDA lesioned D1R/D2R-DARPP-32 mice treated chronically with L-DOPA ($149.5\% \pm 11.9\%$ of control, $p < .05$, Fig. 3.8c, left panel). It has been suggested that supersensitivity of D1R-mediated signaling may underlie the development of L-DOPA induced dyskinesia (Corvol et al., 2004; Gerfen, 2003). In support of this hypothesis we found that T34 phosphorylation was strongly increased in D1R neurons in response to chronic L-DOPA while there was no change in T34 phosphorylation in D2R neurons ($334.3\% \pm 39\%$ of control, $p < .05$ in D1R neurons, Fig. 3.8c). Additionally, T75 phosphorylation was significantly decreased in D1R neurons with no change in phosphorylation at this site in D2 neurons ($73.5\% \pm 3.9\%$ of control, $p < .01$ in D1R neurons, Fig. 3.8c).

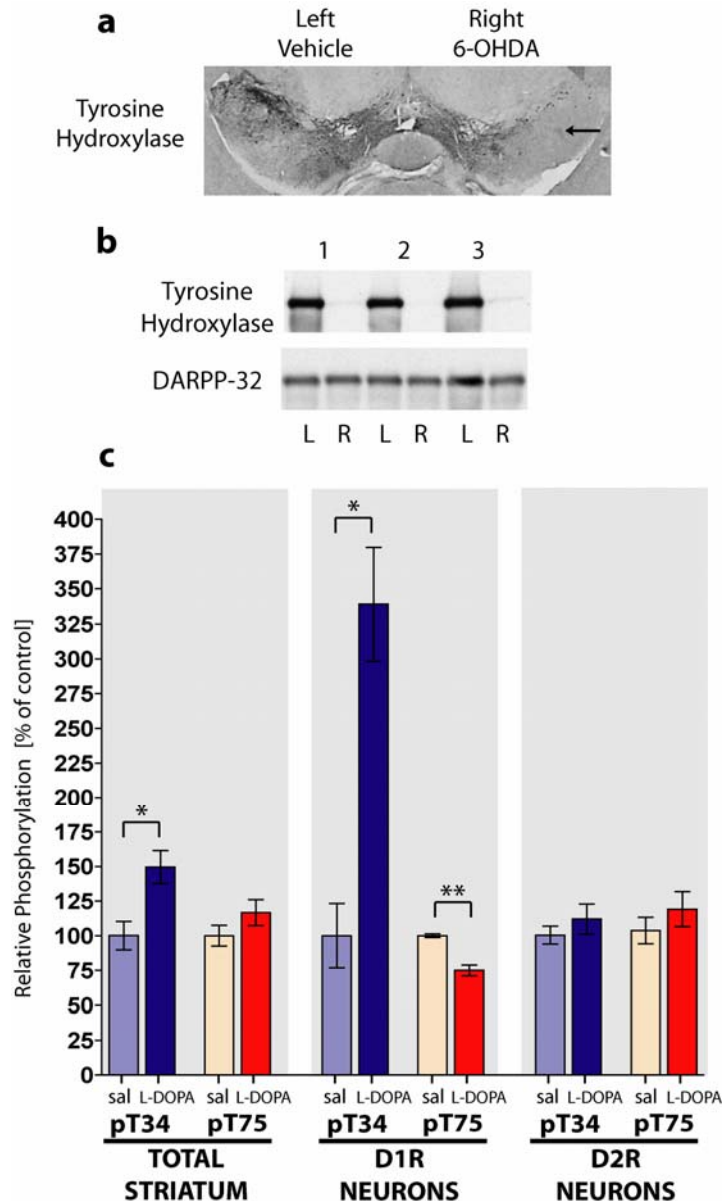


Figure 3.8 Chronic L-DOPA Increases T34 Phosphorylation Selectively in D1R Neurons

(a) Tyrosine hydroxylase (TH) stained coronal section showing the substantia nigra (SN) and ventral tegmental area (VTA). Arrow points to the right 6-OHDA injected side showing the selective loss of TH stained cell bodies in the SNc 3 weeks post-lesion. (b) Western blot of striatal homogenates from 3 individual D1R/D2R-DARPP-32 mice injected unilaterally with 6-OHDA showing the loss of striatal TH immunoreactivity on the right lesioned side with no change in total DARPP-32 protein. L = left side, sham injection; R = right side, 6-OHDA injection. (c) Lesioned D1R/D2R-DARPP-32 mice were injected i.p. with either saline or L-DOPA (20mg/kg)/benserazide (12mg/kg) for 11 days and sacrificed 30 minutes after the last injection. Phospho-T34 and T75 data were normalized to total DARPP-32 levels and expressed as percent of lesioned saline-treated control. The left panel represents data from non-tagged endogenous DARPP-32 (Total Striatum), the center panel represents data from Flag IP'd DARPP-32 (D1R Neurons), and the right panel represents data from Myc IP'd DARPP-32 (D2R Neurons). Error bars represent SEM. *, $p < .05$, **, $p < .01$; unpaired, two-tailed student's t-test. Data from b & c were generated in collaboration with Emanuela Santini and Dr. Gilberto Fisone, Karolinska Institutet, Sweden.

Discussion

In order to study second messenger signaling in striatonigral and striatopallidal neurons we developed a new BAC transgenic mouse method whereby epitope tagged protein is expressed in selective neuronal populations using cell-type specific promoters. The efficacy of this approach relies on the specificity of the promoters to accurately drive expression of tagged protein as well as an immunoprecipitation protocol that allows selective purification of the protein. This method could theoretically be used to study any protein in any cell type *in vivo*. We used this technique to study DARPP-32 phosphorylation selectively in D1R-striatonigral and D2R-striatopallidal neurons in response to various agents which target this brain area. We demonstrate cell-type specific patterns of phosphorylation for several classes of drugs which were not possible to discern with conventional techniques.

D1R and D2R agonists: DARPP-32 phosphorylation in slices and *in vivo*

We first tested the effects of direct D1 and D2 receptor agonists on DARPP-32 phosphorylation in slices and *in vivo* to confirm the validity of our system. The results in striatal slices largely matched our expectations and showed that we could detect changes in DARPP-32 phosphorylation in response to a D1 or D2 receptor agonist which occurred specifically in D1R or D2R neurons. Interestingly, when we looked at the effects of the same drugs *in vivo* we found different patterns of phosphorylation. The D2R agonist, quinpirole, caused a decrease in T34 phosphorylation and an increase in T75 phosphorylation in both D1R and D2R neurons. This can be explained by the fact that D2 receptors are not only expressed post-synaptically on MSNs but are also expressed

pre-synaptically as autoreceptors on the terminals of dopaminergic neurons. When these autoreceptors are stimulated, there is a decrease in the frequency and amount of dopamine released (Cubeddu and Hoffmann, 1982; Pothos et al., 1998; Schmitz et al., 2002). Decreased dopamine stimulation of D1 receptors would result in decreased T34 phosphorylation and increased T75 phosphorylation in D1R neurons as seen here. The small increase in T75 phosphorylation seen in D1R neurons in slices may reflect the presence of some functional nerve terminals in the striatal slice preparation which are responsive to quinpirole. In D2R neurons both *in vivo* and in slices, the effects of decreased dopamine are overridden by direct activation of post-synaptic D2 receptors by quinpirole.

There were also effects in both cell types observed with the D1R receptor agonist SKF 81297 *in vivo*. In this case we observed the expected increase in T34 in D1R neurons but we also observed significant increases in both T34 and T75 phosphorylation in D2R neurons. Since these results were observed *in vivo* and not in dissociated striatal slices, we conclude that it must have been due to stimulation of D1 receptors outside of the striatum. In addition to the striatum, D1 receptors are expressed in the cortex, hippocampus and amygdala all of which have projections to the striatum (Jin et al., 2001; Voorn et al., 2004). These projections are glutamatergic and glutamate has been shown to result in complex changes in both T34 and T75 phosphorylation in post-synaptic striatal neurons (Nishi et al., 2005). D1 receptors are also located on striatonigral terminals and can affect GABA release to the VTA (Cameron and Williams, 1993). Stimulation of these receptors would decrease dopamine input and might account for the increase in T34 phosphorylation seen in D2R neurons. This kind of regulation is not

unprecedented as a previous study showed that a D1 agonist injected *in vivo* resulted in cFos activation in both dynorphin-expressing striatonigral and enkephalin-expressing striatopallidal neurons (Le Moine et al., 1997).

Targeting striatopallidal neurons using the A_{2A} receptor

In addition to dopamine signaling via D1 and D2 receptors, striatal neurons also respond to the neuromodulator adenosine. Application of the A_{2A}R antagonist caffeine *in vivo* caused a selective increase in T75 phosphorylation in D2R neurons, likely due to inhibition of the phosphatase PP-2A. The specificity of this effect may be due to the restricted expression of A_{2A} receptors in the striatum compared with other brain areas and the fact that A_{2A}Rs are selectively expressed in striatopallidal neurons. A recent study comparing mRNA levels of active transcripts in D1R and D2R neurons found that one of the most differentially expressed transcripts in these cells was the A_{2A} receptor which showed robust enrichment in D2R striatonigral neurons (Heiman et al., 2007). For these reasons, A_{2A} receptors may be a more selective target than D2Rs for modification of striatopallidal cells. An example of this is the recent emergence of A_{2A}R antagonists as non-dopaminergic therapies to treat Parkinson's disease (PD) (Schwarzschild et al., 2006). Traditional therapies such as L-DOPA or dopamine receptor agonists target D1 and/or D2 receptors. As demonstrated here, *in vivo* injection of dopamine receptor agonists results in complex changes in DARPP-32 phosphorylation in both striatonigral and striatopallidal neurons. Added to the further array of adaptations in these pathways associated with dopamine loss in PD, these agents may have broad effects that are difficult to predict or control. Therefore, data presented here showing the specificity of

A_{2A}R antagonists *in vivo* support the idea that A_{2A} receptors may provide a more selective target for modification of striatopallidal neurons which could be beneficial in PD.

The opposing effects of cocaine on striatonigral and striatopallidal neurons

The benefit of using a cell-type targeted approach to study DARPP-32 phosphorylation is exemplified by the results obtained with cocaine. Cocaine caused a robust increase in T34 phosphorylation in D1R neurons and a decrease in T34 phosphorylation in D2R neurons. Since the increase in T34 phosphorylation in D1R neurons was larger than the decrease in D2R neurons, the total striatal sample showed a moderate increase in T34 phosphorylation reflecting an averaging of these two effects. The same is true for T75 phosphorylation whereby cocaine treatment resulted in opposing changes in D1R and D2R neurons, and data obtained in the total striatal sample was an average of these effects. This differential regulation of striatonigral and striatopallidal neurons by cocaine is important, however, as it reveals how dopamine can modulate both striatal output pathways in a way that results in a synergistic effect on the basal ganglia's control of motor function.

Activation of the D1R striatonigral pathway results in disinhibition of thalamocortical neurons and increased motor activity, whereas activation of the D2R striatopallidal pathway enhances the inhibition exerted by the basal ganglia and depresses motor function (see Fig. 1.3). Cocaine increases activation of G_i-coupled D2 receptors which decreases PKA activity and T34 phosphorylation in D2R striatopallidal neurons. This decrease in PKA/T34 phosphorylation has the overall effect of decreased

excitability in these cells (Yan et al., 1999). This shuts down the inhibitory activity of the striatopallidal pathway allowing strong stimulation of motor activity by the direct pathway, which is a hallmark cocaine response (Nestler, 2001). Thus, the study of DARPP-32 phosphorylation in D1R and D2R neurons in response to a dopamine potentiator, cocaine, has revealed a molecular mechanism for the synergistic actions of striatonigral and striatopallidal neurons.

DARPP-32 phosphorylation in response to antipsychotics

In contrast to the psychostimulant cocaine, the “typical” antipsychotic haloperidol causes behavioral sedation and catalepsy (Meltzer, 1999). Despite these opposing behavioral effects, cocaine and haloperidol both increase T34 phosphorylation in total striatal samples. Here we show that this increase is restricted to D1R neurons with cocaine and to D2R neurons with haloperidol. Haloperidol is expected to act on D2R neurons since it is a strong antagonist at D2 receptors. D2R striatopallidal neurons also express A_{2A} receptors which counteract D2R signaling by increasing cAMP and PKA activity (Fredholm and Svenningsson, 2003). Haloperidol blocks the inhibitory effect of D2 receptor activation, shifting the balance towards positive regulation of cAMP by endogenous adenosine (Fredholm et al., 2005). This results in an increase in T34 phosphorylation in D2R neurons.

Clozapine is an example of an “atypical” antipsychotic which has little D2R activity but has affinity for 5-HT_{2(a-c)}, 5-HT₃, 5-HT₆, 5-HT_{1a}, adrenergic1 & 2, and mACh receptors. Although haloperidol and clozapine both increased T34 phosphorylation selectively in D2R neurons, the increase was much larger with haloperidol than with

clozapine. Clozapine had additional effects on T75 phosphorylation which were present in both D1R and D2R cell types. The regulation of T34 phosphorylation in D2R neurons may represent a common mechanism of action of antipsychotics which counteracts the overactive striatal D2R signaling associated with schizophrenia (Guillin et al., 2007). The very high levels of T34 achieved with haloperidol may exceed the beneficial level and reach levels associated with unwanted locomotor side effects. In agreement with this, clinical studies show that *in vivo* D2R occupancy by antipsychotics exceeding 80% is often associated with extra-pyramidal side effects (Farde et al., 1992). The more limited increase in T34 phosphorylation balanced by increases in T75 seen with clozapine could reflect this drug's ability to target several different pathways which may be beneficial in treating a disorder as complex as schizophrenia.

Striatal neurons express 5-HT_{2a&c}, 5-HT₃, 5-HT₄ and 5-HT₆ receptors and DARPP-32 has been shown to mediate responses to serotonin in the striatum (Svenningsson et al., 2002a). One possible mechanism for clozapine's effects on T75 phosphorylation could be via antagonism of G_s-coupled striatal 5-HT₄ or 5-HT₆ receptors which would decrease PKA and PP-2A activity leading to an increase in T75 phosphorylation in both cell types. Future studies could test clozapine in combination with different receptor agonists and antagonists to determine the contribution of these signaling pathways to the clozapine effect on DARPP-32 phosphorylation.

L-DOPA and D1 receptor supersensitivity

L-DOPA is one of the most commonly prescribed drugs used in the treatment of Parkinson's disease. However, treatment with L-DOPA does not fully mimic the

endogenous physiological pattern of dopamine stimulation (Chase and Oh, 2000). This long-term non-physiological stimulation of DA receptors combined with secondary adaptations due to decreasing endogenous dopamine is thought to result in the expression of motor fluctuations and abnormal movements known as dyskinesias (Chase and Oh, 2000). It has been suggested that selective hypersensitivity of the D1R striatonigral pathway might account for these motor symptoms. Several studies have shown that signaling molecules downstream of the D1R are sensitized after chronic treatment with L-DOPA which could result in overactivity of the direct pathway (Aubert et al., 2005; Corvol et al., 2004; Gerfen, 2003).

It has also been shown that there is an increase in DARPP-32 phosphorylation at T34 which is correlated with the severity of dyskinesia (Santini et al., 2007). We show here that the increase in T34 phosphorylation seen with chronic L-DOPA treatment is restricted to D1R striatonigral neurons. In addition to the increase in T34 phosphorylation, we also observed a significant decrease in T75 phosphorylation selective to D1R neurons. This pattern of DARPP-32 phosphorylation amplifies PKA signaling whereby increased T34 phosphorylation inhibits the dephosphorylation of down-stream targets by PP-1 and decreased T75 phosphorylation relieves any inhibition of PKA signaling by DARPP-32 (Nishi et al., 2000). An amplification of PKA signaling in response to L-DOPA is supported by the finding that PKA/PP-1 targets such as AMPA receptors and mitogen-activated protein kinase/ERK kinase (MEK) also show increased phosphorylation in response to L-DOPA (Santini et al., 2007). The fact that L-DOPA produced robust changes in DARPP-32 phosphorylation in D1R cells with no effect on

D2R cells supports the hypothesis that D1 receptor signaling pathways are selectively prone to sensitization resulting from chronic L-DOPA treatment.

Conclusions

In addition to the drugs tested here, there are a variety of additional agents which have been shown to regulate DARPP-32 phosphorylation in striatal neurons (see Table 1). For many of these factors it is unclear whether they equally target both D1R and D2R neurons, or whether they have cell-type specific effects. We expect that further studies utilizing the cell-type targeted approach presented in this chapter will be important in gaining more detailed insights into striatal signaling.

Chapter 4

BEHAVIORAL ANALYSIS OF CONDITIONAL DARPP-32 KNOCK-OUT MICE

Introduction

Medium spiny neurons (MSNs) of the striatum receive and process input from a variety of sources including glutamate from the cortex and dopamine from the mid-brain. MSNs project from the dorsal striatum to the output nuclei of the basal ganglia via two pathways. The direct striatonigral pathway sends GABA-ergic projections to the GPi/SNr which in turn projects back to the cortex by way of the thalamus. The indirect striatopallidal pathway sends GABA-ergic projections to the GPe which then projects to the STN which in turn innervates the GPi/SNr. Stimulation of the direct pathway has the net effect of stimulating locomotion while excitation of the indirect pathway suppresses locomotor activity. Dopamine exerts opposing effects on these pathways; it stimulates the direct pathway and inhibits the indirect pathway. This opposing regulation is due to the presence of different sub-classes of dopamine receptors on direct and indirect pathway neurons. Direct pathway striatonigral neurons preferentially express type 1 dopamine receptors (D1R) which upon stimulation by dopamine positively regulate the cAMP/PKA/DARPP-32 T34 cascade. Indirect striatopallidal neurons express type 2 dopamine receptors (D2R) which couple to Gi proteins and negatively regulate the cAMP/PKA/DARPP-32 T34 signaling pathway.

In the previous chapter it was shown that agents which cause an increase in locomotion, i.e. cocaine and L-DOPA, increase the phosphorylation of DARPP-32 at T34 selectively in D1R-expressing direct pathway striatal neurons. Conversely, antipsychotics such as haloperidol and clozapine increase T34 phosphorylation specifically in D2R-expressing indirect pathway striatal neurons. Increased T34 phosphorylation amplifies PKA signaling by inhibiting the dephosphorylation of PKA

targets by protein phosphatase 1. Enhanced PKA activity is associated with an increase in excitability in MSNs due in part to its ability to phosphorylate glutamate receptors, which positively regulates receptor activity. Through this mechanism, T34 phosphorylation can be viewed as a biochemical marker for the relative activation state of striatonigral and striatopallidal neurons. Inhibition of T34 phosphorylation in either cell-type should therefore result in a decrease in excitability in that cell-type and a decrease in the behavioral effect mediated by the corresponding pathway.

To directly test this hypothesis, we generated conditional knock-out mice in which DARPP-32 protein was selectively deleted in either direct or indirect pathway striatal neurons. To achieve a conditional cell-type specific deletion we utilized the Cre-LoxP system. We chose the D1R and D2R promoters to drive Cre recombinase expression as we have shown previously that these promoters selectively target direct and indirect pathway neurons respectively. We studied these mice to examine the relative contribution of the direct and indirect pathways to basal open field locomotor behavior as well as drug induced locomotor activity.

Results

Generation of conditional DARPP-32 knock-out mice

In order to generate a conditional knock-out (cKO) of DARPP-32, we made a mouse line in which exons 1-4 of the DARPP-32 gene were flanked by loxP sites (floxed DARPP-32 mice) (Fig. 4.1a). Through homologous recombination, the floxed DARPP-32 gene was inserted into the endogenous DARPP-32 gene locus. The presence of floxed versus wild-type alleles was identified by PCR genotyping using primers in which the

presence of the wild-type allele resulted in a 376bp band and the floxed allele gave a 486bp band (Fig. 4.1b). The introduction of the loxP sites into the mouse DARPP-32 gene had no effect on the expression of DARPP-32 protein in the striatum of floxed-DARPP-32 mice (Fig. 4.1c).

After confirming normal expression of striatal DARPP-32 protein in the homozygous floxed DARPP-32 mice, we bred these mice to D1R-Cre and D2R-Cre BAC mice generated by the GENSAT project. The expression of Cre recombinase in these BAC mice has previously been characterized (Gong et al., 2007a). The D1R-Cre line shows high levels of expression in the striatum, specifically in neurons which project to the SNr. There is also Cre expression distributed in other regions consistent with the known pattern of endogenous *drd1a* gene expression. The D2R-Cre line used here was the ER44 line which shows expression of Cre in striatal neurons projecting to the GPe, as well as in additional regions known to express the *drd2* gene, such as the SNc.

To determine the presence of D1R- or D2R-driven Cre expression in our cKO DARPP-32 mice, we generated PCR primers in which the 5' primer sequence was located in the D1 or D2 receptor promoter and the 3' primer sequence was in the Cre recombinase gene. Figure 4.1d shows a schematic of the location of these primers in the D1R-Cre and D2R-Cre transgenes, and figure 4.1e shows the detection of D1R- or D2R-driven Cre in the cKO DARPP-32 mice using PCR genotyping.

The D1R-Cre and D2R-Cre mice were made on an FVB genetic background, while the floxed DARPP-32 mice were made on a C57/Bl6 genetic background. Since the background strain of knock-out mice has been shown to impact their behavioral responses (Kelly et al., 1998), we used the following breeding strategy to generate cKO

DARPP-32 mice. Homozygous floxed-DARPP-32 C57/Bl6 mice were first bred to either D1R-Cre or D2R-Cre FVB mice. The heterozygous floxed-DARPP-32 D1R- or D2R-Cre positive offspring were then bred back to homozygous floxed-DARPP-32 C57/Bl6 mice. We selected homozygous floxed-DARPP-32 D1R- or D2R-Cre positive offspring to breed again to the homozygous floxed-DARPP-32 C57/Bl6 mice. Using this type of cross, subsequent generations were all homozygous floxed and either positive or negative for D1R- or D2R-Cre. With this breeding strategy, the mice were continually backcrossed to the C57/Bl6 background and littermate controls could be used for all behavioral analysis. Mice negative for Cre were considered wild-types (WT) while mice positive for Cre were considered conditional knock-outs (D1R-D32-KO or D2R-D32-KO).

Expression of DARPP-32 protein in D1R-D32-KO and D2R-D32-KO mice

We determined the efficiency of Cre-mediated deletion of DARPP-32 by examining striatal DARPP-32 protein levels by western blotting (Fig. 4.1f). There was a $69.8\% \pm 3.3\%$ ($p < .001$) reduction in total DARPP-32 protein in the D1R-D32-KO mice and a $41.3\% \pm 6.1\%$ ($p < .001$) reduction in DARPP-32 in the D2R-D32-KO mice (Fig. 4.1g). The residual DARPP-32 expression in the conditional knock-out mice reflects intact DARPP-32 protein expression in the other cell type (see Fig. 4.2 below).

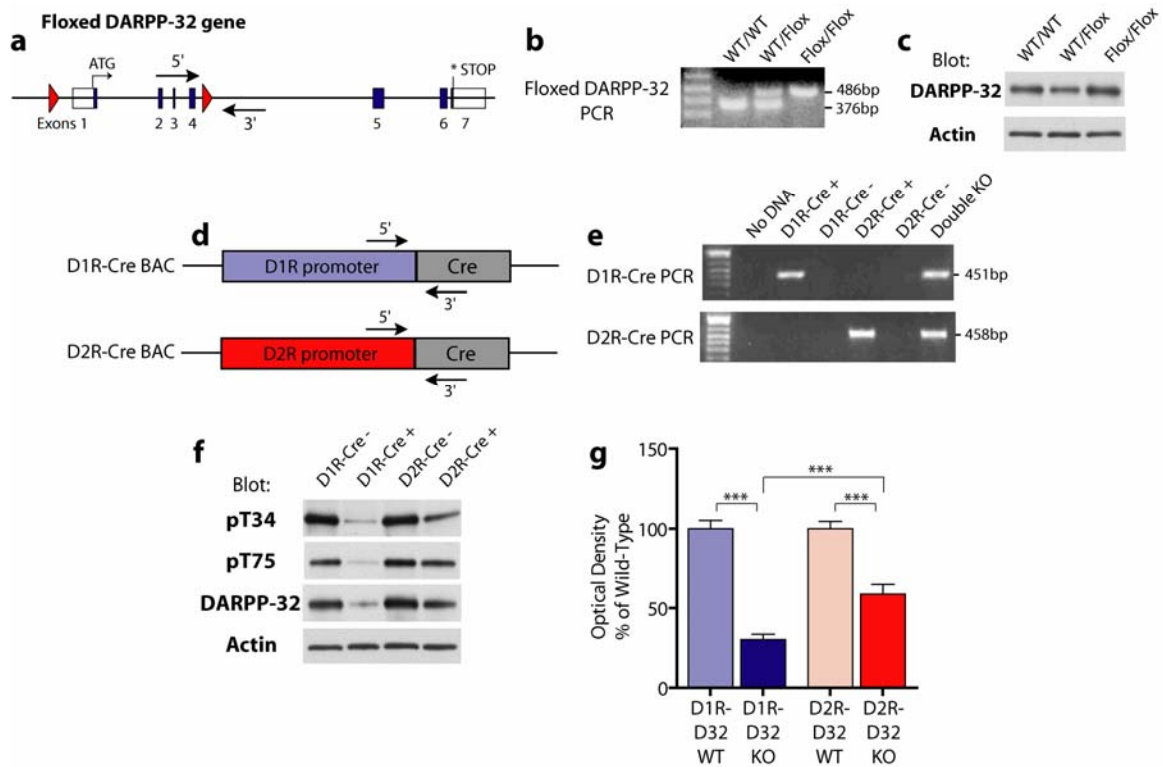


Figure 4.1 Generation of Conditional DARPP-32 Knock-out Mice

(a) Schematic showing targeting of the DARPP-32 gene. Blue boxes represent exons, red triangles show the position of the loxP sites flanking exons 1-4. Black arrows indicate the location of the genotyping primers used in the PCR shown in (b). (b) Genotype PCR from floxed DARPP-32 mice revealing a 376bp wild-type (WT) band and a 486bp Floxed band. (c) Western blot showing normal expression levels of DARPP-32 protein in striata from floxed DARPP-32 mice with genotypes indicated. (d) Schematic of the D1R- and D2R-Cre BAC transgenes. Colored arrows indicate the position of primers used for genotyping in (e). (e) Genotype PCR from conditional knock-out mice using D1R-Cre and D2R-Cre primers to selectively identify Cre expression in D1R and D2R cells. Double knock-out (KO) mice were generated by breeding D1R-D32-KO mice with D2R-D32-KO mice. (f) Western blot of striata from wild-type and cKO DARPP-32 mice comparing protein levels of phospho-T34, phospho-T75, and total DARPP-32. Actin antibody (bottom panel) was used to show equal loading. (g) DARPP-32 protein levels were quantified and compared between the cKO DARPP-32 mouse lines. Optical density was measured from total DARPP-32 western blots and averages of 12 mice from each genotype were expressed as percent of wild-type protein level. Error bars represent SEM. ***, $p < .001$; unpaired, two-tailed, student's t-test.

To further confirm the cell-type specific deletion of DARPP-32 in the conditional knock-out mice, we examined expression of DARPP-32 protein in striatal cells using immunofluorescence (Fig. 4.2). As expected based on previous reports, 93.2% (165/177) of striatal neurons, identified by staining with the neuronal marker NeuN, were positive for DARPP-32 in wild-type mice (Fig. 4.2a). In the D1R-D32-KO mice, only 34.2% (122/357) of striatal neurons were positive for DARPP-32 (Fig. 4.2b). In the D2R-D32-KO mice, 55.8% (106/190) of striatal neurons were positive for DARPP-32 (Fig. 4.2c). The D1R-D32-KO mice were crossed with the D2R-D32-KO mice to generate double D1R/D2R-D32-KO mice. Striatal sections from these mice showed no striatal DARPP-32 immunoreactivity indicating that the neurons absent for DARPP-32 in the D1R and D2R conditional knock-out mice were segregated to different striatal cell populations (Fig. 4.2d).

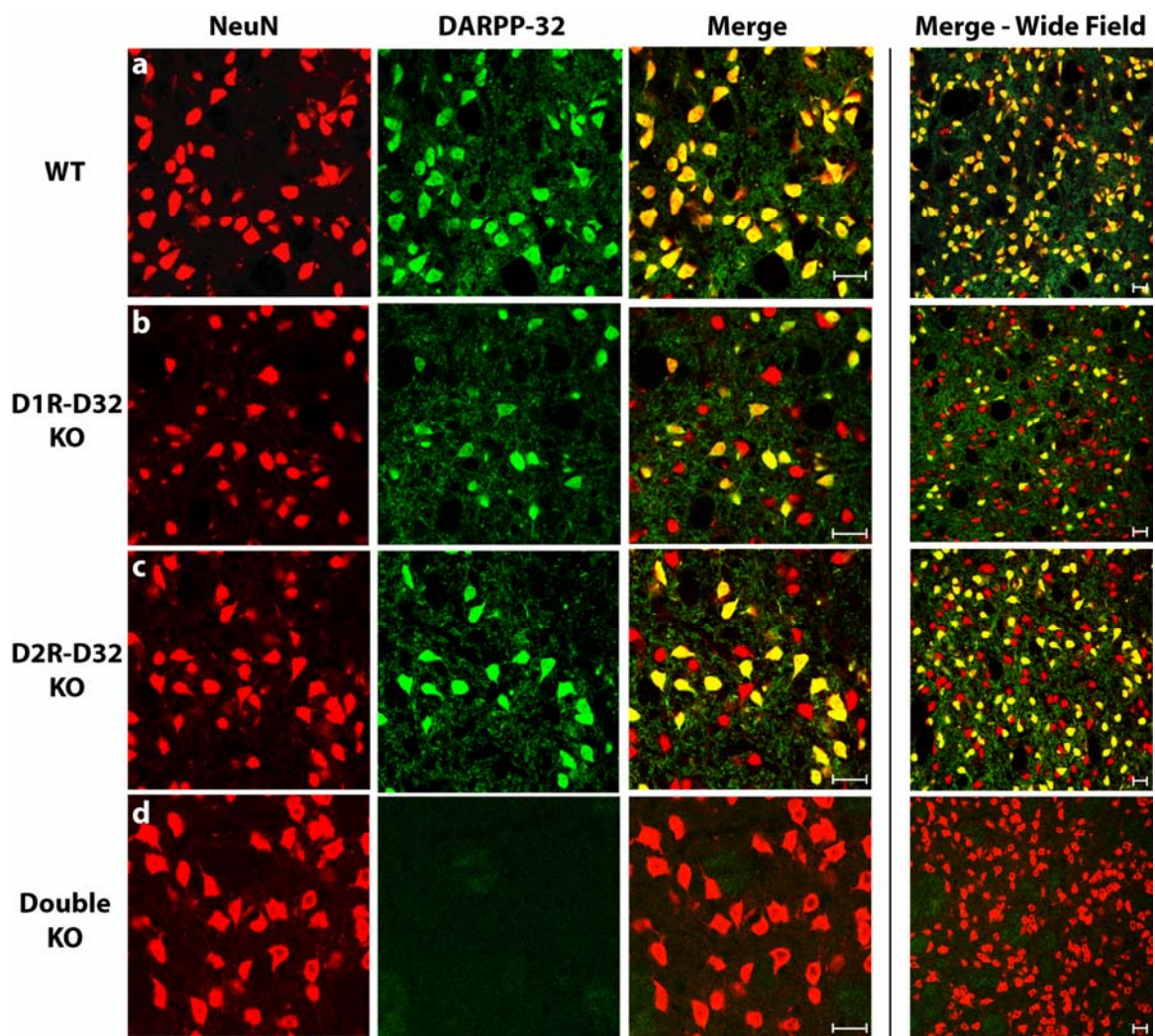


Figure 4.2 DARPP-32 Protein Expression in Conditional Knock-out Mice

Immunohistochemistry showing localization of the neuronal marker NeuN (red) and DARPP-32 (green) in striatal sections. **(a)** Striatal sections from a wild-type mouse **(b)** Striatal sections from a D1R-D32-KO mouse **(c)** Striatal sections from a D2R-D32-KO mouse **(d)** Striatal sections from a double knock-out mouse generated by breeding D1R-D32-KO and D2R-D32-KO mice together.

Scale bars represent 20µm.

Basal locomotor activity in conditional DARPP-32 KO mice

After confirming the selective knock-out of DARPP-32 protein in striatal D1R and D2R neurons, we studied the basal locomotor activity of the conditional knock-out mice. We measured the distance traveled by these mice in the open field over a period of 60 minutes. The time course of basal locomotor activity is shown in Figure 4.3a&c. D1R-D32-KO mice exhibited reduced activity compared to wild-type littermates at all times points (Fig. 4.3a). Conversely, D2R-D32-KO mice exhibited significantly increased locomotor activity compared to wild-type littermates at all time points (Fig. 4.3c). Quantification of the total distance traveled in 60 minutes revealed that D1R-D32-KO mice exhibited a 20% ($p < .01$) reduction in locomotor activity while D2R-D32-KO mice showed a 42.2% ($p < .001$) increase in activity compared to wild-type mice (Fig. 4.3b,d).

To rule out the possibility of major physiological changes in the knock-out mice which might account for the observed changes in locomotor activity, we compared the body weights of the wild-type and cKO mice at 8 weeks of age. There was no difference in the average weight of cKO male or female mice compared to wild-type littermates in either line (Table 3). This suggests that the locomotor phenotype in the cKO mice was not due to major changes in appetitive behavior or overall health.

Table 3 Body Weights of Conditional DARPP-32 Knock-out Mice

	D1-D32-KO		D2-D32-KO	
	WT	KO	WT	KO
Males	23.05 ± 0.25	23.57 ± 0.60	24.32 ± 0.33	24.62 ± 0.38
Females	19.55 ± 0.72	19.0 ± 0.51	19.97 ± 0.52	19.33 ± 0.61

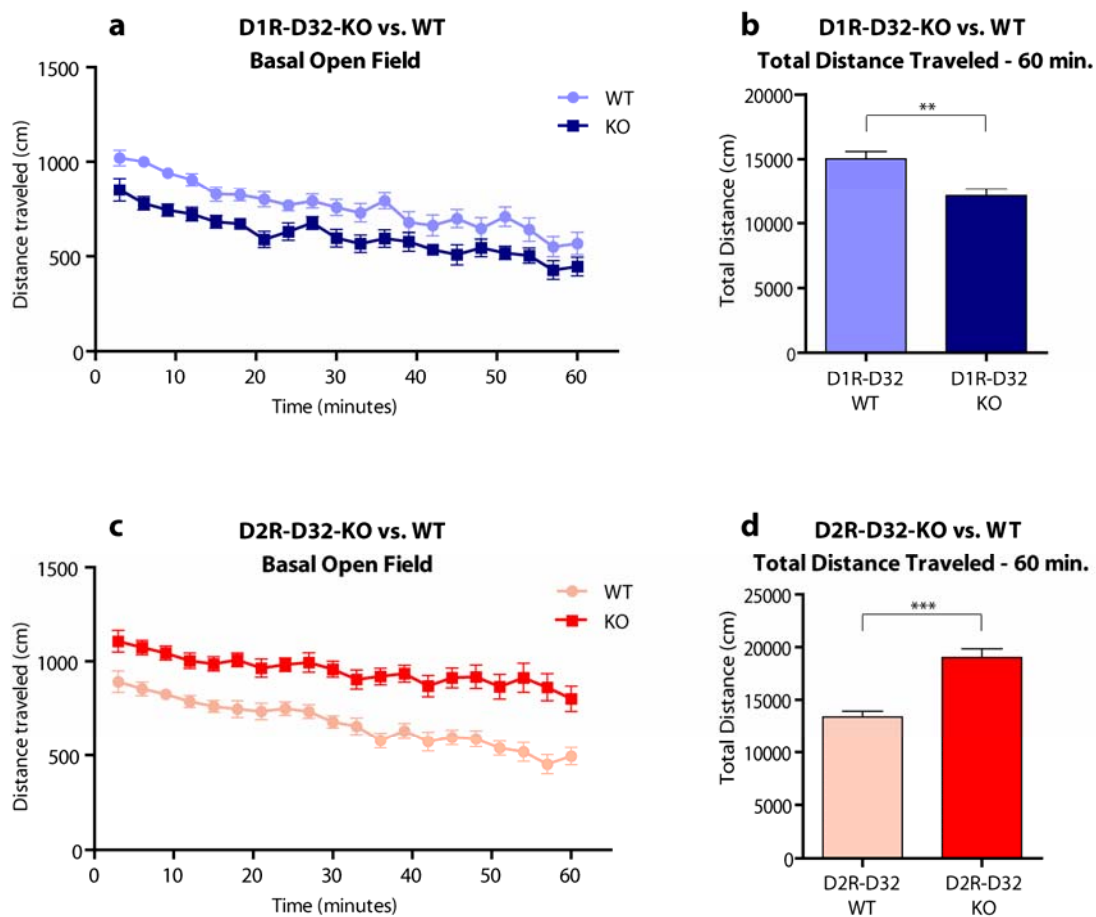


Figure 4.3 Basal Locomotor Activity in D1R-D32-KO and D2R-D32-KO Mice

Locomotor activity was measured by placing mice in an open field chamber and recording the distance traveled using an overhead camera and EthoVision software. **(a,c)** Line graphs show the distance traveled over a 60 minute period by wild-type and conditional knock-out mice, reported in centimeters. Each point represents the total distance traveled during each 3 minute time interval. **(a)** The light blue line corresponds to D1R-D32 wild-type mice ($n=22$), the dark blue line corresponds to D1R-D32-KO mice ($n=16$). **(c)** The pale red line corresponds to D2R-D32 wild-type mice ($n=17$), the dark red line corresponds to D2R-D32-KO mice ($n=25$). **(b,d)** Bar graphs show the average distance traveled over the entire 60 minute test period by mice from each genotype. Error bars represent SEM. **, $p<.01$; ***, $p<.001$; unpaired, two-tailed student's t-test.

Behavioral responses to acute cocaine and haloperidol in conditional DARPP-32 knock-out mice

After establishing the presence of a basal locomotor phenotype in the conditional DARPP-32 knock-out mice, we then asked whether deletion of DARPP-32 in direct or indirect neurons would have an effect on cocaine or haloperidol mediated locomotor behavior. As shown in the previous chapter, acute cocaine injection resulted in differential changes in both T34 and T75 phosphorylation in D1R direct pathway and D2R indirect pathway neurons. Previous studies have also suggested a role for both D1R and D2R neurons in mediating behavioral responses to cocaine (Self et al., 1996; Xu et al., 2000). In line with these findings we found that acute locomotor responses to cocaine were altered in both the D1R-D32-KO and D2R-D32-KO mice (Fig. 4.4).

As expected, acute injection of cocaine resulted in a robust induction of locomotor activity in wild-type mice (Fig. 4.4a-d). In D1R-D32-KO mice, this increase was significantly reduced across all time points (Fig. 4.4a,b). We quantified the early phase locomotor response to cocaine since the peak of DARPP-32 T34 phosphorylation occurs at approximately 15 minutes after cocaine injection. Results revealed that although cocaine significantly increased locomotor activity in the D1R-D32-KO mice compared to vehicle treated D1R-D32-KO mice ($p < .05$), this increase was significantly lower than the increase seen in wild-type mice ($6485 \pm 990\text{cm}$ for WT; $3555 \pm 472\text{cm}$ for D1R-D32-KO, $p < .05$, Fig. 4.4b).

In contrast to the D1R-D32-KO mice, D2R-D32-KO mice exhibited enhanced locomotor activity in response to cocaine compared to wild-type mice (Fig. 4.4c,d). This enhancement was most pronounced in the early response to cocaine, 15-20 minutes post-

injection. Quantification of the response in this time period revealed a significant difference between D2R-D32-KO mice and wild-type mice (5598 ± 771 cm for WT; 7795 ± 709 cm for D2R-D32-KO, $p < .05$, Fig. 4.4d).

The antipsychotic haloperidol is a strong antagonist of D2 receptors. In previous experiments we showed that haloperidol caused a selective increase in T34 phosphorylation in D2R-expressing neurons with no changes in DARPP-32 phosphorylation in D1R-expressing neurons. This pattern of phosphorylation is expected to activate the indirect pathway and result in inhibition of locomotor behavior. In wild-type mice, haloperidol significantly reduced locomotor activity (Fig. 4.4a-d). In D2R-D32-KO mice, however, there was a decrease in the degree of locomotor suppression induced by haloperidol in the first 30 minutes following drug injection (Fig. 4.4c). Quantification of the early locomotor response to haloperidol indicated that wild-type and D2R-D32-KO mice had a significantly different response (707 ± 47 cm for WT; 1842 ± 191 cm for D2R-D32-KO, $p < .001$, Fig. 4.4d). In contrast, there was no difference between wild-type and D1R-D32-KO mice with regard to their locomotor response to haloperidol (1371 ± 258 cm for WT; 1228 ± 283 cm for D1R-D32-KO, Fig. 4.4b).

Since the vehicle-treated D2R-D32-KO mice exhibited higher locomotor activity compared to vehicle-treated wild-type mice (2302 ± 436 cm for vehicle treated WT; 3303 ± 244 cm for vehicle treated D2R-D32-KO, $p < .05$, Fig. 4.4d), we calculated the percent decrease in locomotor activity compared to vehicle control 15 minutes following acute haloperidol injection. We found that even accounting for the higher baseline activity, the D2R-D32-KO mice showed a significantly reduced response to haloperidol treatment as measured by the percent decrease in activity compared to vehicle control ($30.7\% \pm 2\%$ of

control for haloperidol treated WT mice; $55.8\% \pm 5.8\%$ of control for haloperidol treated D2R-D32-KO mice, $p < .001$, Fig. 4.4e).

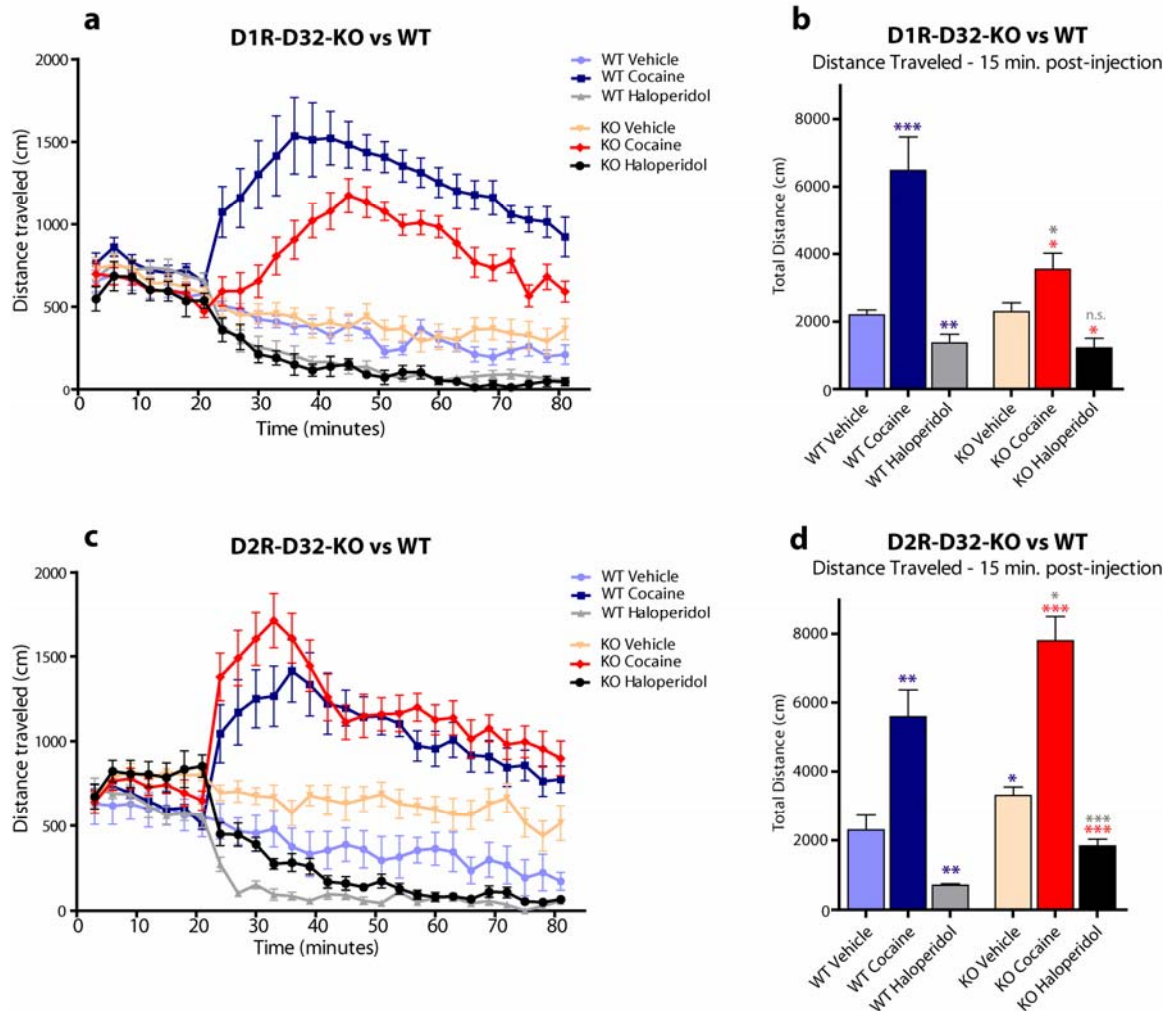


Figure 4.4 Locomotor Responses to Acute Cocaine and Haloperidol in Conditional DARPP-32 KO Mice

Locomotor activity was measured by placing mice in an open field chamber and recording the distance traveled over time using an overhead camera and EthoVision software. (a,c) Line graphs show the distance traveled over an 81 minute period by wild-type and conditional knock-out mice, reported in centimeters. Each point represents the total distance traveled during each 3 minute time interval. Mice were injected with either vehicle, cocaine (20mg/kg), or haloperidol (1mg/kg) at 21 minutes and locomotor activity was recorded for 60 minutes following the drug treatments. The light blue lines correspond to vehicle injected (a) D1R- or (c) D2R-D32 wild-type mice (n=11,6), the dark blue lines correspond to cocaine injected (a) D1R- or (c) D2R-D32 wild-type mice (n=10,9), the grey lines correspond to haloperidol injected (a) D1R- or (c) D2R-D32 wild-type mice (n=10,6), pale red lines correspond to vehicle injected (a) D1R- or (c) D2R-D32 knock-out mice (n=10,12), dark red lines correspond to cocaine injected (a) D1R- or (c) D2R-D32 knock-out mice (n=7,10), black lines correspond to haloperidol injected (a) D1R- or (c) D2R-D32 knock-out mice (n=5,9). (b,d) Bar graphs show the sum of the distance traveled in the 15 minute period following drug injection by mice in each genotype and treatment group. Error bars represent SEM. Blue asterisks indicate comparisons between the group indicated and wild-type vehicle treated mice. Red asterisks indicate comparisons between the group indicated and knock-out vehicle treated mice. Grey asterisks indicate comparisons between the group indicated and the corresponding treatment group in wild-type mice. *, $p < .05$; **, $p < .01$; ***, $p < .001$; unpaired, two-tailed student's t-test. n.s. = no significant difference.

Discussion

In order to better understand the role of DARPP-32 signaling in direct and indirect pathway neurons, we generated mice in which DARPP-32 was conditionally deleted in either D1R-expressing or D2R-expressing neurons. These mice allowed us to directly compare the behavioral consequences of selective alteration of these pathways at a basal level and in response to drug treatments. We chose to knock-out DARPP-32 as it is a key mediator of signaling in both D1R and D2R medium spiny neurons (Greengard, 2001). Global deletion of DARPP-32 results in decreased cellular and behavioral responses to a variety of therapeutic agents and drugs of abuse (Fienberg and Greengard, 2000). Interestingly, basal physiology is generally intact in DARPP-32 knock-out mice. This makes DARPP-32 a good choice for manipulation of direct and indirect pathway neurons as there may be fewer adaptive responses than would be seen by knocking out another molecule in these neurons.

DARPP-32 is an amplifier of signaling in MSNs and one interpretation is that removal of DARPP-32 in these neurons generates a “knock-down” of these cells. By removing DARPP-32, we remove the amplifying mechanism and therefore responses to dopamine and other stimuli are blunted. D1R-D32-KO mice for example, can be thought of as a knock-down of the direct pathway and therefore responses to dopamine in these mice are primarily mediated by signaling in the indirect pathway. In accordance with this idea, we found that the basal activity of the D1R-D32-KO mice was reduced compared to wild-type littermates. This would be expected if the D1R-expressing direct pathway neurons are turned off upon deletion of DARPP-32. This would shift the balance towards decreased locomotion due to stronger inhibitory signaling by intact indirect pathway

neurons. Likewise, the enhanced basal locomotor activity seen in the D2R-D32-KO mice can be interpreted as due to the relief of the inhibitory tone of the D2R indirect pathway neurons allowing strong activation of locomotor pathways by direct pathway neurons.

This type of interpretation can also be used to explain the behavioral responses to acute cocaine and haloperidol in the conditional DARPP-32 knock-out mice. The blunted response to cocaine in the D1R-D32-KO mice suggests that DARPP-32 signaling in D1R neurons is required for the full locomotor response to acute cocaine. The fact that there is an increase in locomotion in response to cocaine in the D2R-D32-KO mice corroborates the idea that deletion of DARPP-32 in these neurons results in a silencing of the indirect pathway. Acute cocaine treatment causes a decrease in T34 phosphorylation and PKA activity in D2R neurons which shuts down these cells and relieves inhibition by the indirect pathway. Removal of DARPP-32 should further dampen activity in this pathway which may explain the enhancement of cocaine induced locomotion seen in the D2R-D32-KO mice. It would be interesting to see how deletion of DARPP-32 directly affects the excitability or the firing rate of these neurons to see if this could be the mechanism underlying the behavioral effects seen here.

In the case of haloperidol, we observed a decrease in the behavioral response of the D2R-D32-KO mice compared to wild-type mice. This was expected since haloperidol has strong affinity for the D2 receptor and we have previously shown that acute haloperidol causes an increase in DARPP-32 T34 phosphorylation selectively in D2R- but not D1R-expressing neurons. It has also been shown that the total DARPP-32 knock-out mice have a reduced behavioral response to another antipsychotic drug, raclopride (Fienberg et al., 1998). We saw a reduction in the ability of acute haloperidol

to inhibit locomotor activity in the D2R-D32-KO mice while responses in the D1R-D32-KO mice were equivalent to wild-type mice. This provides further evidence that both the biochemical and behavioral effects of haloperidol are primarily mediated by changes in signaling in D2R-expressing indirect pathway neurons.

One interesting observation regarding the differences in the drug induced locomotor responses in cKO mice, was that the differences between WT and cKO mice were largest in the first 15-30 minutes following drug treatment. This supports the idea that the function of DARPP-32 is to amplify signaling which may be most relevant in the early phase of drug-induced signaling changes. In line with this, we have observed that in response to drugs injected *in vivo*, the peak of DARPP-32 T34 phosphorylation occurs within the first 15-30 minutes after injection. It is possible that at later time points, the intact signaling pathways “catch-up” in the conditional DARPP-32 knock-out mice or that other pathways are responsible for mediating the behavioral effects at later time points.

Additionally, we reported a basal difference in locomotor activity in both the D1R-D32-KO and D2R-D32-KO mice, however, only D2R-D32-KO mice exhibited a basal difference in the drug treatment experiment. This may have been due to the fact that the basal activity trial was the first exposure to the open field chamber. In the drug treatment trial conducted a week later, the environment was no longer novel. It has been shown that mice show increased locomotor activity and exploratory behavior in novel environments and that individual differences in behavior can be masked after subsequent exposures to the environment (Piazza et al., 1990). Since the phenotype in the D1R-D32-KO mice was a reduction in locomotion, there may have been a “floor” effect whereby

the difference between knock-out and wild-type mice was obscured by an overall decrease in activity due to the second exposure to the testing chamber. Another possibility is that the reduction in locomotor activity in D1R-D32-KO mice is a more subtle and variable effect. A greater number of mice were used in the basal locomotor analysis (n=22 WT mice and n=16 D1R-D32-KO mice) than in the vehicle treated locomotor analysis (n=11 WT mice and n=10 D1R-D32-KO mice). Also both male and female mice were tested and there may be sex differences which contribute to the variability. Future studies using a larger and more homogenous population of mice could test the effects of these variables.

The results presented here reveal differences in the regulation of locomotor behavior by direct and indirect pathway neurons. Despite clear predictions based on the classical model of basal ganglia circuitry, it has been a challenge to directly show differences in direct pathway and indirect pathway signaling due to the difficulty in targeting these cells. Within the striatum, these cell populations are morphologically indistinguishable and anatomically intermixed. Studies with selective dopamine receptor agonists and antagonists have the disadvantage that these agents can have indirect effects in the striatum resulting from expression of D1 and D2 receptors in neurons other than MSNs (e.g. D1 receptors in the cortex and D2 autoreceptors on striatal dopaminergic nerve terminals). Additionally, studies with receptor knock-out mice have the disadvantage that there are often compensatory or adaptive changes which are difficult to control. This also could be the case with the conditional DARPP-32 knock-out mice, however it is likely that the removal of DARPP-32 has less of an impact on basal physiology than removal of a receptor type.

The advantage of using a cell-type specific approach is evident when comparing our results to results obtained with the total DARPP-32 knock-out (KO) mice. In terms of locomotor behavior, there was no change in basal activity observed in the total DARPP-32 KO mice (Fienberg et al., 1998). Since we saw an increase in activity in the D2R-D32-KO mice and a decrease in activity in the D1R-D32-KO mice, there would be no net change in activity if these effects were summed, as was observed in the total DARPP-32 KO mice.

It was reported that the total DARPP-32 KO mice exhibited reduced responses to cocaine both biochemically and behaviorally (Fienberg et al., 1998). At low doses of cocaine there was a significant reduction in the acute locomotor response (10 minutes following injection) in total DARPP-32 knock-out mice; however, at a higher 20 mg/kg dose, there was no effect of the knock-out (Fienberg et al., 1998). We tested behavioral responses to the 20mg/kg dose, and found that D1R-D32-KO mice had a reduced locomotor response while the D2R-D32-KO mice exhibited an enhanced response. The sum of these effects is again zero which accounts for the lack of an observable effect in the total DARPP-32 KO mice. By looking at the average of striatal signaling events, important differences between the two output pathways are missed.

Taken together, these findings support the classical model of basal ganglia circuitry showing the opposing regulation of locomotor behavior by direct and indirect pathway neurons. These results also directly demonstrate how dopamine can modulate both pathways in a synergistic manner to influence locomotor activity, as seen with acute cocaine treatment. It will be interesting to examine responses of these conditional knock-out mice to other factors known to affect striatal signaling and behavior. It would be

especially valuable to look at additional behaviors such as pre-pulse inhibition in response to antipsychotics or conditioned place preference in response to chronic cocaine to determine the relative contributions of the two cell types to other basal ganglia-mediated behaviors.

Chapter 5

CONCLUSIONS AND FUTURE DIRECTIONS

The striatum is a site of integration for diverse neurotransmitters and neuromodulators. These signaling molecules originate from within the basal ganglia and from a variety of brain regions including the cortex, mid-brain, and limbic regions. This diversity of signaling makes the striatum unique. Medium spiny neurons of the striatum receive the majority of these inputs and send efferent projections out of the striatum to other basal ganglia structures. MSNs express a large number of neurotransmitter receptors and are a model system for studying the integration of fast and slow synaptic signaling pathways. DARPP-32 is a phosphoprotein highly enriched in MSNs which has been shown to regulate, or be regulated by, nearly all of the signaling molecules in the striatum. Global deletion of DARPP-32 protein results in markedly reduced or abolished biochemical, behavioral and electrophysiological responses to a variety of agents confirming its central importance in striatal signaling.

In addition to the integration of striatal inputs, medium spiny neurons also play an essential role in regulating the output signaling of the basal ganglia. The two types of striatal projection neurons comprising the direct and the indirect pathways differentially modulate the output activity of the basal ganglia. Stimulation of the direct pathway results in an inhibition of the GPi/SNr which increases the excitation of cortical neurons by the thalamus. Stimulation of the indirect pathway which projects via the GPe and STN, enhances the activity of the GPi/SNr which ultimately inhibits stimulation of the cortex. Excitation of the direct pathway stimulates locomotion while activation of the indirect pathway inhibits locomotion. Dopamine synergistically regulates these pathways by exciting the direct pathway and inhibiting the indirect pathway resulting in an additive facilitatory effect on locomotion.

Direct and indirect striatal neurons are known to differ in their expression of several proteins including dopamine receptors and neuropeptides. A recent study using a novel technique to profile differences in gene expression between these two cell types has revealed hundreds of additional gene transcripts which are differentially expressed between direct and indirect pathway neurons (Heiman et al., 2007). Despite the overall similarity between these cells in terms of their anatomical and morphological features, the selective differences between these neurons allow them to differentially respond to signaling factors within the striatum. Having two different efferent pathways with differential signaling properties allows for more options and greater flexibility in the ability of the basal ganglia to respond to varying signaling environments.

Traditional biochemical and behavioral studies have mainly reported changes in overall striatal signaling or function in response to different factors. While those studies are informative, they report an average of signaling events which obscures differences in responses between direct and indirect pathway neurons. This has presented problems in the interpretation of data exemplified by the paradox presented in chapter 3 concerning the regulation of DARPP-32 phosphorylation by psychostimulants and antipsychotics. We have attempted to address these limitations by studying striatal signaling in a cell-type specific manner. These studies rely on the ability to accurately target different cell-types which has been aided by the BAC transgenic mouse methodology established by the GENSAT project (Gong et al., 2003). We have generated transgenic mice which use cell-type specific promoters to drive expression of tagged DARPP-32 or Cre recombinase in direct and indirect pathway neurons. This has allowed selective purification or deletion of proteins in specific striatal populations. By using these approaches we have

been able to study striatal signaling in more detail and have provided direct evidence confirming several predictions based on the classical model of basal ganglia circuitry.

Figure 5.1 summarizes the circuitry of the basal ganglia with regard to the direct and indirect pathways. This schematic can be used to visualize how biochemical changes in striatal neurons can ultimately affect behavioral responses. In one series of experiments presented here we studied the selective changes in DARPP-32 phosphorylation in direct and indirect pathway striatal neurons in response to various signaling molecules. In subsequent experiments we examined the relative contributions of the direct and indirect pathways to behavioral responses by studying mice with a selective deletion of DARPP-32 in striatonigral or striatopallidal neurons. These studies are integrated in the model shown in figure 5.1 which provides a framework for studying how changes in striatal biochemistry could be translated into behavioral responses.

The utility of this model is exemplified by our results obtained with cocaine. In D1 receptor-expressing direct pathway neurons, acute cocaine treatment increased T34 phosphorylation and decreased T75 phosphorylation. This pattern of phosphorylation is expected to inhibit PP-1, enhance PKA signaling, and excite striatonigral neurons of the direct pathway. Cocaine caused a concomitant decrease in T34 phosphorylation and an increase in T75 phosphorylation in D2R neurons which has the effect of shutting down these neurons and inhibiting the indirect pathway. The simultaneous stimulation of the direct pathway and inhibition of the indirect pathway resulted in a robust induction of locomotor activity in response to cocaine in wild-type mice. Selective deletion of DARPP-32 in direct pathway neurons decreased the locomotor response to cocaine while deletion of DARPP-32 in indirect pathway neurons enhanced this response. Using this

model it may be possible to make testable predictions about the behavioral effects of drugs by examining the phosphorylation pattern of DARPP-32 in direct and indirect pathway neurons.

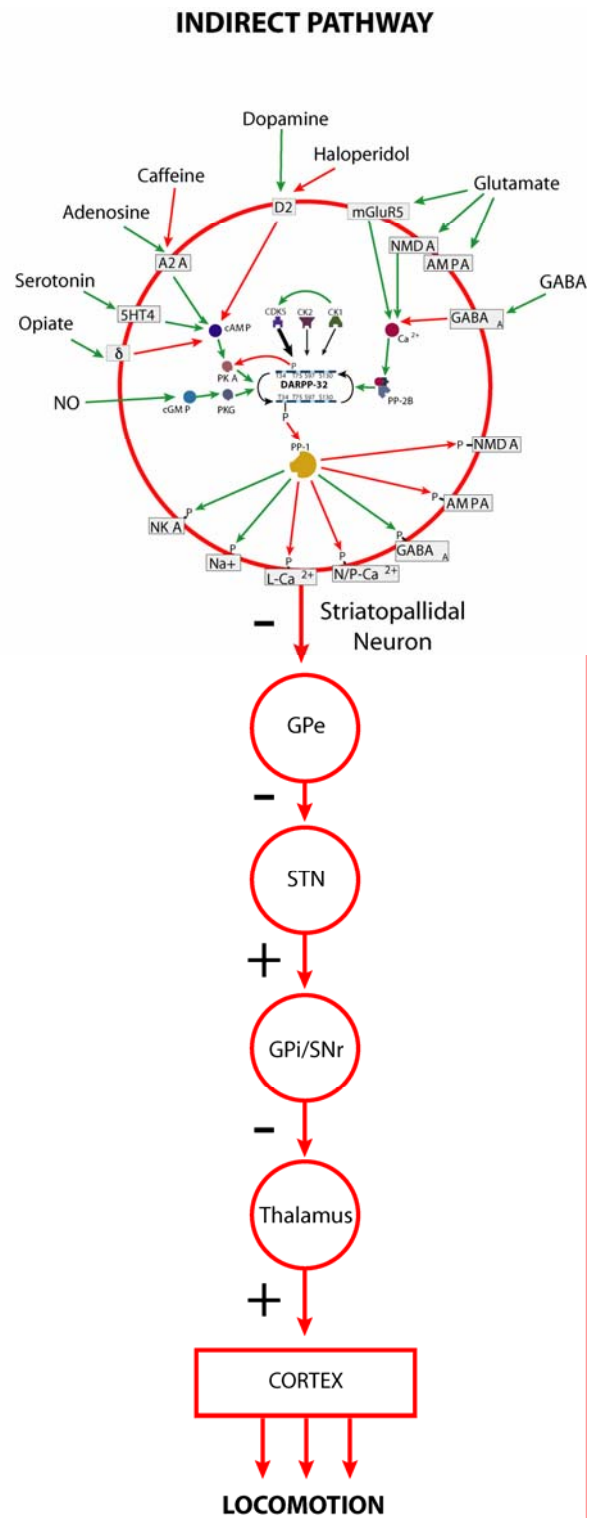
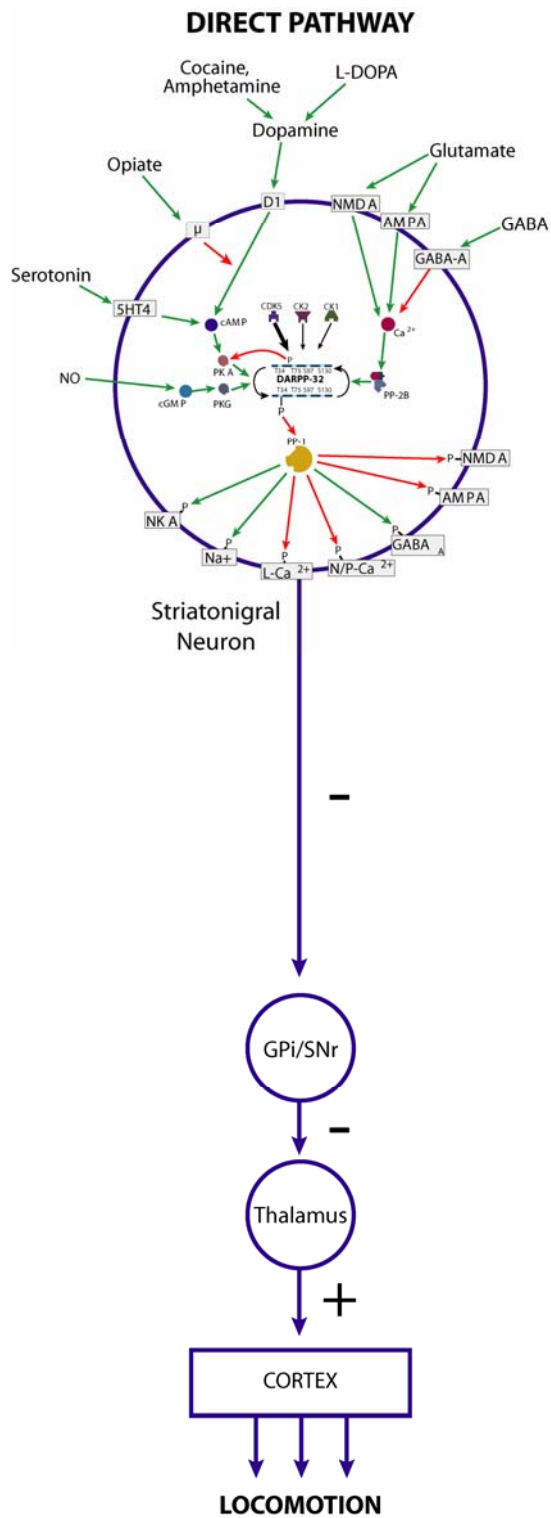
We have examined the initial signaling responses and the final behavioral responses to drugs which appear to be highly correlated, at least with respect to acute cocaine and haloperidol-mediated changes in locomotion. Thus far we have focused on the nigrostriatal dopamine pathway which has a well known role in mediating locomotor behavior. This dopaminergic pathway projects largely to the dorsal striatum where it is clear that D1R and D2R-expressing neurons have segregated projections comprising the direct and indirect pathways. The circuitry of this system is relatively well defined which makes it feasible to correlate changes in striatal biochemistry with clear-cut behavioral effects. However, the acute locomotor effects of psychostimulant drugs can be dissociated from their addictive effects and it has been well established that addiction results from changes in the VTA-nucleus accumbens reward circuitry (Kauer and Malenka, 2007). We have not yet examined behaviors which are mediated by the mesolimbic dopamine system which are important aspects of chronic drug use.

It has been shown that in the ventral striatum/nucleus accumbens, D1R and D2R are largely segregated to different sub-populations of neurons, however the projections of these neurons are more complex and overlapping than in the dorsal striatum (Lee et al., 2006; Lu et al., 1998). In addition, other dopamine receptors such as D3R are highly expressed in the ventral striatum which makes dopamine signaling more complex in this area. Furthermore, the division of the nucleus accumbens into the core and shell regions which play selective roles in different aspects of addiction and which have different

efferent projections further increases the complexity of this region. Studies have already demonstrated differences between D1R and D2R-expressing neurons in the nucleus accumbens with regard to changes in spines occurring with chronic drug use (Lee et al., 2006). It will be very interesting to study behaviors known to be indices of addiction and reward processes in our mouse models to understand how D1R and D2R-expressing neurons differentially contribute to these processes.

Figure 5.1 (on the following page) Striatonigral and Striatopallidal Neurons Integrate Afferent Signals and Modulate Behavior via the Direct and Indirect Pathways

Schematic of the direct and indirect pathways highlighting the integration of diverse afferent signals by striatonigral and striatopallidal neurons. Striatonigral neurons preferentially express type 1 dopamine receptors while striatopallidal neurons express type 2 dopamine receptors. There are additional differences between these neurons in the expression of adenosine, opioid, and metabotropic glutamate receptor subtypes. All of the signaling pathways shown here can regulate DARPP-32 phosphorylation which in turn inhibits PP-1 or PKA depending on the relative level of phosphorylation at T34 or T75. Phosphorylation at T34 turns DARPP-32 into a potent inhibitor of PP-1 which regulates the excitability of these neurons by modifying the state of phosphorylation of AMPA receptors, NMDA receptors, and ion channels. Activation of direct pathway striatonigral neurons stimulates locomotor behavior whereas activation of indirect pathway striatopallidal neurons inhibits locomotor activity. Minus signs indicate GABA-ergic inhibitory connections and plus signs indicate glutamatergic excitatory connections. Abbreviations: GPi, globus pallidus internal segment; SNr, substantia nigra pars reticulata; GPe, globus pallidus external segment; STN, subthalamic nucleus.



Future studies

In the studies presented here we have described the initial characterization of new mouse tools which allow the study of striatal signaling in a cell-type specific manner. We have used these tools to understand acute responses to drugs with respect to the direct and indirect pathways. Thus far we have studied agents which have previously been well characterized and therefore we were able to make predictions and test these using our model systems. Our results have largely confirmed the hypothesized mechanism of action of these drugs. It will be interesting to use these tools to look at new pathways or signaling molecules which are less well characterized. It would also be beneficial to study the regulation of other DARPP-32 phosphorylation sites in addition to T34 and T75, as less is known about how these sites might be differentially regulated in the two pathways.

Here we studied acute biochemical responses to drugs at a single time-point. It would be informative to study these signaling changes over time to gain a better understanding of the temporal regulation of these processes. It would be especially interesting to use these mice to understand the adaptive biochemical changes that occur in response to chronic drug use. Studies have shown that although acute cocaine increases DARPP-32 phosphorylation at T34 and decreases phosphorylation at T75, chronic cocaine use is associated with an increase in T75 phosphorylation and a decrease in T34 phosphorylation (Bibb et al., 2001; Nishi et al., 2000). It would be interesting to see what cell-type this increase in T75 phosphorylation occurs in and when the shift in DARPP-32 signaling takes place. Additionally, further studies using the conditional DARPP-32 knock-out mice could examine behavioral responses other than locomotion such as

conditioned place preference or behavioral sensitization to understand the relative contributions of the two cell-types to these correlates of drug addiction.

In addition we would also like to study the long-term changes in signaling that occur in neurodegenerative disease models such as Parkinson's disease. It would be informative to do a time course study after dopamine depletion and subsequent L-DOPA treatment to understand the temporal changes in signaling and adaptations that occur over time within the direct and indirect pathways. In the current study we have shown that the increase in T34 phosphorylation in response to chronic exposure to L-DOPA occurs selectively in D1 receptor-expressing direct pathway neurons. This has provided further evidence that signaling pathways downstream of the D1 receptor become sensitized following chronic exposure to L-DOPA. Previous studies have shown that dyskinesia is reduced in the total DARPP-32 knock-out mouse (Santini et al., 2007). It would be interesting to test whether the expression of L-DOPA-induced dyskinesia (LID) is reduced upon the selective loss of DARPP-32 in D1R direct pathway neurons. If this is the case then it might point to a possible target for intervention to prevent maladaptations in the D1R direct pathway which underlie LID.

Much remains to be understood about the direct and indirect pathways and how they contribute to striatal signaling and behavior. Even less is known about the other striatal cell populations such as striatal interneurons, the patch and matrix regions, and the subregions of the ventral striatum. Additional cell-type specific promoters selective for these compartments could be used to study responses in these cell populations. This selective cell-type targeted approach will be essential to provide a complete understanding of basal ganglia function in the normal and diseased states.

MATERIALS AND METHODS

All procedures involving animals were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Rockefeller University Institutional Animal Care and Use Committee.

Purification of DARPP-32 from mouse striatum

Six to eight week-old wild-type and DARPP-32 knock-out mice were sacrificed by focused microwave irradiation (FMI) (4.0-4.5 kW for 1.35 seconds using a small animal microwave, Muromachi Kikai, Japan). Brains were removed and bilateral striata were quickly dissected on a cold plate and stored in liquid nitrogen or at -80°C until processing. Striata from 2-3 wild-type or knock-out mice were pooled and sonicated in sodium citrate buffer (10mM Na₂HPO₄, 10mM citric acid, pH2.8, 0.2% Tween-20, phosphatase inhibitors 1:100 – Phosphatase Inhibitor Cocktail Sets I and II, Calbiochem). Samples were centrifuged at 15,000 rpm for 15 minutes to remove insoluble material. The supernatant was adjusted to pH 6.0 using 0.5M Na₂HPO₄ and 20ug of a non-phosphorylation state specific total DARPP-32 antibody was added (Hemmings and Greengard, 1986). Samples were rotated at 4°C for 1 hour and 200μL of protein G magnetic beads (Dynal Biotech) were added for an additional hour. Bound protein was isolated using a magnetic particle concentrator (Dynal Biotech), washed three times in 1x PBS, and eluted by boiling in 2x NuPAGE sample buffer. Ten percent of the final immunoprecipitated sample was run on an SDS-PAGE gel and analyzed by western blotting (see procedure below) using a phospho-specific polyclonal T34 antibody

(Valjent et al., 2005). The remaining sample was reduced with 10mM DTT at 70°C for 10 min. and then alkylated with 50mM iodoacetamide at room temperature in the dark for 30 min. Samples were separated on an SDS-PAGE gel and stained with GelCode blue coomaassie staining reagent (Pierce).

For quantitative mass spectrometry analysis of DARPP-32 phosphorylation in drug treated samples, wild-type C57Bl/6 mice were injected with saline, cocaine (30mg/kg, Sigma), amphetamine (7.5mg/kg, Sigma), or SKF 81297 (5mg/kg, Tocris) and sacrificed by FMI 15 minutes later. Brains were removed and striata were rapidly dissected and processed using the acid extraction/immunoprecipitation protocol described above.

Mass spectrometry analysis of DARPP-32 phosphorylation

Analysis of DARPP-32 phosphorylation by mass spectrometry using the HMS-MS approach was performed as previously described (Chang et al., 2004; Jin et al., 2005). Following SDS-PAGE of immunoprecipitated DARPP-32 samples and staining of the gel with GelCode blue reagent (Pierce), bands corresponding to DARPP-32 were excised from the gel. Protein in the gel band was oxidized with 0.01% performic acid reagent in acetonitrile/water (1:1) for 15 min. at room temperature, destained in acetonitrile/50 mM NH_3HCO_3 (1:1) and dried. Trypsin (250ng) in 50 mM NH_4HCO_3 was added, and the digestion was allowed to proceed for 6 hours at 37°C. For quantitative analysis of DARPP-32 phosphorylation in drug treated samples, propionylation and peptide extraction was achieved by sequentially treating the gel with the following solutions: (1) 50uL of 1% *d*₀- or *d*₁₀-propionic anhydride (Sigma) in acetonitrile/50mM NH_4HCO_3

(1:1); (2) 30uL of 1% *d*₀- or *d*₁₀-propionic anhydride in acetonitrile; (3) twice with 50uL of 0.1% TFA in acetonitrile/water (1:1); (4) 30uL of acetonitrile. The samples were sonicated for 15 min. in each solution, and the supernatant from each extraction step was combined and dried in a Speedvac.

Single-stage and MS/MS mass spectra were collected on a MALDI-QqTOF mass spectrometer and MALDI-ion trap mass spectrometer, respectively, both fitted with custom made ion sources (Krutchinsky et al., 2001; Krutchinsky et al., 2000). The MALDI matrix used was 2,5-dihydroxybenzoic acid (DHB) (Sigma). MS/MS spectra were collected for 0.5-2 min., using injection times of 100-1000ms, activation time of 300ms, and relative collision energy of 30-40%. The isolation width was 15-20 *m/z* units, where the range was set to include all isotope peaks of the hypothesized phospho-peptide pair of interest.

Generation of recombinant DARPP-32 protein and serine-to-alanine point mutant proteins

C-terminal 6xHis tagged mouse DARPP-32 cDNA was generated by PCR using a 6xHis tag reverse primer from a plasmid template containing mouse DARPP-32 cDNA. After verifying the correct DNA sequence, 6xHis tagged DARPP-32 was cloned into the pET-3a (Novagen) vector and transformed into BL21 (DE3) pLys E. coli (Novagen) for protein expression. Transformed cultures were grown at 37°C until they reached an optical density of 0.6. Cultures were induced with 1mM IPTG and incubated at 37°C for an additional 4 hours. Cultures were spun down at 6000 rpm for 15 minutes and resuspended in cold buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole).

Bacterial homogenates were lysed using a french press and lysates were centrifuged at 45,000 rpm for 45 minutes at 4°C. Supernatants were applied to an Ni-HiTrap FPLC column (Amersham Biosciences) washed, and bound protein was eluted by using a linear gradient starting with 100% Buffer A (300mM NaCl, 50 mM NaH₂PO₄, 20mM imidazole) and ending with 100% Buffer B (300mM NaCl, 50mM NaH₂PO₄, 200mM imidazole). Protein content of the eluted fractions was analyzed by SDS-PAGE followed by coomassie staining of the gel with GelCode blue reagent (Pierce). Fractions containing the largest amount of protein at 32kD (corresponding to DARPP-32) with the least amount of contaminating protein were pooled, concentrated to 1-2mg/mL and stored at -80°C until use.

Serine-to-alanine point mutant proteins for the S45, S52, and S192 residues were generated from the 6xHis tagged mouse DARPP-32 cDNA template using the Quickchange Site-directed mutagenesis kit (Stratagene). Point mutations were verified by DNA sequencing and 6xHis tagged point mutant S45A, S52A, and S192A protein was generated and purified as described above.

***In vitro* kinase assays**

To measure the ability of brain kinases to phosphorylate DARPP-32 protein *in vitro* we added ~5ug of wild-type, S45A, S52A, or S192A recombinant protein to a 50uL reaction mixture containing reaction buffer, ddH₂O, 5uL of ATP/MgCl₂ cocktail (Upstate #20-113), 3uL kinase, and 0.5mCi of ³²P ATP. Kinases, their corresponding reaction buffers, and co-factors were obtained from the following sources: PKA, purified enzyme previously described in (Kaczmarek et al., 1980), PKA reaction buffer (500mM HEPES,

100mM Mg(OAc)₂, 10mM EGTA final concentration); CamKIV, Upstate; CKI, New England Biolabs; CKII, New England Biolabs and Upstate; CamKII, Upstate; PKG, Calbiochem ; cdk5/p25, Upstate; GSK-3a, Upstate; MAPK 1 (Erk1), Upstate; p42 MAPK (Erk2), New England Biolabs; PKC, Upstate; Trk-B, Upstate; CamKI, Upstate; ROK α /ROCK-II, Upstate. Reactions were carried out at 30°C and 10uL aliquots of the reactions were removed at each time point. Reactions were stopped by boiling in 4x sample buffer and samples were stored at -20°C until use. Individual samples were run out on a 10.5-14% Tris-HCL SDS-PAGE gel (Criterion, Bio-Rad), coomassie stained with GelCode blue reagent (Pierce), and dried overnight. Dried gels were exposed to autoradiography film and bands corresponding to DARPP-32 protein were cut out and ³²P incorporation was measured on a Beckman LS 6500 scintillation counter.

Generation of tagged DARPP-32 BAC transgenic mice

D1R-DARPP-32/Flag and D2R-DARPP-32/Myc BAC mice were generated according to the GENSAT BAC modification protocol (Gong et al., 2002). Mouse DARPP-32 cDNA was amplified from a mouse brain cDNA library (Stratagene). For the D1R-DARPP-32/Flag mouse, a c-terminal Flag tag (DYKDDDDK) was added to the mouse DARPP-32 cDNA by PCR and the Flag tagged DARPP-32 construct was inserted into the S247 shuttle vector using the AscI/NotI restriction sites. A SmaI site was placed 5' to DARPP-32 and a 532bp segment of the Drd1a promoter corresponding to the region right before the start codon (D1 Abox) was cloned into the shuttle vector using the AscI and SmaI sites. This modified shuttle vector was recombined into the bacterial artificial chromosome (BAC) containing the full Drd1a promoter. Positive co-integrates

were double selected by chloramphenicol and ampicillin and verified by PCR. BAC DNA was then purified and injected into pronuclei from FVB/N oocytes. Detailed protocols for these procedures can be found at www.gensat.org.

To generate the D2R-DARPP-32/Myc mouse, c-terminal Myc tagged (SEQKLISEEDL) mouse DARPP-32 cDNA was inserted into the S247 vector using Asc1/Not1. A Swa1 site was placed 5' to the DARPP-32 cDNA and a 484bp segment of the Drd2 promoter corresponding to the region right before the start codon (D2 Abox) was cloned into the vector using Asc1/Swa1. Venus fluorescent protein cDNA was excised from the vector and replaced with ECFP cDNA using Nco1/Sal1. This modified vector was recombined in to the Drd2 BAC and prepared as described above.

Litters were screened for positive transgenics by PCR genotyping from tail DNA using the primers listed in the following section. Positive mice were backcrossed 10 generations to the C57Bl/6 background. D1R-DARPP-32/Flag and D2R-DARPP-32/Myc were then crossed to obtain double transgenic mice expressing both Flag and Myc tagged DARPP-32. Male mice homozygous for Flag and/or Myc were generated by interbreeding double transgenic males and females. These homozygous male mice were subsequently bred to C57Bl/6 females and the heterozygous double transgenic offspring were used for biochemical experiments.

PCR genotyping from tail DNA

A 0.5cm piece of tail was cut from three week old mice. Tails were lysed overnight in 500ul of lysis buffer (For 500ml: 50mL 1M Tris, pH 8.8; 25mL of 4M NaCl; 500uL of 1M CaCl₂; 5mL of 20% SDS; 419.5mL ddH₂O). Lysates were spun down for

10 min. at 13,000rpm and the supernatant was transferred to a new tube. 500uL of isopropanol was added to the supernatant, mixed gently, and centrifuged for 5 minutes at 13,000rpm. The supernatant was discarded and 1mL of 70% ethanol was added to the DNA pellet, mixed gently and centrifuged for 3 minutes at 13,000rpm. The supernatant was discarded and DNA pellets were dried for 1 hour at 50°C. Pellets were resuspended in 150uL of TE buffer (For 500mL: 5mL of 1M Tris, pH 8.0; 1mL of 0.5M EDTA, pH 8.0; 494mL ddH₂O).

For PCR genotyping, 0.25uL of purified DNA was added to 8-strip reaction tubes containing 25uL of Platinum PCR Supermix (Invitrogen) plus 2.5pmol of forward and reverse primers (see below).

Genotyping PCR primers:

<u>D1R-DARPP-32/Flag mice</u>	FOR: AGGTCCTGAAAGGCAGCAG REV: CTTATCGTCGTCGTCCTTG TAGTC
<u>D2R-DARPP-32/Myc mice</u>	FOR: GAGGATGAAGAGGAGGACGA REV: CAGAAATCAATTTTTGTTCAGAGG
<u>D1R/D2R-DARPP-32 mice</u>	Two separate PCR reactions using the Flag and Myc primers listed above
<u>Floxed DARPP-32 mice</u>	FOR: CTAGTTGTCCTAGGTGTCCTGTGCTCTGTC REV: CCCTTCAACCTACTGTTAAGACCCTAGCTG
<u>D1R-Cre mice</u>	FOR: AGGGGCTGGGTGGTGAGTGATTG REV: CGCCGCATAACCAGTGAAACAGC
<u>D2R-Cre mice</u>	FOR: AGCATGCCTTGAAAACACTCCTG

REV: CACCGGCATCAACGTTTTCTTTTC

D1R-D32-KO mice

Two separate PCR reactions using the Floxed DARPP-32 and D1R-Cre primers listed above

D2R-D32-KO mice

Two separate PCR reactions using the Floxed DARPP-32 and D2R-Cre primers listed above

In situ hybridization

12µm thick sagittal sections of fresh frozen brain from wild-type, D1R-DARPP-32/Flag and D2R-DARPP-32/Myc single transgenic mice were processed for in situ hybridization with a ³⁵S labeled antisense riboprobe against full-length GFP using a protocol described previously (Svenningsson et al., 1998a). Briefly, slides were fixed in 4% paraformaldehyde, and washed twice in 4x SSC buffer. Sections were acetylated for 10 minutes, dehydrated in a series of ethanols, and incubated in a humidity chamber overnight at 55°C with 2 x 10⁶ cpm of probe per slide mixed in hybridization buffer. The next day slides were washed 4 times in 4xSSC and incubated with RNase A for 15 minutes at 37°C. Slides were then washed with a series of SSC buffers of decreasing concentration starting with 2x SSC and ending with 0.1x SSC. Sections were dehydrated, dried, and placed on Kodak MR film for 10 days.

To visualize GFP labeling at the cellular level, slides were dipped in Kodak NTB emulsion and left in the dark for 6 weeks. Slides were developed and counterstained with cresyl violet. Pictures of labeled cells were taken on a Zeiss microscope using a 63x water objective and AxioVision software.

Fluoro-Gold retrograde labeling

6-8 week old D1R-DARPP-32/Flag and D2R-DARPP-32/Myc mice were anesthetized with a 1:5 dilution of sodium pentobarbital, 9ul/gram i.p. The mice were placed in a stereotaxic apparatus (Kopf) and injected bilaterally with 1.0ul of 0.8% Fluoro-Gold (Fluorochrome, LLC) into the SNpr at a rate of 250nl/minute. Coordinates for injection into the SNpr were A/P -2.9, M/L +/- 1.5, D/V -4.7 from Bregma. Mice were perfused 5 days following the injection.

Immunohistochemistry

Mice were perfused transcardially with 1x PBS and 4% paraformaldehyde. Brains were removed and post-fixed in 4% paraformaldehyde for one hour. Brains were cryoprotected in sucrose and placed in a mounting block with Neg50 mounting medium (Richard Allen) and frozen at -80°C until sectioning. 12µm coronal sections were blocked for one hour in 2% normal serum in 1x PB with 0.25% Triton X-100 (PB-T). Sections were incubated overnight at 4°C with antibodies against Fluoro-Gold (rabbit 1:3500, Chemicon), GFP (goat 1:1000, gift of Dr. Myriam Heiman), Flag (mouse 1:2000, Sigma), Myc (goat 1:2500 or 1:10,000, Novus), Tyrosine hydroxylase (TH) (rabbit 1:1000, Chemicon), GFAP (mouse 1:1000, Abcam), DARPP-32 (rabbit 1:2000 Novus), or NeuN (mouse 1:5000, Chemicon). Slides were washed and incubated with fluorescent secondary antibodies (1:200) at room temperature for one hour (Fluoro-Gold and TH, Cy2 conjugated Donkey anti-Rabbit; GFP, Cy3 conjugated Donkey anti-Goat; GFAP, Cy3 conjugated Donkey anti-Mouse; Flag, FITC conjugated Donkey anti-Mouse; Myc (Fig. 2c) Cy3 conjugated Donkey anti-Goat; Myc (Fig. 2d) Rhodamine-Red conjugated

Donkey anti-Goat; DARPP-32, Cy2 conjugated Goat anti-Rabbit; NeuN, Cy3 conjugated Goat anti-Mouse; all from Jackson ImmunoResearch). Fluorescent images were taken on a Zeiss confocal microscope using 40x and 63x oil objectives.

Flag and Myc-tagged DARPP-32 immunoprecipitations

Bilateral striata from one mouse (*in vivo* experiments) or six striatal slices (slice experiments) were sonicated in 500ul IP lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, Complete Mini protease inhibitors (Calbiochem), and Halt phosphatase inhibitors (Pierce)). Homogenates were spun down for 20 minutes at 4°C at 13K rpm. Soluble supernatant was removed and 50 uL/IP of washed EZView Red anti-Flag M2 affinity gel (Sigma) was added along with 45 uL/IP of anti-Myc antibody (Novus Biologicals #NB600-338) coupled to magnetic beads (Dynabeads M-280 Tosylactivated, Invitrogen #142-03). The homogenate/antibody mixture was gently rotated overnight at 4°C. The Myc antibody was coupled to the Dynabeads following the protocol in the product manual; 3ug of Myc antibody was added for every 5ul of magnetic beads.

Following the overnight incubation, the homogenate/antibody mixture was placed on a magnetic particle concentrator (MPC, Invitrogen #120-20D) for 1-2 minutes to separate the Myc magnetic beads from the Flag affinity gel. The supernatant containing the unbound homogenate and Flag affinity gel was removed to a new tube and kept on ice. The Myc magnetic beads were washed three times in 1x PBS using the MPC to separate the beads each time. After the final wash, 30uL of non-reducing sample buffer (Pierce, #1859594) was added and the beads were boiled for two minutes. Eluted supernatants

were removed from the beads and added to 7ul of sample buffer reduced with B-mercaptoethanol. Samples were stored at -80°C until used for immunoblotting.

Flag IP/homogenate mixtures were spun down for 30 seconds at 13K rpm and unbound supernatant was removed to a fresh tube. This represented the total striatum sample. 1ul of this unbound supernatant was used in a BCA protein assay (Pierce) to determine protein concentration for the total striatum sample. Flag affinity gel was washed three times in 1x PBS and applied to the MPC to remove any residual magnetic beads. Flag IP's were eluted and stored at -80°C as described above.

In addition to the anti-Myc-coupled Dynabeads, anti-Myc-coupled agarose (Novus Biologicals #NB600-342) was also used for the IP's with identical results. For this protocol, Myc-agarose was incubated with the striatal homogenate for 6 hours at 4°C, and the Flag-affinity gel was subsequently incubated with the unbound supernatant from the Myc IP overnight at 4°C.

Immunoblotting

Samples were loaded onto 10.5-14% Tris-HCl Criterion gels (Bio-Rad), separated by electrophoresis and transferred to PVDF membranes. Membranes were blocked for one hour in 5% dry milk in TBS with 0.15% Tween-20 and incubated overnight at 4°C with phospho-specific primary antibodies against T34 (1:5000, (Valjent et al., 2005) or T75 (1:7500, (Bibb et al., 1999), or total protein primary antibodies against DARPP-32 (1:10,000, (Hemmings and Greengard, 1986), Flag (1:4000, Sigma), Myc (1:500, Cell Signaling), Tyrosine hydroxylase (1:1000, Chemicon) or Actin (1:15,000, Abcam). Antibody binding was revealed by HRP conjugated goat anti-rabbit, goat anti-mouse, or

donkey anti-goat IgG (1:10,000) and the ECL detection method (PerkinElmer) followed by exposure on Kodak BioMax film. Membranes blotted previously with phospho-T34 and -T75 antibodies were stripped (Chemicon, Re-blot plus strong stripping buffer) and re-probed using a monoclonal antibody against DARPP-32 which is not phosphorylation state specific (Hemmings and Greengard, 1986). Quantification of bands was done by densitometry, using NIH Image software, version 1.52.

Striatal slice preparation

6-8 week old D1R/D2R-DARPP-32 BAC mice were killed by decapitation. The brains were rapidly removed and placed in ice-cold, oxygenated Krebs-HCO₃⁻ buffer (124 mM NaCl, 4 mM KCl, 26 mM NaHCO₃, 1.5 mM CaCl₂, 1.25 mM KH₂PO₄, 1.5 mM MgSO₄ and 10 mM d-glucose, pH 7.4). Coronal slices (350 µm) were prepared using a vibrating blade microtome, VT1000S (Leica Microsystems). Striata were dissected from the slices in ice-cold Krebs-HCO₃⁻ buffer. Each slice was placed in a polypropylene incubation tube with 2 ml fresh Krebs-HCO₃⁻ buffer containing adenosine deaminase (10 µg/ml). The slices were preincubated at 30 °C under constant oxygenation with 95% O₂/5% CO₂ for 60 min. The buffer was replaced with fresh Krebs-HCO₃⁻ buffer after 30 min of preincubation. Slices were treated with drugs as specified in each experiment. All drugs were obtained from Sigma-Aldrich. After the drug treatments, slices were transferred to Eppendorf tubes, frozen on dry ice, and stored at -80 °C until processed for western blotting.

In vivo drug treatments – D1R/D2R-DARPP-32 BAC mice

6-8 week old male D1R/D2R-DARPP-32 double transgenic mice were used for all experiments. Mice were injected i.p. with drug or vehicle and sacrificed 15 minutes later using focused microwave irradiation. Brains were removed and striata were rapidly dissected and frozen at -80°C until processing. Drugs used: SKF81297 5mg/kg (Tocris); Quinpirole 0.2 mg/kg, Caffeine 7.5 mg/kg, Cocaine 25 mg/kg, Haloperidol 1mg/kg, Clozapine 5 mg/kg (all from Sigma).

Unilateral 6-OHDA lesions and L-DOPA treatment

The procedure used for generating unilateral 6-OHDA lesions in mice has been previously described (Santini et al., 2007). Mice were anesthetized with a mixture of midazolam 5 mg/ml (Alpharma) and Hypnorm (Janssen Pharmaceutica) and mounted in a stereotaxic frame (David Kopf Instruments) equipped with a Cunningham mouse-adaptor (Stoelting). 6-OHDA-HCl (Sigma) was dissolved in 0.02% ascorbic acid in saline at the concentration of 3.0 µg of freebase 6-OHDA/µL. Mice received unilateral injections (2 x 2 µl) of vehicle or 6-OHDA into the right striatum at the following coordinates according to the mouse brain atlas: anteroposterior +1.0 mm, mediolateral – 2.1 mm, dorsoventral –3.2 mm; and anteroposterior +0.3 mm, mediolateral –2.3 mm, dorsoventral –3.2. Each injection was performed at a rate of 0.5 µL/min using a glass capillary with an outer diameter of ~50 µm attached to a 10 µL Hamilton syringe. After the injection, the capillary was left in place for an additional 3 min before slowly retracting it. Mice recovered in their home cage for 3 weeks before beginning drug treatment. Lesions were assessed at the end of the experiments by determining the striatal

levels of tyrosine hydroxylase (TH) using Western blotting. Only mice with greater than a 75% reduction in striatal TH compared to the unlesioned side were used in the final analysis.

Three weeks post-lesion, mice were injected with either saline or a combination of L-DOPA (20mg/kg, Sigma) and the peripheral DOPA decarboxylase inhibitor benserazide (12mg/kg, Sigma) daily for 10 days. On the 11th day, mice were injected with saline or L-DOPA/benserazide and sacrificed 30 minutes later by focused microwave irradiation. Brains were removed and unilateral striata were dissected. Left and right sides of the striatum were kept separate and analyzed individually.

Tyrosine hydroxylase DAB immunohistochemistry

Unilateral 6-OHDA injected mice were perfused 3 weeks following the injection as described above. 20µm coronal sections were washed in 1x PB and incubated for 10 min. in hydrogen peroxide (180uL of 30% H₂O₂/10mL 1x PBS). Sections were washed in 1x PB with 0.1% Triton-X 100 (PB-T) and blocked for one hour at room temperature in 2% normal goat serum in PB-T. Sections were then incubated overnight at 4°C with a tyrosine hydroxylase antibody (1:1000, Chemicon). The next day, slides were washed in PB-T and incubated with biotinylated anti-rabbit secondary antibody (Vector Laboratories) for one hour at room temperature. Sections were washed again in PB-T and incubated with Vectastain ABC reagent (Vector Laboratories) for one hour at room temperature. Sections were washed in 1x PBS, incubated for 5-10 minutes in DAB reagent (Sigma), washed again in 1x PBS, and coverslipped using Permount mounting medium (Fisher).

Generation of Floxed DARPP-32 mice

Floxed DARPP-32 mice were generated by Cell & Molecular Technologies, Inc. Briefly, a targeting vector was generated in which loxP sites were inserted surrounding exons 1-4 of the mouse DARPP-32 gene. An FRT flanked Neo cassette was inserted between exon 4 and the 3' loxP site. The targeting vector included right and left homology arms which were used to target the construct to the endogenous DARPP-32 locus. The targeted construct was electroporated into ES cells derived from C57Bl/6 mice. Neomycin resistant clones were screened by southern blot for correct homologous recombination using a 3' external and a 5' internal probe and EcoRV digestion. The Neo cassette was excised *in vitro* by expression of FLPe recombinase to generate the final conditional floxed allele. A southern blot strategy was used to detect cells with correct excision of the Neo cassette. These cells were then used to generate chimeras by injection into C57Bl/6 blastocysts using standard techniques. Germ line transmission was confirmed by PCR genotyping and heterozygous floxed DARPP-32 mice were interbred to generate homozygous floxed DARPP-32 mice. These mice were crossed to either D1R-Cre or D2R-Cre mice to generate conditional DARPP-32 knock-out mice as discussed below.

Generation of conditional DARPP-32 knock-out mice

D1R- and D2R-Cre (ER44) lines were generated by the GENSAT project and characterized previously (Gong et al., 2007b). We obtained male breeders for both of these lines and bred them with homozygous floxed DARPP-32 female mice. We bred the heterozygous floxed DARPP-32 D1R- or D2R-Cre positive offspring back to

homozygous floxed DARPP-32 mice. The resulting homozygous floxed DARPP-32 D1R- or D2R-Cre positive mice were bred again to the homozygous floxed DARPP-32 mice. The resulting offspring were all homozygous floxed and positive or negative for D1R- or D2R-Cre. Mice negative for Cre were considered wild-types and mice positive for Cre were considered D1R- or D2R-D32 conditional knock-outs. We continued this breeding strategy for subsequent generations and mice which were backcrossed at least 4 generations (>90% C57/Bl6) were used in the behavioral analyses.

Open field locomotor behavior

Mice were brought in to the testing room in their home cages at least 1 hour prior to testing. Female and male mice were kept separate and tested on different days. Mice were placed in the open field chambers which consisted of 38cm (L) x 25cm (W) x 17cm (H) white plastic boxes covered with a clear plexiglass lid left open at the edge for ventilation. Four mice were tested simultaneously using four identical side-by-side chambers. Locomotor activity was recorded using an overhead Panasonic digital camera connected to a Dell computer. The mice were tracked using EthoVision software version 3.1 (Noldus) which sampled the x/y location of the mice in the chambers 6 times per second and recorded the total distance traveled (in centimeters) by each mouse in a given time period.

For the basal measurement we recorded open field locomotor activity of the mice for 60 minutes and reported the distance traveled in 3 minute bins. After testing, mice were removed from the chambers and returned to their home cages. 7 days later, mice were brought back into the testing room and allowed to habituate for at least one hour.

Mice were first weighed and then reintroduced to the testing chambers where locomotor activity was recorded for 21 minutes. After 21 minutes mice were injected i.p. with either vehicle, cocaine (20 mg/kg, Sigma), or haloperidol (1 mg/kg, Sigma). Recording was resumed after drug injection for an additional 60 minutes after which mice were returned to their home cage.

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