DOPAMINE DEPLETION ALTERS THE BALANCE BETWEEN CA^{2+}/CALMODULIN-DEPENDENT PROTEIN KINASE II AND PROTEIN PHOSPHATASE I

By

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DEDICATION

To my husband Steven Brown

and my parents Robert and Susan Gragg

who have given me endless encouragement and support.

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INTRODUCTION

Parkinson's Disease

General Overview

Parkinson's disease (PD) is a progressive neurodegenerative disorder present in ~1-2% of the population in the United States over 60 years of age, with symptoms that include a progressive decline in motor function, tremor, bradykinesia, rigidity, and postural instability. PD is characterized by the preferential loss of the nigrostriatal dopaminergic neurons, whose axons terminate within the striatum and release dopamine (DA) onto medium spiny neurons (MSNs). The neuropathological findings in PD include a decrease in spine density of striatal MSNs, and patients with PD display a decreased MSN dendrite number and length (McNeill et al., 1988; Zaja-Milatovic et al., 2005).

Concurrent with the morphological changes in MSNs, nigrostriatal dopaminergic neurons undergo a variety of changes including the development of cytoplasmic, eosinophilic inclusions known as Lewy bodies (soma) or Lewy neurites (neuronal processes), and prematurely die (Braak and Braak, 2000). Lewy bodies are composed of polymerized α-synuclein, abnormally phosphorylated neurofilaments, ubiquitin, proteasome subunits, heat-shock proteins, and neurofilaments (Lotharius and Brundin, 2002). To definitively diagnose PD, a combination of clinical evaluation and post-

mortem confirmation of the presence of Lewy bodies is required. As post-mortem delay affects the stability of many proteins, the use of human tissue in studies examining the biochemical mechanisms underlying progression of PD may be difficult to interpret.

Known Mechanisms of PD

Both genetic and environmental factors influence the development of PD. Mutations in the following genes have been linked to either sporadic or familial PD cases: UCH-L1 (ubiquitin carboxyl-terminal hydrolase L1), PINK1 (PTEN-induced kinase-1), DJ1, PARK 2 (parkin), LRRK 2 (leucine-rich repeat kinase 2), and SNCA (α-synuclein) (Warner and Schapira, 2003; Farrer, 2006). It is unclear how each of the mutations leads to the PD phenotype, but altered protein processing appears to be a common theme. First, mutant α-synuclein more readily oligomerizes and accumulates at possibly toxic doses (Moore et al., 2005). Second, mutations that reduce enzymatic activity of parkin, a ubiquitin E3 ligase, may contribute to the accumulation of putative parkin substrates, such as α-synuclein, CDCrel-1, CDCrel-2, Pael-R, cyclin-E, p38/JTV-1, FBP-1, and EPS-15 (Moore et al., 2005; Fallon et al., 2006; Savitt et al., 2006).

Environmental factors also play a role in the development of many cases of PD. Exposure to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), originally an inadvertent byproduct of meperidine, can cause parkinsonism in humans within 7-14 days. After systemic injection, MPTP is converted to MPP^+ by monoamine oxidase B. MPP^+ enters dopaminergic neurons via the DA transporter, and accumulates in the mitochondria. An inhibitor of mitochondrial complex I, MPP^+ increases free radical release and oxidative damage, ultimately resulting in the degeneration of DA neurons.

Consistent with this finding, exposure to other mitochondrial complex I inhibitors such as various herbicides, pesticides, and natural compounds have been reported to increase PD risk (Greenamyre et al., 2001; Warner and Schapira, 2003). For many sporadic cases of PD, no known causative factor has yet been identified.

Current Therapies

The most commonly used therapy for PD patients attempts to replace DA by providing the direct precursor to DA, L-DOPA (Schapira, 2005). Treatment with L-DOPA is effective in controlling motor symptoms of PD, but typically becomes less effective with long-term use and can eventually cause unwanted motor complications, such as dyskinesias and a "wearing-off" phenomenon (Martignoni et al., 2003). Wearing-off is a phenomenon that may be related to the pulsatile administration of L-DOPA, and describes the re-emergence or worsening of parkinsonian symptoms prior to taking the next scheduled dose of L-DOPA. Unfortunately, the numerous side effects of dopaminergic agonists may complicate clinical management.

Surgical implantation of a 'pacemaker' device in the subthalamic nucleus is an increasingly used therapeutic strategy to suppress the hyperactivity of this brain region. High frequency stimulation of the subthalamic nucleus dramatically, but not completely, reduces many of the motor symptoms associated with PD (Benabid et al., 2005). While this type of deep brain stimulation results in an improved quality of life for many patients, there are potential surgical complications, along with reports of neuropsychological alterations (Volkman, 2004; Benabid et al., 2005). The long-term effectiveness of this type of intervention is unknown, and it may not halt the cellular and

subcellular pathologies that are hallmarks of PD.

Model System: Unilateral 6-OHDA Lesioned Rat

Several animal models have provided invaluable insight into the mechanisms underlying PD and were considered for use in the experiments described in this dissertation. Many of these other models use toxins such as reserpine, methamphetamine, MPTP, or rotenone to either temporarily or permanently deplete dopaminergic neurons in the rat (Beal, 2001; Betarbet et al., 2002). However, the most widely used model is the unilaterally 6-OHDA (6-hydroxydopamine) lesioned rat. This model is generated by unilateral injection of the neurotoxin 6-OHDA into the substantia nigra, and has three main advantages over other models. First, degeneration of the nigrostriatal dopaminergic neurons is confined to one hemisphere, leaving the intact contralateral hemisphere as an internal control. Second, the decrease in MSN spine density on the side ipsilateral to the 6-OHDA lesion resembles that observed in human PD patients (McNeill et al., 1988; Stephens et al., 2005; Zaja-Milatovic et al., 2005). One drawback to using this model is that the DA neuron degeneration occurs within 10-14 days after injection of L-DOPA, more rapid than the slow nigrostriatal degeneration recorded in PD. In addition, most published reports that use 6-OHDA lesioned rats use the rats at a relatively young age (typically 3-6 months of age), within a few weeks of the lesion surgery. Thus, this short-term lesion in younger animals may not accurately recapitulate the complex interaction between gradual loss of DA and aging in human PD. Finally, the use of an animal model such as the 6-OHDA lesioned rat, allows examination of the evolving biochemical and structural changes at many different time points

following DA depletion, an undertaking not possible in studies using human postmortem tissue.

Basal Ganglia

General Overview: Function and Anatomy

The major function of the basal ganglia is to process incoming signals from throughout the cortex, and funnel the information through the thalamus prior to returning to various regions of the frontal lobe of the cortex. This cortical-basal ganglia circuit allows the execution, coordination, and control of voluntary movement. Four parallel circuits exist within the basal ganglia that control different sets of functions. Some functions regulated by the basal ganglia circuits include motivation / emotion (limbic loop), coordination of extrapersonal space maps (association loop), limb movements (skeletomotor loop), and eye movements (occulomotor loop). Diseases that impact the basal ganglia, such as PD, can modify the normal functioning of any of these circuits. The motor and limbic circuits are particularly affected in PD, and thus result in a characteristic behavioral phenotype that includes akinesia, bradykinesia, resting tremor, shuffling gait, and rigidity (Gelb et al., 1999; Suchowersky et al., 2006).

The striatum is the major input region of the basal ganglia that processes signals from other parts of the brain. Roughly 95% of the neurons within the striatum are MSNs that synthesize and release the inhibitory neurotransmitter, γ-aminobutyric acid (GABA, see Fig. 1). The remaining 5% of striatal neurons are aspiny interneurons, which contain acetylcholine, somatostatin, NADPH-diaphorase, GABA, parvalbumin, or calretinin.

Cortical and thalamic glutamatergic neurons provide the major excitatory inputs into the three divisions of the striatum: the caudate nucleus, the putamen, and the nucleus accumbens (Figs. 1, 2). Corticostriatal glutamatergic afferents typically terminate on the MSN spine heads, forming asymmetric synapses as defined by the presence of the postsynaptic density (PSD) (Arbuthnott et al., 2000). The dopaminergic innervation from the substantia nigra pars compacta is thought to function by modulating both the excitatory glutamatergic input, as well as the output of the striatum.

Output nuclei of the basal ganglia transmit signals to the thalamus, which in turn communicates these signals to different areas of the frontal cortex. Output nuclei include the internal segment of the globus pallidus, ventral pallidum, and the substantia nigra pars reticulata. The neurons within these nuclei synthesize and release GABA, and thus the major output from the basal ganglia is inhibitory.

The intrinsic nuclei make connections and relay information from the input nuclei primarily to other regions of the basal ganglia. The four intrinsic nuclei are the external segment of the globus pallidus, the subthalamic nucleus, the substantia nigra pars compacta, and the ventral tegmental area. The neurons within these nuclei synthesize and release a variety of neurotransmitters, such as GABA (external segment of the globus pallidus), glutamate (subthalamic nucleus), and dopamine (substantia nigra pars compacta and ventral tegmental area).

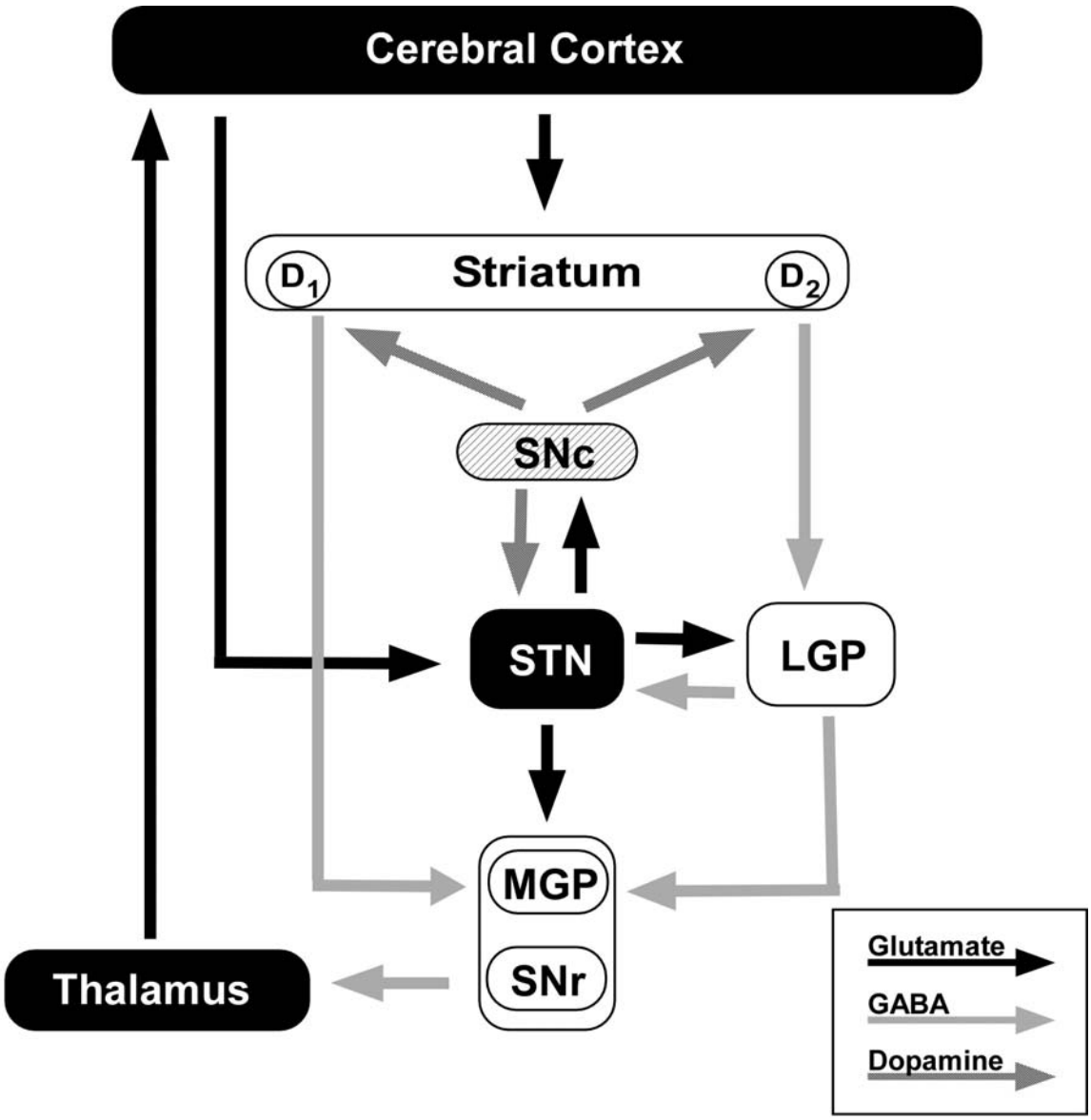

Figure 1. Schematic diagram illustrating the functional connections of the basal ganglia.
MGP: medial globus pallidus; LGP: lateral globus pallidus; SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; STN, subthalamic nucleus. The different colors of the boxes and arrows indicate the neurotransmitters (glutamate, GABA, or dopamine) released by neurons in each specific anatomical region. (adapted from Blandini et al., 2000)

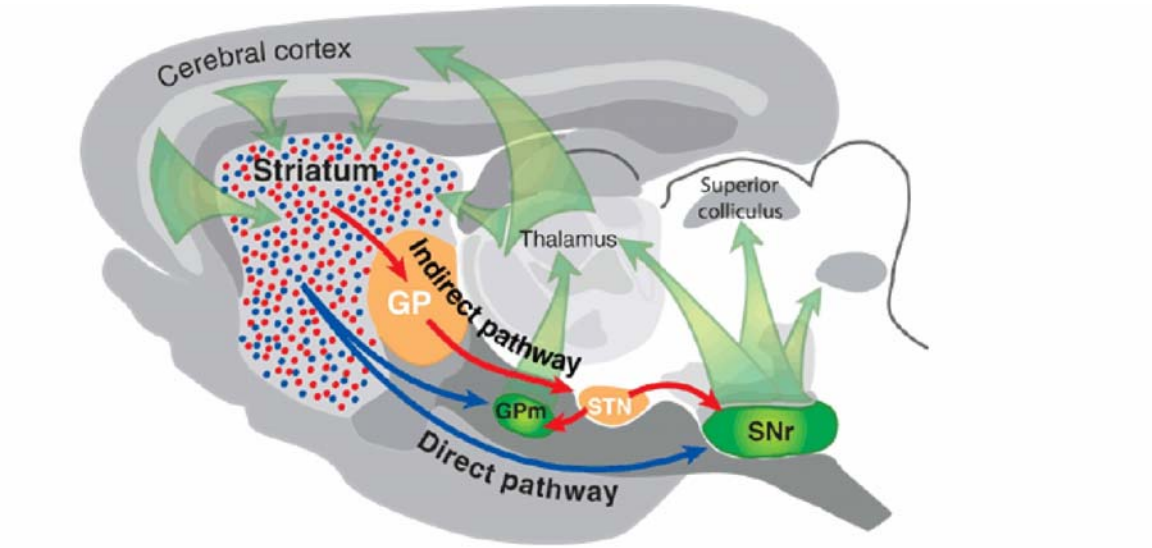

Figure 2. Functional anatomy of the basal ganglia.
Both the cerebral cortex and thalamus provide excitatory, glutamatergic input to the striatum (green arrows). The medial globus pallidus (mGP) and the substantia nigra pars reticulata (SNr) provide the major inhibitory, GABAergic output from the basal ganglia to the thalamus. This diagram indicates how striatal neurons either directly or indirectly modulate the output of the GPm and SNr. The striatal direct pathway neurons send projections (blue arrows) directly to the GPm and SNr. Striatal indirect pathway neurons (red arrows) send projections to the globus pallidus (GP), which in turn projects to the subthalamic nucleus (STN). STN neurons project to the GPm and SNr. (adapted from Gerfen, 2006)

Striatum: Direct and Indirect Pathways

Striatal MSNs are divided into two different subtypes that project either directly or indirectly to the output nuclei of the basal ganglia (Fig. 2). The direct pathway MSNs contain substance P and dynorphin in addition to GABA, and project directly to the substantia nigra pars reticulata. Indirect pathway MSNs contain enkephalin in addition to GABA, and project to other intrinsic nuclei which in turn communicate information to the output nuclei. PD causes an imbalance between the normal signaling through the direct and indirect pathways, in favor of the indirect pathway (Blandini et al., 2000) (Fig. 3). Mechanisms leading to this imbalance are discussed in depth in the later section on dopamine receptors (p. 15). This imbalance results in excessive excitation of the output nuclei, inhibiting thalamic signaling to the frontal cortex. Overall, the relatively enhanced signaling via the indirect pathway reduces motor-dependent behavior, producing the hypokinetic state observed in PD.

6-OHDA Lesioned Rat: Behavior

Systemic administration of L-DOPA exposes receptors in both the intact and lesioned hemispheres to DA. Thus, DA receptors in the lesioned hemisphere are supersensitive to this DA and are no longer in balance with the opposite, intact hemisphere. Therefore, unilaterally 6-OHDA lesioned rats with a greater than ~85% reduction in striatal tyrosine hydroxylase (TH) that receive an injection of L-DOPA will rotate in a direction contralateral to the lesioned hemisphere (Schwarting and Huston, 1996; Lundblad et al., 2002). The final result of this imbalance is an elevated inhibitory GABAergic output from the basal ganglia to the thalamus and motor cortex in only the

lesioned hemisphere (Schwarting and Huston, 1996; Blandini et al., 2000). The motor cortex regulates motor responses in limbs on the opposite side of the body. Therefore, the motor cortex ipsilateral to the lesion is less active, resulting in decreased contralateral limb movement and contralateral rotations in response to L-DOPA challenge (Schwarting and Huston, 1996). Chronic, intermittent L-DOPA administration (every 12 hrs.)

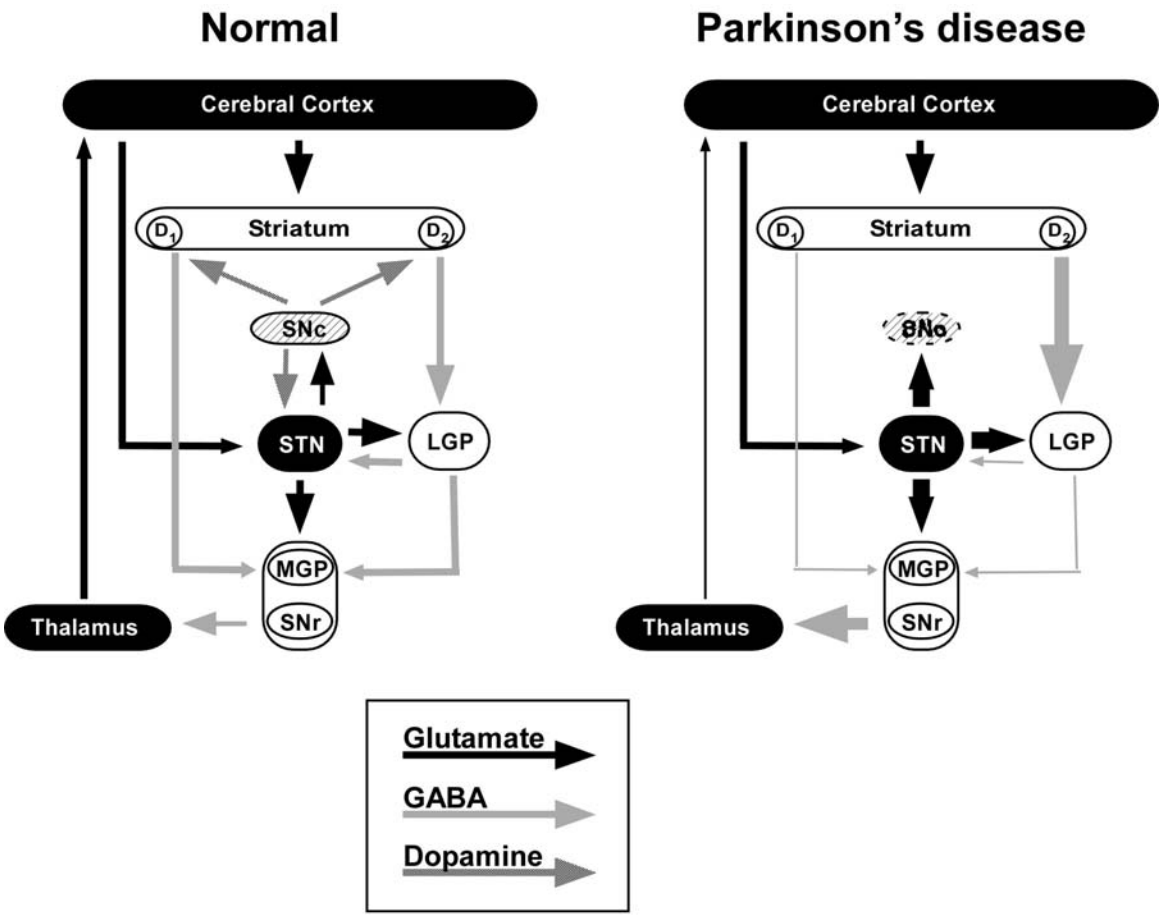

Figure 3. Schematic diagram illustrating the changes in functional connections of the basal ganglia.
Basal ganglia functional connections in the normal brain (left) and in a parkinsonian brain (right). The degeneration of nigrostriatal dopaminergic neurons in Parkinson's disease is depicted by the loss of these neurons in the figure on the right. The arrow thickness indicates the relative degree of neurotransmitter release from each region, in both the normal brain and in Parkinson's disease. MGP: medial globus pallidus; LGP: lateral globus pallidus; SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; STN, subthalamic nucleus. The different colors of the boxes and arrows indicate the neurotransmitters (glutamate, GABA, or dopamine) released by neurons in each specific anatomical region. (adapted from Blandini et al., 2000)

results in faster rotation (sensitization) and the acute behavioral effects of L-DOPA are maintained for a shorter period of time, reminiscent of the "wearing-off" observed after long-term L-DOPA therapy in human PD patients (Schwarting and Huston, 1996; Olanow et al., 2006).

Synaptic Plasticity

General Overview

Corticostriatal glutamatergic synapses are the major excitatory input to striatal medium spiny neurons. These synapses are therefore a key site for dopaminergic modulation of incoming excitatory signals into the basal ganglia. The importance of dopaminergic modulation is highlighted in PD, in which the loss of DA results in development of a number of characteristic motor pathologies (see p. 1, 8-9). Therefore, a closer examination of the mechanisms underlying the dopaminergic modulation of corticostriatal synapse function will help in understanding the significance of the biochemical changes that occur in the striatum following DA depletion. To date, the mechanisms underlying the bidirectional modulation of synaptic function have been most clearly defined in the hippocampus.

The hippocampus is a region of the brain involved in learning and memory, and has been the focus of many studies examining synaptic plasticity. Changes in synaptic strength are examined by electrically stimulating excitatory glutamatergic axons and recording the postsynaptic responses in the hippocampus CA1 region. Stimulation of these synapses with low frequency stimulation (LFS, 1-5 Hz) leads to a long-lasting

depression of synaptic transmission. In contrast, high frequency stimulation (HFS, 10-100 Hz) or chemical stimulation of excitatory synapses in the hippocampus leads to a long-lasting enhancement of synaptic transmission. These persistent changes in synaptic strength are referred to as long-term depression (LTD) and long-term potentiation (LTP), respectively (Stanton, 1996; Malinow and Malenka, 2002; Daoudal and Debanne, 2003).

Molecular Mechanisms

Hippocampal LTD weakens synapses, at least in part through the activation of phosphatases. LTD is dependent on activation of postsynaptic N-methyl-D-aspartate receptors (NMDAR), which allow influx of extracellular Ca^{2+} in response to LFS. As the phosphatase calcineurin (PP2B) has a higher affinity for Ca^{2+} than opposing kinases, PP2B is preferentially activated by LFS. Furthermore, LTD produces increases in activities of the protein phosphatases 1 (PP1, transient increase) and 2A (PP2A, long-lasting increase) (Thiels et al., 1998; Thiels et al., 2000). The subcellular localization of PP1 is critical for the induction of LTD, as PP1 localization away from the synapse prevents LTD induction (Feng et al., 2000; Morishita et al., 2001). LTD induction results in the dephosphorylation of the GluR1 subunit of the α-amino-3-hydroxy-5-methyl-4-isoxazole proprionic acid receptor (AMPAR), presumably as a consequence of the LTD-induced elevation in phosphatase activity. Dephosphorylation of GluR1 results in both an acute decrease in responsiveness of AMPA receptors to glutamate, and receptor internalization, a topic of a later section in this chapter on glutamate receptors. Thus, the outcome of LTD is a decreased postsynaptic response to subsequent glutamate stimulation.

Hippocampal LTP is also NMDA receptor-dependent but strengthens synapses, so that subsequent exposure to glutamate results in an increased excitability of the postsynaptic cell. HFS induces a strong activation of the NMDA receptor, resulting in a postsynaptic influx of extracellular Ca^{2+} that is sufficient to activate downstream kinases, such as CaMKII, which phosphorylate key postsynaptic substrates. One mechanistic explanation for the long-lasting effect of LTP is that LTP-inducing protocols and NMDA-dependent postsynaptic Ca^{2+} transients drive AMPA receptor subunits into the synapse (Shi et al., 1999; Hayashi et al., 2000; Shi et al., 2001). A higher number of synaptic AMPA receptors should directly increase the amount of postsynaptic excitability in response to presynaptic glutamate release. The role of CaMKII in hippocampal LTP will be discussed in a later section (p. 29-32).

Striatal Synaptic Plasticity

Mechanisms of striatal synaptic plasticity appear to be quite different from those used in the hippocampus and are relatively poorly understood. The striatum processes both glutamatergic and dopaminergic input, and determines the overall output of the basal ganglia. The intrinsic membrane properties of striatal MSNs influence the output of the striatum. *In vivo*, MSNs spontaneously transition from a resting "downstate" membrane potential close to -80 mV to an "upstate" depolarized potential near -50 mV (Wilson and Kawaguchi, 1996). Action potentials are generated from the more highly depolarized upstate. Transition between upstate and downstate membrane potentials is thought to modulate the overall output of the striatum. More specifically, transitions to the upstate enhance dendritic and spine L-type voltage-gated calcium channel (VGCC),

and decrease T-type VGCC contributions to the overall Ca^{2+} current (Carter and Sabatini, 2004). In addition, the upstate transition changes the dominant postsynaptic Ca^{2+} source from Ca^{2+}-permeable AMPA receptors to NMDA receptors (Carter and Sabatini, 2004).

State transitions in MSNs may mediate changes in striatal synaptic plasticity in the acute striatal slice (Picconi et al., 2005). A high frequency stimulus can produce either long-lasting increases or decreases in synaptic transmission at corticostriatal synapses. High-frequency stimulation of corticostriatal synapses results in LTD, the prominent form of HFS-induced synaptic plasticity in the adult dorsolateral striatum (Calabresi et al., 1992a; Calabresi et al., 1992b; Choi and Lovinger, 1997). In contrast, non-physiological conditions such as the exclusion of extracellular magnesium increase synaptic efficacy, resulting in an NMDA-receptor-dependent, HFS-induced LTP (Calabresi et al., 1992c). The regional heterogeneity within the striatum as well as the age-related changes in synaptic plasticity may contribute to the production of differing forms of synaptic plasticity by the same stimulus (Lovinger et al., 1993; Partridge et al., 2000).

Neuronal Disruptions in PD

Disruptions in Medium Spiny Neuron Morphology

Changes in MSN spine density and morphology have been reported in PD (McNeill et al., 1988; Stephens et al., 2005; Zaja-Milatovic et al., 2005). The decrease in spine density of MSNs is paralleled in the 6-OHDA lesioned rat, ranging from 14-19%, with a specific 14-17% loss of spines that form asymmetric synapses (Ingham et al.,

1998; Arbuthnott et al., 2000; Day et al., 2006). Moreover, a 43% decrease in asymmetric synapses specific to indirect pathway MSNs (Day et al., 2006) provides an explanation for the decreased signaling through the indirect pathway. Further examination of ultrastructural changes in the spine following DA depletion indicate both an increased area of asymmetric terminals and an increased number of perforated PSDs (Meshul et al., 2000). In combination, these findings suggest that while fewer corticostriatal glutamatergic terminals synapse directly on MSNs in the indirect pathway, the functional signaling of these remaining synapses may be altered.

Disruptions in Striatal Plasticity

The loss in dopaminergic and glutamatergic synapses formed by MSNs can have profound effects on striatal synaptic plasticity. Several forms of corticostriatal synaptic plasticity are disrupted following DA depletion (Calabresi et al., 1992a; Centonze et al., 1999; Partridge et al., 2000; Tang et al., 2001). Specifically, neurons in the dorsolateral striatum of the normal adult rat exhibit LTD in response to HFS, which is ablated following a unilateral 6-OHDA lesion. LTD in the striatum contralateral to the lesion remains normal (Partridge et al., 2000). LTD in the DA depleted region can be restored by application of dopamine or by co-application of D1 and D2 receptor agonists (Calabresi et al., 1992b).

Striatal Dopamine Receptors

Nigrostriatal dopaminergic afferents typically synapse on the spine and/or dendrite shaft of the striatal MSN (Bouyer et al., 1984; Freund et al., 1984). This DA

input is ideally positioned to modulate the activity of the MSNs, and thus the output of the entire basal ganglia. Striatal MSNs contain DA receptors of the D1 class (D_1, D_5) and the D2 class (D_2, D_3, and D_4).

D_1 receptors are located in the spine head and neck of postsynaptic asymmetric synapses (Bergson et al., 1995). Activation of D1 receptors activates adenylyl cyclase, which elevates cAMP levels, and results in activation of protein kinase A (PKA). Active PKA phosphorylates the cytosolic protein DARPP-32 (dopamine and cAMP-regulated phosphoprotein 32 kDa), at Thr^{34}, which renders DARPP-32 a potent inhibitor of PP1 *in vitro* (Hemmings et al., 1984; Greengard et al., 1999). The hypothesized final effect of D_1 receptor pathway activation *in vivo* is a decrease in PP1 activity, although changes in striatal PP1 activity have not been directly measured following D_1 stimulation.

Activation of D2 receptors inhibits adenylyl cyclase and stimulates the release of Ca^{2+} via IP_3 receptors on the endoplasmic reticulum (Hernandez-Lopez et al., 2000) (Fig. 4). The release of intracellular Ca^{2+} activates a Ca^{2+}-dependent phosphatase, PP2B, which dephosphorylates phospho-Thr^{34}-DARPP-32 (Nishi et al., 1997). Thus, the final effect of D_2 pathway activation is likely a disinhibition of PP1, which is expected to promote the net dephosphorylation of PP1 substrates.

Striatal Distribution of Dopamine Receptors

Historically, the colocalization of DA receptors in MSNs has been controversial due to the poor quality of subtype-specific antibodies. Recently, bacterial artificial chromosome (BAC)-D1 and BAC-D2 receptor transgenic mice have been created that express EGFP under the control of the D_1 (or D_2) receptor promoter (Heintz, 2001). In

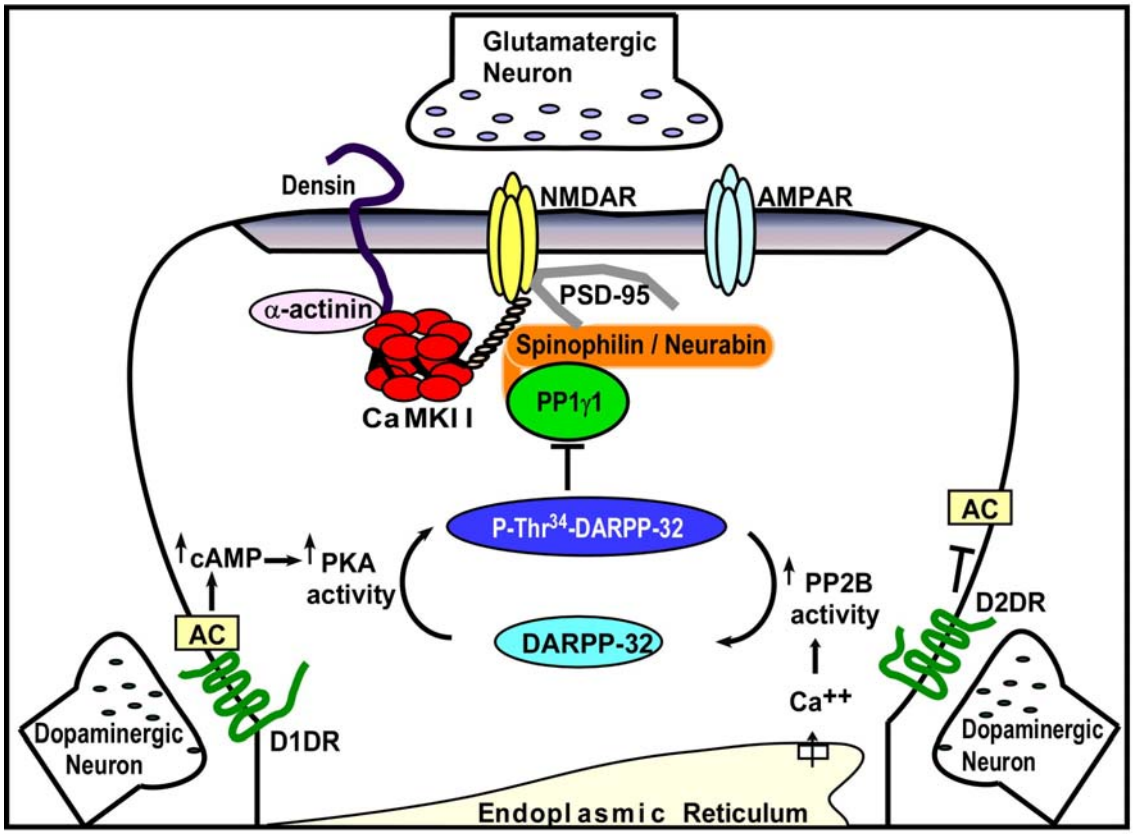

Figure 4. Intracellular signaling pathways of synaptic striatal D1-like (D1DR) and D2-like (D2DR) dopamine receptors. D1DR and D2DR are located on separate neurons within the striatum, but are depicted here on the same neuron for ease of presentation. Stimulation of D1DR activates adenylyl cyclase, which increases cAMP levels, thus activating PKA. PKA-mediated phosphorylation of DARPP-32 on Thr^{34}, renders DARPP-32 an effective inhibitor of PP1 *in vitro*. Activation of D2DR both inhibits adenylyl cyclase and stimulates IP_3-mediated intracellular Ca^{2+} release. Elevated intracellular Ca^{2+} activates calcineurin (PP2B)-mediated dephosphorylation of DARPP-32 at Thr^{34}.

these mice, cells that normally express D_1 (or D_2) receptor also produce EGFP. BAC-D1 transgenic mice express EGFP exclusively in MSN direct pathway projection neurons. In contrast, BAC-D2 receptor transgenic mice express EGFP exclusively in indirect pathway neurons. These data suggest that direct pathway neurons express D_1 receptors, while indirect pathway neurons express D_2 receptors (Gerfen, 2006). However, other D2 receptors may be present on neurons expressing D_1 receptors, and vice versa.

D2 receptors are also present on non-MSN striatal neurons. Cholinergic interneurons express functional D2 receptors, and release acetylcholine in response to stimulation by quinpirole, a D2 agonist (DeBoer et al., 1996). In turn, some MSNs lacking D_2 receptors possess synaptic muscarinic M1 receptors (Hersch et al., 1994), and can thus indirectly respond to a D2 agonist. This indirect cellular signaling explains why MSNs in the direct pathway respond to D2 agonist stimulation, even though they do not express D_2 receptors. D2 receptors also are present on a population of presynaptic corticostriatal terminals, where they function to inhibit neurotransmitter release from a subset (~85%) of corticostriatal terminals in acute striatal slices (Bamford et al., 2004b).

Effects of Dopamine Depletion

As a result of a unilateral 6-OHDA lesion, the striatum ipsilateral to the lesion becomes supersensitive, altering the output of the basal ganglia in this hemisphere. Up to 8 weeks following a unilateral 6-OHDA lesion, there is an increase in the number of D_2 receptors (Araki et al., 1998), D_2 mRNA (Gerfen et al., 1990; Xu et al., 1992), and D_2 maximal binding (B_{max}) (Cai et al., 2002) in the dorsolateral striatum ipsilateral to the lesion, while the D_1 number and mRNA levels remain constant. More recently, Day et al.

(Day et al., 2006) have shown that DA depletion results in a decreased spine density on only indirect pathway (D_2), and not direct pathway (D_1) MSNs.

Glutamate Receptors

Striatal Glutamate Receptor Types and Distribution

Glutamate is the major excitatory neurotransmitter in the central nervous system. Many types of glutamate receptors are more abundant in the striatum than in other regions of the basal ganglia (Blandini et al., 2000), underscoring the role of the striatum as the main excitatory input nucleus of the basal ganglia. Members of the major families of glutamate receptors are present in the striatum: the ionotropic receptors (AMPAR and NMDAR), and group I metabotropic glutamate receptors (mGluR1 and mGluR5).

Subcellular localization of both AMPA and NMDA receptors both at the PSD and peri-synaptically enables rapid detection of presynaptic glutamate release. AMPA receptors are tetrameric transmembrane receptors, composed of subunits GluR1-4. In the mature hippocampus, AMPA receptors exist as tetramers, containing GluR1 + GluR2 or GluR3 + GluR2 subunits (Wenthold et al., 1996; Malinow and Malenka, 2002). In the hippocampus, GluR4 receptor expression is limited largely to the first postnatal week (Zhu et al., 2000). The GluR2/3 AMPA receptors constitutively recycle in and out of the plasma membrane, but synaptic activity is required for synaptic insertion of GluR1/2 AMPA receptors (Shi et al., 1999). These receptors are found at the PSD, peri-synaptically, and in intracellular vesicles close to the synapse. Activation of AMPA receptors by glutamate results in the influx of Na^+ and sometimes Ca^{2+}, enabling

membrane depolarization. Many striatal AMPA receptors contain GluR2 subunits that prevent Ca^{2+} entry (Jayakar and Dikshit, 2004).

AMPA receptor phosphorylation plays an important role in receptor function. Phosphorylation of GluR1 at Ser^{831} increases channel conductance (Barria et al., 1997; Barria et al., 1997; Derkach et al., 1999), while phosphorylation at Ser^{845} increases channel open probability (Banke et al., 2000). In AMPA receptors that contain the GluR2 subunit, these effects of phosphorylation at Ser^{831} appear to be lost, as the conductance of GluR1/2 receptors remains low, even after phosphorylation of GluR1 at Ser^{831} (Oh and Derkach, 2005). Phosphorylation at these sites is regulated by several kinases and phosphatases during synaptic plasticity in the hippocampus. During LTD, both $Ser^{831/845}$ sites become dephosphorylated (Lee et al., 2000). In contrast, LTP results in phosphorylation at Ser^{831}, and reversal of LTD results in phosphorylation of Ser^{845} (Barria et al., 1997; Lee et al., 2000). Thus, in response to an excitatory stimulus, AMPA receptors contribute to changes in postsynaptic excitability by increased synaptic insertion.

NMDA receptors are heterotetrameric transmembrane receptors, composed of different combinations of subunits NR1, NR2A, NR2B, and NR2C. When activated by concomitant membrane depolarization and glutamate binding, postsynaptic NMDA receptors allow influx of extracellular Na^{+} and Ca^{2+}. Striatal NMDA receptor subunits are thought to be tetrameric complexes, composed of either NR1/NR2A, NR1/NR2B, or NR1/NR2A/NR2B (Dunah and Standaert, 2003). NR3A and NR3B expression is largely restricted to the immature brain (Sucher et al., 1995) or spinal motor neurons (Nishi et al., 2001), respectively. The NR1/NR2A/NR2B complexes are exclusively in the

synaptosomal membrane fraction (Dunah and Standaert 2003), consistent with a synaptic localization. However, the NR1/NR2A and NR1/NR2B complexes are present in three membrane-associated fractions: light membranes, synaptosomal membranes, and synaptic vesicles. It is unknown whether these latter receptor complexes are in transit for insertion or removal from the plasma membrane.

Metabotropic glutamate receptors function to modulate synaptic plasticity in many brain regions, including the striatum. In the striatum, group I metabotropic receptors are present peri-synaptically on MSNs and on cholinergic interneurons, whereas group II and group III mGluRs are present on presynaptic corticostriatal terminals and glial cells (Gubellini et al., 2004). Activation of mGluR1 is necessary for striatal LTD induction (Sung et al., 2001). The expression of mGluR subtypes on nigrostriatal dopaminergic terminals is less well defined. A family of G-protein coupled receptors, the group I mGluRs are coupled to phospholipase C (PLC). Activation of group I mGluRs results in both the release of intracellular Ca^{2+} from the ER via an IP_3-mediated pathway, and enhances L-type VGCC conductance (Hermans and Challiss, 2001).

Glutamate Receptor Modulation by Dopamine Depletion

Modulation of MSN glutamate receptors following DA depletion may alter the detection of corticostriatal glutamatergic signals within the striatum. There are conflicting reports as to whether D_1 activation modulates AMPAR subcellular redistribution, an effect that may be dependent on the specific D1 agonist used in the studies, or the use of fresh brain slices vs. cultured neurons (Dunah and Standaert, 2001;

Mangiavacchi and Wolf, 2004). While it is unclear if DA modulates the subcellular localization of AMPA receptors, it may modify their function. In striatal slices, D_1 activation stimulates PKA-mediated phosphorylation of GluR1 at Ser^{845}, suggesting an increased channel open probability (Price et al., 1999; Banke et al., 2000; Snyder et al., 2000).

Dunah and Standaert have demonstrated a D_1-dependent redistribution of NR1, NR2A, and NR2B subunits from light membrane and synaptic vesicle-enriched membranes to synaptosomal membranes, suggesting synaptic insertion (Dunah and Standaert, 2001). Moreover, this DA-dependent redistribution of NMDA receptor subunits is dependent on fyn protein tyrosine kinase (Dunah et al., 2004), and can be induced by tyrosine phosphatase inhibitors (Hallett et al., 2006). Together, these findings suggest that an imbalance between tyrosine phosphatase and tyrosine kinase activities may play a role in NMDA receptor subcellular redistribution in the striatum.

Group I mGluR antagonists alleviate motor symptoms in DA-depleted rats, possibly by reducing corticostriatal glutamate release (Ossowska et al., 2006). In addition, DA depletion results in increased number and B_{max} of group II mGluRs, which is reversible by L-DOPA (Picconi et al., 2002; Gubellini et al., 2004).

Voltage-Gated Channels

Plasma membrane depolarization activates voltage-gated calcium channels (VGCC) to allow an influx of Ca^{2+} ions. Based on sequence homology and function, VGCC are divided into three classes: Cav1 (high-voltage activated, L-type channels), Cav2 (low-voltage activated P/Q, N, and R-type channels) and Cav3 (low-voltage

activated, T-type channels) (Trimmer and Rhodes, 2004). VGCC consist of (1) a transmembrane voltage-sensing α-subunit that forms the channel pore, (2) transmembrane γ and δ subunits, and (3) an intracellular β-subunit. Each of these subunits plays a unique role in modulating Ca^{2+} influx (Catterall, 2000).

L-type Calcium Channels: Modulation by Dopamine Depletion

Each of the classes of VGCC is present in the striatum. Two types of L-type channels are present in MSNs of both the direct and indirect pathways, the Cav1.3α1 and the Cav1.2α1. Functional data have localized the L-type channels to the MSN synapse (Calabresi et al., 1994; Cepeda et al., 1998; Carter and Sabatini, 2004). In addition, immunoelectron microscopy has identified a long splice variant of the Cav 1.3α1 subunit in MSN spines (Day et al., 2006), where it is likely targeted by the scaffolding protein, Shank (Olson et al., 2005).

L-type channels can be modulated by signaling via DA-associated signaling pathways. D1 activation enhances L-type channel conductance via a PKA-mediated mechanism (Surmeier et al., 1995), and D2 or M1 muscarinic receptor activation inhibits L-type channel conductance via a calcineurin-mediated mechanism (Hernandez-Lopez et al., 2000). This dopaminergic and cholinergic modulation of striatal L-type channels is dependent on the targeting of L-type channels by Shank (Olson et al., 2005). One possible function of L-type channels is in the regulation of MSN morphology, as mice lacking Cav1.3α1 have an increased MSN spine density (Day et al., 2006). Moreover, chronic treatment with an L-type antagonist, nimodipine, rescues the 6-OHDA lesion-induced decrease in MSN spine density (Day et al., 2006). Following DA depletion, the

loss of D_2-mediated inhibition of L-type channels may allow unregulated Ca^{2+} influx through these channels. An unregulated influx of Ca^{2+} is expected to have multiple consequences on intracellular signaling pathways, specifically on Ca^{2+}-dependent enzymes such as CaMKII.

CaMKII

General Overview

Isoforms of mammalian CaMKII (α, β, γ, δ) are encoded by four separate genes; each has unique patterns of mRNA alternative splicing and protein expression throughout the body. Calcium/calmodulin-dependent protein kinase II (CaMKII) is one of the most abundant proteins in the brain, accounting for 1-2% of total protein. In the brain, CaMKII holoenzymes are predominantly found as either heteromultimers of both the α and β isoforms, or homomultimers of either isoform. The two major CaMKII isoforms in the striatum, α and β, are expressed in a 3:1 ratio. CaMKIIα is one of the most abundant proteins expressed in dendritic spines, comprising anywhere from 2-10% of the total protein in PSDs (Hanson and Schulman, 1992; Colbran, 2004a). A multifunctional enzyme (Fig. 5), CaMKII (1) is activated by increases in intracellular Ca^{2+}, (2) phosphorylates proteins at serine or threonine residues, (3) translocates from the cytosol to the PSD in response to neuronal activity, (4) serves as a "molecular memory" molecule, propagating signals long after the elevated Ca^{2+} subsides, and (5) may help serve as a scaffold for AMPA receptors to the plasma membrane (Lisman and Zhabotinsky, 2001; Lisman et al., 2002; Colbran and Brown, 2004).

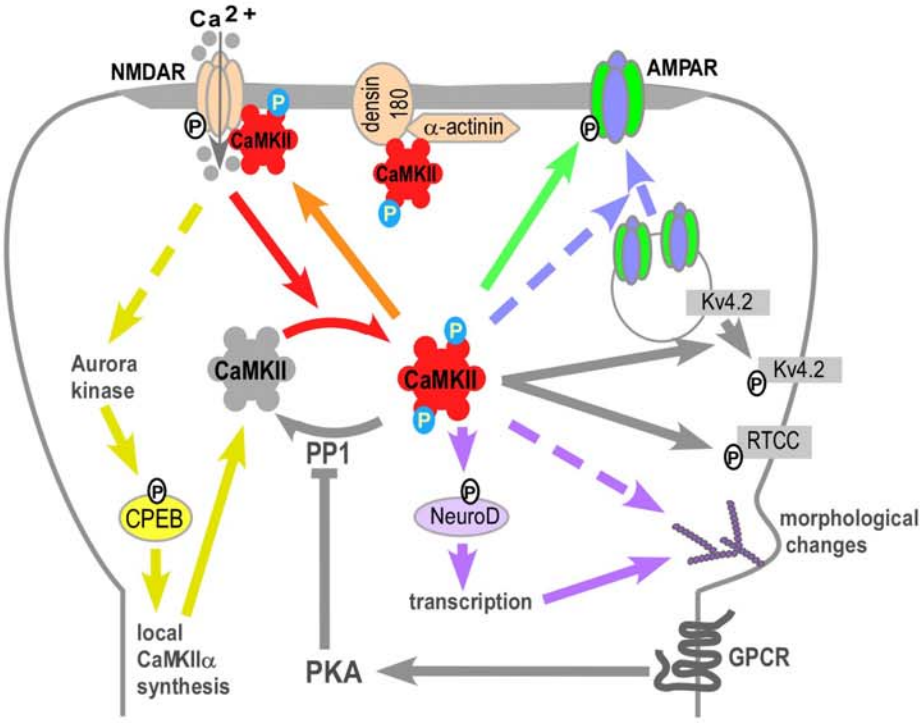

Figure 5. CaMKII signaling in mammalian synaptic plasticity. Synaptic plasticity is often dependent on Ca^{2+} influx via the NMDAR. The unique Thr^{286} autophosphorylation properties permit CaMKII to integrate information conveyed by the frequency, duration and amplitude of synaptic stimulation and provide a signal that is critical to normal synaptic plasticity. PP1 opposes Thr^{286} autophosphorylation thereby increasing the positive cooperativity for Ca^{2+}-dependent activation of CaMKII. PP1 is inhibited in response to activation of G-protein coupled receptors (GPCR) coupled to cAMP and PKA, such as D1DR. Activated CaMKII translocates to postsynaptic densities, presumably by binding to a number of different scaffolding proteins, where it regulates synaptic NMDAR and AMPAR. CaMKII also promotes the synaptic insertion of new AMPA receptors and surface expression of Kv4.2 potassium channels, and regulates R-type voltage-gated calcium channels (RTCC). Alterations in dendritic spine morphology are driven by CaMKII-dependent modulation of cytoskeletal proteins and/or gene transcription mediated by the NeuroD transcription factor. In addition, NMDA receptor activation activates local synthesis of new CaMKII subunits and other proteins from local mRNA pools via an Aurora kinase dependent phosphorylation of the cytoplasmic polyadenylation element binding protein (CPEB). (Adapted from Colbran and Brown, 2004)

CaMKII Structure

A dodecameric holoenzyme, each CaMKII monomer contains the catalytic, regulatory, variable, and association domains (Fig. 6). The monomers assemble into two stacked, hexameric rings via the C-terminal association domains (residues 315-478). The N-terminal catalytic domain (residues 1-272) contains both the ATP and the substrate binding sites, and mediates the phosphotransferase activity. The variable domains constitute the major regions of dissimilarity between the different CaMKII isoforms, and as a result of alternative splicing, can contain up to 4 different inserted cassettes. The regulatory domain (residues 273-314) is made up of the autoinhibitory and calmodulin binding regions. In the resting state, the autoinhibitory region binds the catalytic domain, and prevents substrate access, maintaining CaMKII in an inactive state (Lisman et al., 2002; Griffith, 2004; Colbran, 2004a).

CaMKIIα Regulation by Ca^{2+}

When local Ca^{2+} levels are elevated as a result of neuronal activity, Ca^{2+}/calmodulin (Ca^{2+}/CaM) binds to CaMKII (Fig. 6). CaM binding disrupts interactions between the catalytic and autoinhibitory domains, allowing substrates and ATP access to the catalytic sites. Ca^{2+}/CaM binding activates CaMKII and induces a rapid autophosphorylation of CaMKIIα at Threonine 286 (Thr^{286}). Thr^{286} autophosphorylated CaMKII has an enhanced affinity for Ca^{2+}/CaM. Although CaMKII phosphorylation at Thr^{286} is often used as an indirect measure of CaMKII activity, there is not always an exact temporal correlation (Lengyel et al., 2004).

CaMKII is also subject to forms of Ca^{2+}-independent regulation, via

autophosphorylation at Thr^{305} and /or Thr^{306}. First, a slow autophosphorylation at Thr^{306} in the basal state occludes the CaM-binding site, preventing CaM from binding (Colbran, 1993). Autophosphorylation at Thr^{306} maintains CaMKII in an inactive state, unresponsive to changes in Ca^{2+} levels, until this site is dephosphorylated by phosphatases. Second, the removal of Ca^{2+}/CaM after phosphorylation at Thr^{286} enables a rapid autophosphorylation at either Thr^{305} or Thr^{306} and Ser^{314} (Hashimoto et al., 1987; Patton et al., 1990; Hanson and Schulman, 1992). Although phosphorylation at $Thr^{305/306}$ occludes the CaM binding site, the prior phosphorylation at Thr^{286} maintains CaMKII in a constitutively active state. Thus, CaMKII that is phosphorylated at both Thr^{286} and $Thr^{305/306}$ is active, but is unresponsive to subsequent changes in Ca^{2+} concentration.

CaMKII Regulation by Endogenous CaMKII Inhibitors

Endogenous CaMKII inhibitors, CaMKII-Nα and CaMKII-Nβ are present throughout the brain, and bind the catalytic domains of CaMKIIα and β with an IC_{50} of 50 nM (Chang et al., 1998). While CaMKIIα is localized diffusely throughout cultured hippocampal neurons, CaMKII-N seems restricted to the soma, proximal dendrites, and possibly axonal processes (Chang et al., 2001). It is not currently known how CaMKII-N regulates CaMKII activity *in vivo*.

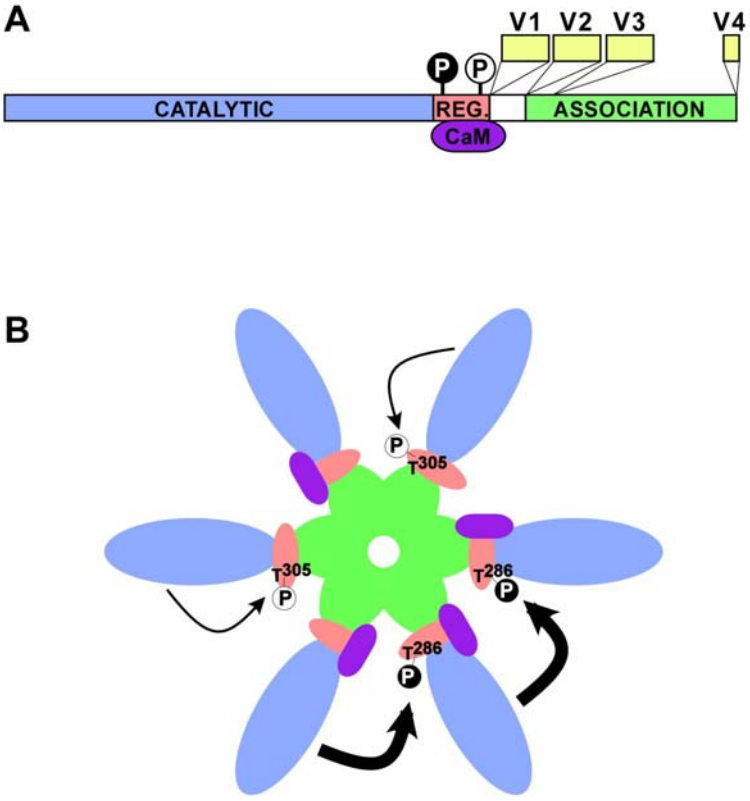

Figure 6. CaMKII structural domains.
(A): Each CaMKII monomer contains a catalytic domain (blue), a regulatory domain (pink), a variable domain region (white / yellow), and an association domain. Ca^{2+}-dependent autophosphorylation sites at Thr^{286} (depicted by white P in black circle) and Ca^{2+}-independent autophosphorylation sites at $Thr^{305/306}$ (depicted by black P in white circle) are shown. (B): CaMKII monomers assemble into hexameric rings, via their association domains. Ca^{2+}/calmodulin binding to two adjacent CaMKII subunits is required for the inter-subunit autophosphosphorylation at Thr^{286}. In contrast, Ca^{2+}/calmodulin binding is not required for the intrasubunit autophosphorylation at $Thr^{305/306}$. (adapted from Colbran, 2004)

CaMKII Regulation by Phosphatases

CaMKII activity is acutely regulated via dephosphorylation at Thr^{286} by phosphatases (Colbran, 2004b). PSD-associated CaMKII is selectively dephosphorylated by PP1, while cytosolic CaMKII is dephosphorylated by PP2A (Strack et al., 1997a). Specifically, the cAMP-pathway regulation of PP1 'gates' CaMKII activity in other brain regions (Blitzer et al., 1998; Brown et al., 2000). Thus, striatal CaMKII may be similarly regulated by D1 receptor signaling.

CaMKII Subcellular Localization

The holoenzyme isoform composition modulates the subcellular localization and activity-driven targeting of the enzyme. The CaMKIIβ isoform binds to actin filaments *in vitro*, a complex that is disrupted by the presence of both Ca^{2+}/CaM (Ohta et al., 1986). Specifically, a region in the association domain of only the β isoform targets CaMKIIβ homomers to F-actin (Shen et al., 1998; Fink et al., 2003). CaMKII is found at PSDs in cytoplasmic tower-like arrays (Petersen et al., 2003), and the predominant alpha isoform is found diffusely throughout neurons (Gleason et al., 2003; reviewed in Fink and Meyer, 2002). One advantage to altering CaMKII subcellular localization is to position the kinase at synaptic sites. CaMKII can bind to several PSD-associated proteins, such as F-actin, α-actinin, densin-180, cdk5, synGAPβ, the NR2B subunit of the NMDA receptor, which may drive translocation to the PSD (Colbran and Brown, 2004; Schulman, 2004; Merrill et al., 2005). It is unknown at this time whether any of these binding partners are found in the CaMKII tower-like structures.

Synaptic activity in the hippocampus increases CaMKII autophosphorylation at

Thr^{286} and Thr^{253} and increases the association of CaMKII with PSDs (Strack et al., 1997b; Migues et al., 2006). Moreover, autophosphorylation of CaMKII increases its affinity for NR2B, providing an activity-dependent regulatory mechanism of CaMKII localization to the PSD (Strack and Colbran, 1998; Strack et al., 2000). The interaction of active CaMKII with the NR2B subunit of the NMDAR is required for normal LTP in hippocampal organotypic cultures (Barria and Malinow, 2005). More specifically, NMDA receptor-mediated Ca^{2+} influx results in CaMKIIα autophosphorylation and rapid translocation to the PSD, close to numerous synapse-associated substrates. CaMKII is necessary and sufficient to drive one of these substrates, GluR1, to the synapse (Hayashi et al., 2000; Lee et al., 2000). Phosphorylation of GluR1 at Ser^{831} by CaMKII enhances conductance through the AMPA receptor, providing a mechanism for a rapid alteration in synaptic plasticity (Barria et al., 1997; Barria et al., 1997; Derkach et al., 1999), as previously discussed on p. 20. In addition, PSD-associated CaMKII is in close proximity to PP1, which dephosphorylates and inactivates CaMKII. Thus, the proteins that interact with CaMKII in this restricted environment allow synaptic activity to be transformed into a localized, specific response in the synapse.

CaMKII Function in vivo

Numerous studies have investigated the role of CaMKII in learning and memory using transgenic mice. These studies have used different strategies, such as manipulation of (1) the total level of CaMKIIα, (2) the level of active (phospho-Thr^{286}) CaMKIIα, or (3) the level of CaMKIIα locally translated in dendrites (Elgersma et al., 2004). The role of CaMKII in behavioral learning and memory has been assessed using various

hippocampal-dependent behavioral assays, such as the Morris water maze. When total protein levels of CaMKIIα are decreased in the hippocampus, CaMKIIα homozygous or heterozygous null mice display impaired spatial learning (Silva et al., 1992; Hinds et al., 1998; Frankland et al., 2001; Elgersma et al., 2002). Furthermore, the ability of CaMKII to undergo normal Ca^{2+}/CaM-dependent and Ca^{2+}/CaM-independent autophosphorylation is also critical, as transgenic or knock-in mice expressing mutant CaMKIIα (T286A, T286D, T305D, and T305/306VA) all have impaired spatial memory (Bach et al., 1995; Mayford et al., 1996; Giese et al., 1998; Elgersma et al., 2002).

The function of CaMKII *in vivo* has also been examined in a model of synaptic learning and memory, LTP. There is an increase in both the Ca^{2+}-dependent and total CaMKII activity after LTP, that is accompanied by a transient increase in CaMKII autophosphorylation at Thr^{286} and an increase in phosphorylation of the CaMKII substrates synapsin I and MAP-2 (Fukunaga et al., 1993; Fukunaga et al., 1995; Lengyel et al., 2004). Active CaMKII is only necessary for the induction, but not the maintenance of LTP, as application of the CaMKII inhibitory peptide, AC3-I, only blocks LTP if applied prior to, but not after induction (Chen et al., 2001). Not only is CaMKII activity important, but adequate CaMKII levels are key for normal synaptic plasticity. LTP is severely impaired in CaMKIIα null mutant mice (-/-), mice partially lacking CaMKIIα (+/-), and in transgenic CaMKIIα (T286A, T286D, T305D, and T305/306VA) mice (Silva et al., 1992; Hinds et al., 1998; Elgersma et al., 2002; Elgersma et al., 2004). Adequate levels of CaMKII may be important in part to bind the NR2B subunit of the NMDA receptor, an event which is required for normal LTP (Barria and Malinow, 2005).

LTP induction in the hippocampus CA1 region elevates total dendritic CaMKIIα

protein levels by an NMDA receptor- and MAP-kinase-dependent mechanism (Ouyang et al., 1999; Giovannini et al., 2001). More recently, high synaptic activity was shown to upregulate CaMKIIα expression in cultured hippocampal neurons (Thiagarajan et al., 2002). Both protein synthesis machinery and CaMKIIα mRNA, have been localized to neuronal dendrites (Steward and Schuman, 2003). Binding of the CPE-binding protein (CPEB) to two cytoplasmic polyadenylation elements (CPEs) in the CaMKIIα 3'-untranslated region (UTR) facilitates efficient transport of CaMKIIα mRNA to dendrites (Huang et al., 2003). NMDA receptor-dependent phosphorylation of CPEB on Ser^{174} appears to initiate local CaMKIIα translation via an Aurora kinase and / or a CaMKII-dependent mechanism (Huang et al., 2002; Atkins et al., 2005).

Local, dendritic synthesis of CaMKIIα is essential for late-phase LTP, as seen in transgenic mice lacking the 3'-untranslated region (UTR) of the CaMKIIα gene (Miller et al., 2002). Disruption of the entire 3'-UTR blocks dendritic mRNA transport and reduces hippocampal CaMKIIα protein levels, resulting in abnormal late-phase LTP and long-term memory. These mice have less CaMKII in the dendrites and at the PSD, impaired late-phase-LTP, and impaired spatial memory. In summary, normal CaMKII levels, CaMKII function (activity, autophosphorylation, targeting), and local dendritic synthesis are all essential for normal synaptic plasticity, learning, and memory.

Role of CaMKII in Disease

Previous studies suggest that DA modulates striatal CaMKII. Chronic administration of either a systemic D1 or D2 agonist to 6-OHDA lesioned rats enhances phosphorylation at unidentified serine(s) in NR2A and NR2B, respectively (Oh et al.,

1999). Intrastriatal injection of the CaMKIIα inhibitor, KN93, reverses these DA agonist-induced changes in NMDA receptor subunit phosphorylation. The reversal of NMDA receptor phosphorylation by KN93 implies that CaMKIIα plays a role in the phosphorylation of these receptor subunits in the 6-OHDA lesioned rat (Oh et al., 1999). Moreover, intrastriatal administration of CaMKII inhibitors KN-93 or Ant-AIP-II normalizes 6-OHDA lesion-induced changes in motor coordination and corticostriatal LTP (Picconi et al., 2004). In total, CaMKII appears to play a critical role in the 6-OHDA lesion-induced alterations of behavior and synaptic plasticity in rodents.

Finally, CaMKII is misregulated in a mouse model of Angelman's mental retardation syndrome (AS) (Weeber et al., 2003). Patients with AS display severe mental retardation, severe learning deficits, frequent seizures, and an ataxic gait. AS results from deletion or truncation of the maternally derived Ube3A gene, which encodes for E6-AP, an E3-ubiquitin ligase. The Ube3A gene is subject to brain specific imprinting, such that only the maternally-derived E6-AP is normally expressed in the hippocampus. A mouse model for AS was created that lacks the maternal Ube3A gene, and thus expresses no E6-AP in the hippocampus (Weeber et al., 2003). The AS phenotype is reflected in the AS mice, which display impaired NMDA-dependent and NMDA-independent LTP, impaired spatial learning, impaired motor coordination, and frequent seizures (Jiang et al., 1998; Miura et al., 2002; Weeber et al., 2003). Although the level of hippocampal CaMKII is unaltered in these mice, the phosphorylation of hippocampal CaMKII at Thr^{286} and Thr^{305} is elevated. Interestingly, preventing CaMKII phosphorylation in AS / Thr305V/306A mice rescues the hippocampal learning and plasticity deficits(van Woerden et al. 2007). The increased phosphorylation of CaMKII may directly result

from the decreased PP1/PP2A activity in the hippocampus of AS mice. This mouse model displays how a disruption in the balance between CaMKII and PP1/PP2A activity in the hippocampus results in a severe neurological disease.

Protein Phosphatase 1

General Overview

The serine / threonine protein phosphatases present in the brain include PP1, PP2A, PP2B, and PP2C. The four PP1 catalytic subunit isoforms, α, β, γ1, and γ2 share at least 70% sequence identity (Ceulemans and Bollen, 2004) and are found at high levels in the striatum, relative to other brain regions (Shima et al., 1993; da Cruz E Silva et al., 1995; Strack et al., 1999). PP1 dephosphorylates numerous neuronal proteins, thereby helping to maintain the balance between kinase and phosphatase activity in the cell.

PP1 Structure

Crystal structures of PP1 catalytic subunits show that the enzyme exists in a single globular domain (Egloff et al., 1995; Kita et al., 2002; Terrak et al., 2004) (Fig. 7). The carboxy- and amino-termini are excluded from this central globular domain. The carboxy-terminus contains threonine residues, that when phosphorylated, reduce PP1 enzymatic activity. The center of the enzyme contains a sandwich of β-sheets and two metal ions (Fe^{2+} and Mn^{2+}), thought to be necessary for phosphatase activity (Egloff et al., 1995). The active site of PP1 is located at the center of a Y-shaped depression in the surface of the enzyme (Fig. 7). The three grooves that make up this Y-shaped depression

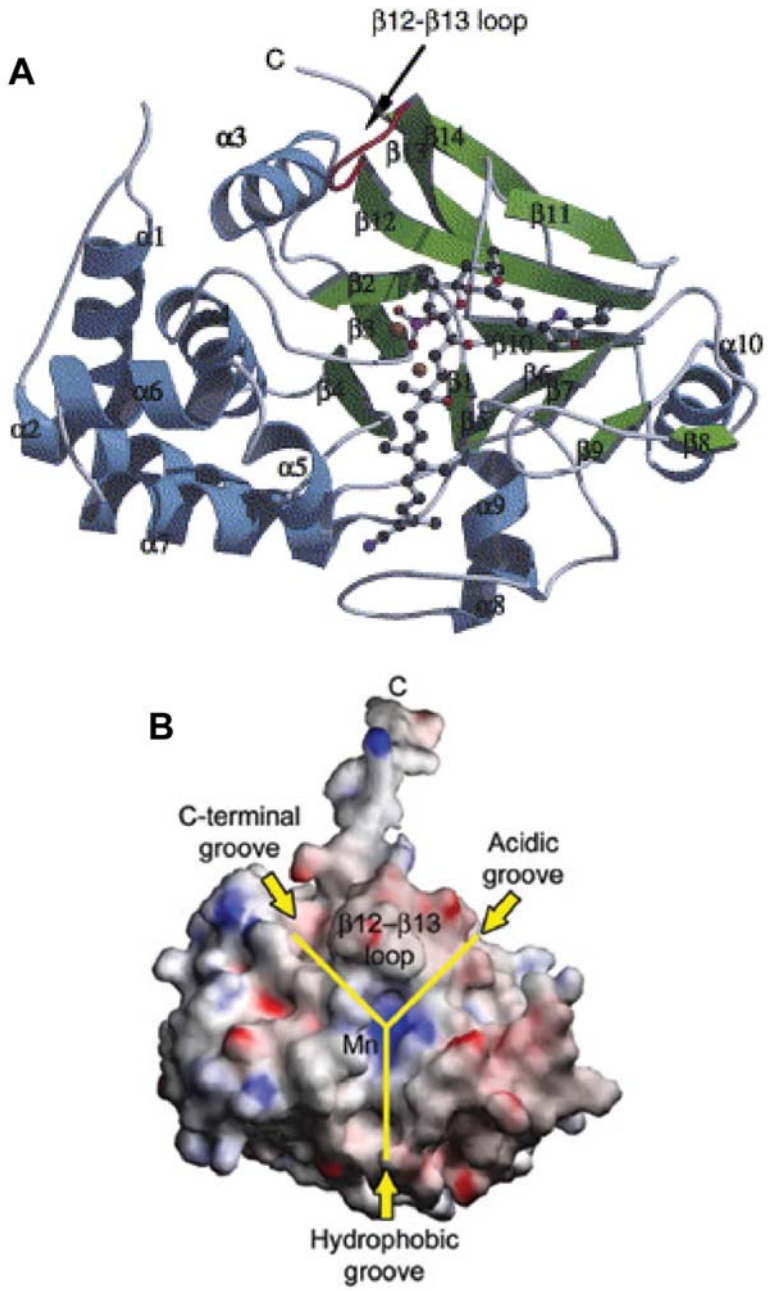

Figure 7. Three-dimensional structure of Protein Phosphatase 1. (A) Ribbon diagram of PP1γ in a complex with calyculin A, represented as a ball and stick molecule (from Kita et al., 2002). (B) Space-filled model of PP1δ, with the Y-shaped groove, made up of the C-terminal, acidic, and hydrophobic grooves (from Terrak et al., 2004).

are referred to as the C-terminal groove, the acidic groove, and the hydrophobic groove. Many PP1 inhibitors used in the experiments described in this dissertation block PP1 activity by interacting with portions of this Y-shaped groove. For example, microcystin-LR interacts with two metal-bound water molecules and binds Cys273 in the β12- β13 loop, which flanks the catalytic site, thus blocking access to the PP1 active site (Ceulemans and Bollen, 2004).

Regulation of PP1 Activity

PP1 activity is thought to be acutely regulated in the brain by the endogenous phosphatase inhibitors DARPP-32 / inhibitor-1 and inhibitor-2, as well as by phosphorylation of PP1 itself at Thr^{320} (Allen, 2004). Previously, DA regulation of striatal PP1 activity has been linked to endogenous PP1 inhibitors, DARPP-32 and inhibitor-1, which become active following phosphorylation by PKA (phospho-Thr^{34}-DARPP-32 / phospho-Thr^{35}-inhibitor-1) (Svenningsson et al., 2004). DARPP-32 knockout mice display numerous deficits, such as an altered phosphorylation of PP1 substrates, a decreased DA-induced GABA release from MSNs, and a deficit in DA-induced striatal Fos expression (Fienberg et al., 1998). In slices from DARPP-32 knockout mice, D1 agonists did not modulate AMPAR current, as shown by the lack of D_1-mediated AMPAR current rundowns. In addition, these mice also displayed a reduced DA / forskolin-induced phosphorylation of the NR1 subunit of the NMDAR. These data indicate that DARPP-32 plays a critical role in mediating the downstream effects of D_1 signaling, presumably via the inhibition of PP1 activity.

A second endogenous PP1 inhibitor, Inhibitor-2, inhibits PP1 activity by forming

a complex with PP1 (Cohen, 1989). GSK-3 phosphorylation of Inhibitor-2 at Thr^{72} changes the conformation of the Inhibitor-2/PP1 complex, relieving PP1 inhibition (Cohen, 1989). Although signaling through D2 receptors activates GSK-3 (Beaulieu et al., 2004), it is unclear whether striatal DA regulates striatal PP1 via Inhibitor-2 phosphorylation.

Phosphorylation of PP1 at the C-terminal Thr^{320} by cdc2 inhibits PP1 activity (Dohadwala et al., 1994). This phosphorylation may inhibit PP1 activity by the binding of phospho-Thr^{320} to the PP1 active site, blocking substrate access (Egloff et al., 1995). As cdc2 is a cyclin-dependent kinase that phosphorylates PP1 in a cell cycle-dependent manner, it is unclear how phosphorylation of striatal PP1 at Thr^{320} *in vivo* might be regulated by cdc2 or a related cyclin-dependent kinase, cdk5.

Subcellular Localization of PP1: Scaffolding Proteins

The three major PP1 isoforms (α, β, and γ_1) are each enriched in distinct subcellular compartments in neurons (da Cruz E Silva et al., 1995; Ouimet et al., 1995; Strack et al., 1999; Bordelon et al., 2005). The subcellular localization of the PP1β and γ1 isoforms is very different, with PP1β largely limited to the soma and dendritic shafts, and PP1γ1 found throughout the neuron, but concentrated at synapses (Ouimet et al., 1995; Strack et al., 1999; Bordelon et al., 2005). Due to it's concentration at synapses, PP1γ_1 is poised to rapidly dephosphorylate synaptic substrates, such as CaMKII and GluR1. The homologous F-actin binding proteins spinophilin and neurabin appear to be responsible for the selective targeting of the γ_1 isoform of PP1 to dendritic spines (MacMillan et al., 1999; Strack et al., 1999; Terry-Lorenzo et al., 2002; Carmody et al.,

2004; Bordelon et al., 2005). Specifically, immunoelectron microscopy has revealed that PP1γ1 is enriched exclusively in the PSD, while PP1α is found both at the PSD, and deeper in the spine head (Bordelon et al., 2005). Spinophilin and neurabin, two PSD-associated scaffolding proteins, selectively interact with the γ_1 isoform of PP1 and preferentially coimmunoprecipitate with PP1γ1 (MacMillan et al., 1999; Terry-Lorenzo et al., 2002; Carmody et al., 2004). In contrast, PP1β is more abundant in dendritic shafts and the soma (Strack et al., 1999; Bordelon et al., 2005). This selective interaction with spinophilin / neurabin may aid in the specific targeting of PP1γ1 to the synapse.

Function of striatal PP1

The balance between kinase and phosphatase activity is critical for normal regulation of cell signaling. In the forebrain, CaMKII is selectively dephosphorylated by PP1 at the PSD (Strack et al., 1997a; Strack et al., 1997b). The regulation of PP1 localization and activity by spinophilin and neurabin is critical for normal striatal synaptic plasticity and morphology (Surmeier et al., 1995; Yan et al., 1999; Feng et al., 2000; Pisani et al., 2005; Terry-Lorenzo et al., 2005; Allen et al., 2006). In the hippocampus, inhibition of PP1 via cAMP-dependent pathways functions as a regulatory 'gate' to allow enhanced CaMKII autophosphorylation at Thr^{286} (Blitzer et al., 1998; Brown et al., 2000). While the exact mechanisms involved in regulation of striatal CaMKII are unknown, it seems likely that the regulation by PP1 may be similar to that found in the forebrain and hippocampus. Many acute effects of DA in the striatum are mediated by the modulation of protein phosphatases, especially via the modulation of PP1 by DARPP-32, spinophilin, and calcineurin (Svenningsson et al., 2004). Although

regulation of PP1 is thought to play a key role mediating the actions of DA in the striatum (Feng et al., 2000; Allen et al., 2006), such alterations of PP1 activity have not been directly demonstrated.

Role of PP1 in Disease

Dysregulation of phosphatase activity has been implicated in a wide variety of human diseases or disease models, such as cancer, diabetes, and neurodegenerative disease. For example, increased phosphorylation of several proteins has been reported in Angelman's mental retardation syndrome (Weeber et al., 2003), amyotrophic lateral sclerosis (Strong et al., 2005), multiple sclerosis (Schneider et al., 2004), Charcot-Marie-Tooth disease (Begley and Dixon, 2005), and Alzheimer's disease (Liu et al., 2005). In particular, studies in a mouse model of Angelman's syndrome suggest that decreased protein phosphatase activity results in increased phosphorylation of hippocampal CaMKII at Thr^{286} and Thr^{305} and disruptions of synaptic plasticity, learning and memory. Thus, the development of strategies to normalize the activity of critical protein phosphatases might be a fruitful therapeutic strategy for treatment of multiple neurodegenerative diseases.

PP1 is thought to play a key role mediating the actions of DA in the normal striatum (Feng et al., 2000; Allen et al., 2006). However, DA depletion disrupts the normal DA receptor signaling and thus is expected to interfere with normal regulation of DARPP-32. This is consistent with the observation that striatal DA depletion results in enhanced phosphorylation of multiple synaptic proteins (Oh et al., 1999; Dunah et al., 2000; Picconi et al., 2004; Brown et al., 2005).

Summary

Striatal DA depletion results in several changes in MSN morphology and disrupts corticostriatal synaptic plasticity. However, the molecular and biochemical mechanisms underlying these consequences of long-term DA depletion are not well understood. Previous reports have largely examined the consequences of short-term DA depletion, and not the chronic, long-term DA depletion seen in PD. Based on prior studies, mostly in other brain regions, I predicted that striatal DA depletion and / or subsequent DA replacement therapy would alter expression and / or function of key striatal synaptic proteins.

Protein phosphorylation and dephosphorylation acutely modulate protein function, protein-protein interactions, and neuronal plasticity. Striatal DA signaling pathways may acutely modulate two critical signaling enzymes, CaMKII and PP1. The goal of this research is to determine how both short-term and more chronic DA depletion modify the regulation of striatal CaMKII and PP1. Changes in these signaling proteins following DA depletion may play a role in the progression of symptoms during Parkinson's disease, as well as in the changing efficacy and debilitating side effects associated with DA replacement therapy.

Hypothesis and Specific Aims

Hypothesis: Removal of nigrostriatal dopaminergic neurons alters the regulation of both CaMKII and PP1, and common downstream targets.

Aim I: Determine the impact of DA depletion on the subcellular localization and activity of striatal CaMKII.

Aim II: Determine the role of DA and glutamate in the regulation of CaMKII in acute striatal slices.

Aim III: Determine the impact of DA depletion on striatal PP1/PP2A levels and activity.

CHAPTER II

MATERIALS AND METHODS

6-OHDA Lesion Surgery

Male Sprague-Dawley rats (Harlan; Indianapolis, IN) were housed under a 12:12 light: dark cycle with food and water freely available. Experiments were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* (NIH), under the oversight of the Institutional Animal Care and Use Committee. Rats (3 months old) were anesthetized with ketamine/xylazine or isoflurane and 6-OHDA HBr (4 μg/μL free base in 0.02% ascorbate) was infused into two sites in the substantia nigra (AP: -5.3, L: 2.3 and 1.0, DV: -8.3) (Paxinos and Watson, 1986), at a rate of 0.25 μl/min. Sham-lesioned rats received injections of vehicle alone. Age-matched non-injected rats were also used in some studies. At the times indicated after surgery, rats were lightly anesthetized with isoflurane, decapitated, and the brain removed. Data reported in Chapters III and V were obtained from batches of 6-8 rats each that were sacrificed 3 weeks, 6-12 weeks, 9 months, 11 months, or 18-20 months after 6-OHDA lesion surgery (as indicated within each chapter). For presentation purposes, we pooled data obtained from animals 3-12 weeks and 9-11 months after surgery. There were no statistically significant differences between batches of animals within each set of pooled data. Moreover, differences observed in the pooled data were observed in both individual groups of animals at each time point**.** Successful lesion surgery was designated by a loss of at least 90% striatal tyrosine hydroxylase (TH), as determined by immunoblot.

L-DOPA Treatment & Behavioral Screening

Rats 6-12 weeks after 6-OHDA lesion were treated with L-DOPA methyl ester / benserazide (50 / 12.5 mg/kg, ip), or benserazide alone every 12 hours for 9 days, then were sacrificed 16 hours after the last injection. Benserazide is a peripheral L-DOPA decarboxylase inhibitor that prevents metabolism of L-DOPA in the periphery. This L-DOPA treatment paradigm induced the characteristic increase in contralateral rotation frequency by the final day of treatment (data not shown), consistent with previous reports (Schwarting and Huston, 1996). This repeated injection paradigm was designed to mimic the effects of repeated, pulsatile L-DOPA administration to PD patients. In these experiments, only rats with ≥90% TH depletion displayed successful contralateral rotation behavior.

Tissue Collection and Preparation

Dorsolateral Striatal Tissue Homogenates: Punches (1.15 mm ID) of dorsolateral striatum were removed from both hemispheres of 1.0 mm thick coronal slices, at the level of the crossing of the anterior commissure, and flash frozen on dry ice within 2 min of decapitation. Tissue punches were stored at –80°C. We confirmed that this procedure minimized postmortem changes in CaMKII phosphorylation (Suzuki et al., 1994; Lengyel et al., 2001).

Whole Striatal Extracts and Subcellular Fractionation: Whole extracts were prepared by sonicating striatal tissue punches in 200-300 µl 2% SDS with 10 µg/ml leupeptin and 1 µg/ml pepstatin. PSD-enriched fractions were prepared by homogenizing frozen striatal punches in ice-cold 7 mM Tris-HCl, pH 7.5 containing 0.2 mM EDTA, 0.2

mM EGTA, 320 mM sucrose, 1 mM benzamidine, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 10 µM pepstatin, 1 µM microcystin, 0.5 nM cypermethrin, and 1 mM $NaVO_4$, using a Kontes tissue homogenizer. After centrifugation (1000 x *g* for 7 min at 4°C) to separate a pellet fraction enriched in nuclei (P1), Triton X-100 (1% final) was added to the supernatant fraction (S1). S1 fractions were mixed gently for 30 min at 4°C, then re-centrifuged (100,000 x *g* for 1 hr at 4°C) to separate a supernatant (S2) enriched in cytosolic/detergent-soluble membrane proteins, and a pellet (P2) enriched in cytoskeletal elements including PSDs. Protein concentrations of samples were determined by the method of Lowry (Lowry et al., 1951).

Subcellular fractionation efficiency was confirmed by evaluating the distribution of phospho-Thr^{286}-CaMKII, total CaMKII, total GluR1, and total DARPP-32 in each fraction. In the P2 fraction, phospho-Thr^{286}-CaMKIIα was ~30-fold more concentrated than in the accompanying S2 fraction. In addition, CaMKIIα was 7-fold and GluR1 was 2-fold more concentrated in the P2 fraction than the S2 fraction. In contrast, DARPP-32 was 10-fold more concentrated in the S2 fraction than in the P2 fraction.

Immunoblot Analysis

Immunoblots: Samples (20-40 µg protein per lane) were fractionated by SDS-PAGE and transferred to nitrocellulose membranes, which were stained with Ponceau-S (Sigma) and then digitally scanned. After blocking (PBST in 2% milk), membranes were probed with the indicated primary antibodies: phosphorylation site-specific primary antibodies were incubated overnight at 4°C, whereas other primary antibodies were incubated for 2 hours at room temperature. Membranes were then washed and incubated

for 1 hr at room temperature with the alkaline phosphatase or horseradish peroxidase conjugated secondary antibodies. Alkaline phosphatase conjugated secondary antibodies were detected with 5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt (BCIP, Pierce) and nitroblue tetrazolium chloride (NTB, Pierce). Horseradish peroxidase-conjugated secondary antibodies were detected with enhanced chemiluminescence (Perkin Elmer) and multiple X-ray film exposures to ensure that signals were within the linear range. In some cases primary and HRP-conjugated secondary antibodies were stripped from the membrane by incubation in stripping buffer (62.5 mM Tris-HCl (pH 7.5), 2% SDS, 0.8% 2-mercaptoethanol) for 1 hr at 50°C. Stripping efficiency was confirmed by subsequent incubation with secondary antibody and development. Membranes were then incubated in PBST blocking buffer for 1 hr prior to reprobing with different primary antibodies.

Antibodies: The following primary antibodies were used for immunoblotting: goat anti-CaMKII α/β (1:4000, (McNeill and Colbran, 1995)), mouse anti-CaMKIIα (ABR, 1:4000), rabbit anti-phospho-Thr^{286}-CaMKIIα (Promega, 1:2500), rabbit anti-phospho-Thr^{305}-CaMKIIα (1:10,000, (Elgersma et al., 2002)), rabbit anti-DARPP-32 (Cell Signaling, 1:4000), rabbit anti-phospho-Thr^{34}-DARPP-32 (Cell Signaling, 1:250), rabbit anti-phospho-Thr^{75}-DARPP-32 (Cell Signaling, 1:500), rabbit anti-GluR1 (Upstate, 1:4000), rabbit anti-phospho-Ser^{831}- GluR1 (Upstate, 1:500), rabbit anti-phospho-Ser^{845}- GluR1 (Upstate, 1:2000), rabbit anti-neurabin (1:2500, (MacMillan et al., 1999)), mouse anti-NR1 (Chemicon, 1:3000), rabbit or mouse anti-NR2B (Molecular Probes, 1:500), sheep and rabbit anti-PP1γ1 and anti-PP1β (Colbran et al., 1997; Strack et al., 1999; Colbran et al., 2003), mouse anti-PP2Ac (Transduction Labs, 1:4000), mouse anti-PSD-95 (Upstate, 1:1000), rabbit anti-spinophilin (1:2000) (MacMillan et al., 1999),

mouse anti-tyrosine hydroxylase (ImmunoStar, 1:1000), and rabbit anti- phospho-PKA substrate (Cell Signaling, 1:1000). Secondary antibodies were from Jackson Immunoresearch (goat anti-rabbit AP, 1:1000), Sigma (rabbit anti-mouse AP, 1:2000), Promega (goat anti-mouse HRP, 1:2000; goat anti-rabbit HRP, 1:4000), Vector Laboratories (rabbit anti-goat AP, 1:2000), or AlphaQuest (rabbit anti-goat HRP, 1:4000).

Characterization of CaMKIIα Phosphorylation Site-Specific Antibodies

In an attempt to confirm the phospho-substrate specificity of phospho-Thr^{286}-CaMKII and phospho-Thr^{305}-CaMKII, purified wild-type mCaMKIIα or mCaMKIIα mutants T286A, T305A, T306A, and T305/306A were autophosphorylated under two different conditions. All autophosphorylation reactions were stopped by addition of SDS-PAGE sample buffer, and boiled at 100ºC for 5 min. Samples were immunoblotted for either phospho-Thr^{286}-CaMKIIα (1:2500, Promega) or Phospho-Thr$^{305/306}$-CaMKIIα (1:10,000, (Elgersma et al., 2002)). All autophosphorylation reactions were carried out using CaMKIIα (wild-type), or the following CaMKIIα mutants: T286A, T305A, T306A, and T305/306A.

Basal autophosphorylation conditions were previously optimized to produce selective phosphorylation primarily at Thr^{306}, but also at Thr^{305} (Colbran, 1993). Briefly, CaMKII (1 μM subunit) was incubated for 60 min at 30°C with 50 mM HEPES pH 7.5, 10 mM magnesium acetate, 1 mM EGTA, and 1mM ATP. Immunoblots of wild-type CaMKII following basal autophosphorylation revealed a band detected by the phospho-Thr^{305}-CaMKII antibody. Furthermore, all but the CaMKII T305/306A mutant also were

detected by the phospho-Thr^{305}-CaMKII antibody after basal autophosphorylation (Fig. 8, Basal). In contrast, neither the wild-type, nor the CaMKII mutants were immunoreactive with the phospho-Thr^{286}-CaMKII antibody. Thus, this antibody recognized CaMKII when phosphorylated at either Thr^{305} or Thr^{306}.

In parallel assays, autophosphorylation conditions (+ Ca^{2+}/CaM) were used to fairly selectively label phospho-Thr^{286}, as previously described (McNeill and Colbran, 1995). Briefly, autophosphorylation at Thr^{286} and other unidentified sites was achieved by incubating 5 μM CaMKIIα at 30ºC for 4 minutes with 50 mM HEPES pH 7.5, 2 mM magnesium acetate, 1.5 mM $CaCl_2$, 10 μM CaM, and 1 mM ATP. Under these conditions, all but the T286A mutant were detected by the phospho-Thr^{286} antibody, as expected. In contrast, the phospho-Thr^{305} antibody detected strong immunoreactivity from wild-type and all CaMKII mutants (Fig. 8, + Ca^{2+}/CaM), indicating that this antibody is not specific for CaMKII phosphorylated at only Thr^{305} or Thr^{306}. It appears that under these conditions, CaMKII is becoming phosphorylated at an immunoreactive site that is not Thr^{305} or Thr^{306}.

Previous studies have used the phospho-Thr^{305} antibody to detect a specific immunoreactive signal using hippocampal homogenates from TT305/306VA or T305D mice as negative controls (Elgersma et al., 2002; Weeber et al., 2003). It is possible that the in vitro phosphorylation conditions used here somehow promote phosphorylation of purified CaMKII at sites in addition to those observed *in vivo*. Candidate phosphorylation sites include sites containing two consecutive threonine residues (Thr401/402 and Thr477/478) or threonine residues surrounded by hydrophobic residues (Thr241, Thr261, Thr443). 2D-electrophoresis combined with mass

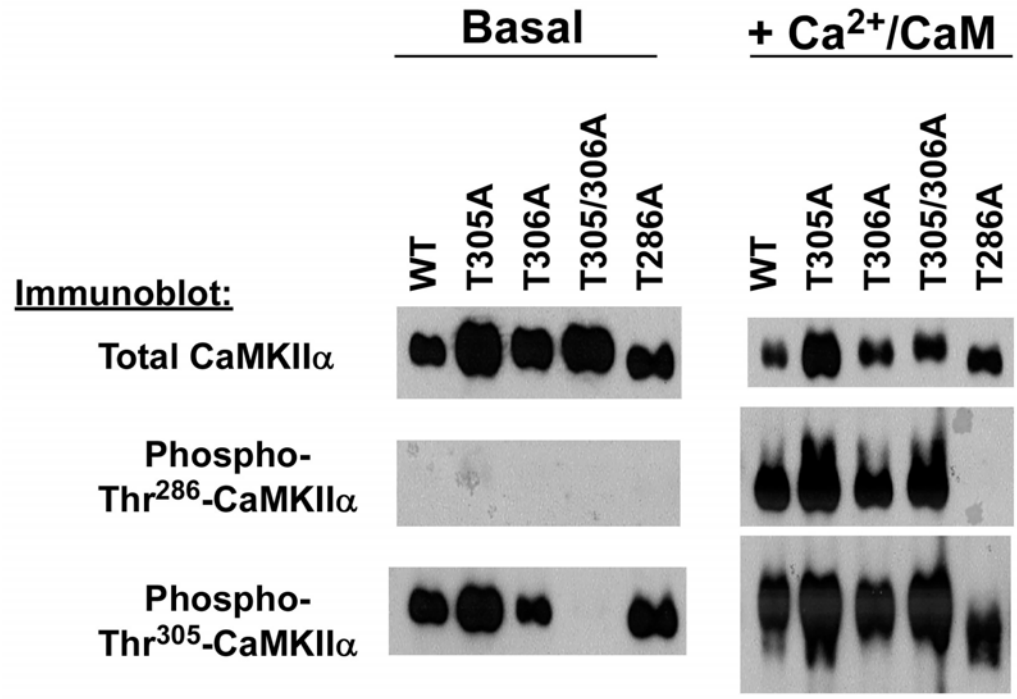

Figure 8. Characterization of phosphorylation site-specific antibodies.
To confirm the phospho-substrate specificity of phosphorylation site-specific antibodies, purified wild-type mCaMKIIα, T305A, T306A, and TT305/306AA and T286A were autophosphorylated under different conditions to induce basal autophosphorylation or Ca^{2+}/CaM-dependent autophosphorylation (see methods for details).

Basal: Basal autophosphorylation conditions were previously optimized to produce selective phosphorylation primarily at Thr^{306}, but also at Thr^{305}. Under these conditions, the phospho-Thr^{305} antibody detected strong immunoreactivity from all but the T305/306A mutant. No signal was detected for WT or and CaMKII mutant with the phospho-Thr^{286} antibody under these conditions.

+ Ca^{2+}/CaM: Autophosphorylation conditions (+ Ca^{2+}/CaM) were used to label phospho-Thr^{286} and other sites. Under these conditions, the phospho-Thr^{305} antibody detected strong immunoreactivity from WT CaMKII and all CaMKII mutants. In contrast, all but the T286A mutant were detected by the phospho-Thr^{286} antibody.

spectrometry will allow precise identification of these sites. Since the specificity of the phospho-Thr^{305} antibody is in question, only the phospho-Thr^{286} antibody was used in subsequent experiments. In the future, affinity purification of the phospho-Thr^{305} antibody may improve the specificity.

Quantitation of Immunoblots and Statistical Analysis

Whole striatal homogenates: Optical densities of total protein loaded in each gel lane (Ponceau S-stained membranes) and specific immunoblotted proteins were measured using NIH Image 1.6 (*http://rsb.info.nih.gov/nih-image*). Immunoblotted protein band densities in each lane were normalized to total protein loaded in the corresponding lane to yield a "normalized immunoblot signal". Statistical comparisons of normalized immunoblot signals were performed using either one or two-way ANOVA with Scheffe's post-hoc tests or post-hoc t-tests, as indicated. For graphing purposes, the mean normalized immunoblot signal obtained from the intact hemisphere of 6-OHDA lesioned rats was set at 100% and values from individual samples were expressed relative to this value.

Subcellular fractions: For analysis of PSD-enriched fractions, the relative "total mass" of the specific protein in each sample was obtained by correcting the "normalized immunoblot signals" for the volume of P2 fraction loaded on each lane and the total volume of the corresponding P2 fraction.

Acute striatal slices: For analysis of data obtained in acute slice experiments, values obtained for all slices from a single animal were normalized to a control slice from

that animal (treated with aCSF alone), then pooled within each experiment. For slices from 6-OHDA lesioned rats, the mean normalized immunoblot signal obtained from the intact hemisphere of 6-OHDA lesioned rats was set at 100% and values from individual samples were expressed relative to this value. Data normalization in this way allowed a more direct comparison between experiments.

All immunoblot signals quantitated were within the linear range of detection. To ensure this, only immunoreactive bands that met the following criteria were quantitated: (1) low immunoreactive signals must be at least 10% higher than the minimum signal detectable by Image J, and (2) strongly immunoreactive signals must fall 10% below the maximal signal detectable by Image J. Any exposures of immunoblots containing bands that fell outside of these criteria were not used in quantitation analysis. If possible, a different exposure of the immunoblot was used, or the data not complying with the above criteria were excluded from further analysis. Finally, only rats with >90% depletion of striatal TH relative to the intact contralateral striatum were included in statistical analyses.

Phosphatase Activity Assays

Punches (1.15 mm ID) of dorsolateral striatum were removed from both hemispheres of 1.0 mm thick coronal slices, at the level of the crossing of the anterior commissure. Tissue was homogenized immediately following dissection in ice-cold homogenization buffer (7 mM Tris-HCl, pH 7.5 containing 0.2 mM EDTA, 0.2 mM EGTA, 320 mM sucrose, 1 mM benzamidine, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μM pepstatin, 50 mM NaF, 50 mM β-glycerophosphate, 20 mM Na pyrophosphate),

using a Kontes tissue homogenizer. Striatal homogenates were immediately frozen at -80ºC, and used for activity assays within two weeks. Whole dorsolateral striatal extracts were assayed for phosphatase activities using [^{32}P]-phosphorylase *a*, [^{32}P]-casein, or [^{32}P-Thr^{286}]-CaMKIIα as previously described (Strack et al., 1997a). Whole striatal extracts (10 µl of 0.1-0.2 mg/ml extract) were incubated (30 min, 30°C) with either [^{32}P]-phosphorylase *a* or [^{32}P]-casein in 50 mM Tris-HCl, pH 7.5, 0.1 M NaCl, 20 mM $Mg(Ac)_2$, 10 mg/ml BSA, 0.2 mM EGTA, 1mM DTT. Assays using [^{32}P]-Thr^{286}-CaMKIIα were incubated at 30°C for 45 min. Assays using [^{32}P]-phosphorylase *a* also included 5 mM caffeine. Assay blanks were incubated without extract. Assays were terminated by addition of 40 µl 40% (w/v) trichloroacetic acid, incubated in ice for 15 min, then centrifuged (10,000 x *g*, 10 min). Supernatants (80 µl) were subjected to liquid scintillation counting to quantify ^{32}P release. PP2A activity was defined as the activity inhibited by 2.5 nM okadaic acid. PP1 activity was defined as the difference in activities measured using 2.5 nM and 2.5 µM okadaic acid. PP1γ_1 activity was defined as the phosphatase activity inhibited by Nb(146-493).

Phosphatase Inhibitor Proteins

Nb(146-453) and Nb(146-493) were prepared as GST fusion proteins and characterized previously (Carmody et al., 2004). Nb(146-453) does not contain the PP1 binding domain (residues 457-460) or the PP1γ1-selectivity domain (residues 473-479) that are present in Nb(146-493). Inhibitor-2 (Calbiochem), and okadaic acid (LC laboratories), were also used in some assays, as indicated.

Immunoprecipitations

Fresh whole striata were homogenized in: 2 mM Tris-HCl, pH 7.5, containing 2 mM EDTA, 2 mM EGTA, 1 mM DTT, 0.2 mM PMSF, 1 mM benzamidine, 40 mg/l soybean trypsin inhibitor, 10 mg/l leupeptin, and 0.5% Triton-X-100. Striatal homogenates were diluted to 1 mg/ml in 500 μl IP buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.5% Triton-X-100), and incubated (rotating, 1 hr., 4°C). Homogenates were centrifuged (10,000 x g, 10 min., 4°C), and the resulting supernatant (homogenate supernatant) was used in immunoprecipitation experiments. Homogenate supernatants were precleared by incubation (rotating 1 hr., 4°C) with gammabind-G Sepharose (Amersham Biosciences) followed by centrifugation. Precleared homogenate supernatants were incubated with either goat preimmune serum, sheep anti-PP1γ1, or sheep anti-PP1β antibodies (rotating, 1 hr., 4°C). Thirty microliters of a 50:50 gammabind-G Sepharose slurry was added, and the incubation continued overnight at 4°C. Immunoprecipitates were sedimented and washed 5 times with 1 ml IP buffer prior to dilution in homogenization buffer (excluding Triton-X-100). 10 μl of the diluted immunoprecipitate pellet was immediately assayed for phosphatase activity, in the presence of 2.5 nM okadaic acid. Aliquots of the immunoprecipitated pellets and supernatants were diluted in SDS-PAGE buffer, fractionated by SDS-PAGE, and immunoblotted.

Acute Striatal Slice Preparation

Acute striatal slices (400 μm thick) were prepared from unlesioned control rats (270-300g), or rats 4 weeks after unilateral 6-OHDA lesion. Following dissection, rat

brains were immediately placed in ice-cold artificial cerebral spinal fluid (aCSF), consisting of 124 mM NaCl, 4.4 mM KCl, 1.2 mM $MgSO_4$, 1.0 mM NaH_2PO_4, 2.5 mM $CaCl_2$, 26 mM $NaHCO_3$, and 10 mM glucose. Coronal slices were made with a vibratome while the brain was submerged in aCSF, oxygenated with 95% oxygen / 5% carbon dioxide. Only slices rostral to the meeting of the anterior commissure were used in these experiments. Slices were transferred to and maintained on submerged nylon mesh chambers in recirculating, oxygenated aCSF at 30ºF, and allowed to equilibrate for 1 hr prior to addition of any drug. Osmolarity of aCSF was monitored at the beginning and end of each experiment, and remained between 290 and 320 mOsm. At the end of each experiment, slices were immediately flash frozen on dry ice, dorsolateral striatum was removed, placed in frozen tubes, and stored at -80ºC until sonicated as indicated above. The use of only dorsolateral striatum compared to the whole striatum resulted in a lower variability in immunoblot signal across different samples. Field recordings (done by Danny Winder) containing N1 (fiber volley response) and N2 (evoked response) verified the health of striatal slices following a 1 hour incubation in aCSF was similar to that seen previously (Norman et al., 2005).

Glutamate agonists / antagonists applied to the striatal slices were as follows: 100 μM NMDA, 5 min. (NMDA receptor agonist) ; 20-200 μM APV, 5 or 20 min. as indicated (selective NMDA receptor antagonist) ; 50 μM AMPA, 5 min. (AMPA receptor agonist); 100 μM ACPD, 20 min. (selective mGluR group I/II agonist). Dopamine agonists applied were: 1 μM SKF81297, 5 min. (D1-like agonist); 1 μM quinpirole, 5 min. (selective D2-like agonist). To inhibit endogenous, spontaneous neuronal activity in striatal slices by blocking voltage-dependent sodium channels, 1 μM TTX was used in

some experiments. Extracellular Ca^{2+} was chelated by application of 3 mM BAPTA for 2-10 min (as indicated).

CHAPTER III

CHRONIC DOPAMINE DEPLETION DISRUPTS NORMAL PHOSPHORYLATION OF INTRACELLULAR SIGNALING PROTEINS

Introduction

Parkinson's Disease (PD) is a neurodegenerative disorder resulting in striatal dopamine depletion that strikes 1-2% of the population over 60 years of age. Normal aging induces morphological changes in striatal medium spiny neurons (MSNs) (Ingham et al., 1989), which constitute ~95% of the total striatal neuron population. However, the loss of nigrostriatal dopamine input results in additional morphological and functional alterations in striatal MSNs. For example, enduring changes in MSN dendritic structure have been reported in Parkinson's Disease and in a rat model of parkinsonism induced by 6-hydroxydopamine (6-OHDA) lesion of the substantia nigra (McNeill et al., 1988; Ingham et al., 1998; Arbuthnott et al., 2000; Meshul et al., 2000; Zaja-Milatovic et al., 2005). Moreover, nigrostriatal dopamine depletion in animal models affects striatal long-term depression (Partridge et al., 2000) and long-term potentiation (Centonze et al., 1999; Picconi et al., 2004; Norman et al., 2005).

Both synaptic and morphological plasticity in hippocampal neurons are modulated by intracellular signaling proteins, such as CaMKII and related proteins (Smart and Halpain, 2000; Winder and Sweatt, 2001; Lisman et al., 2002; Colbran and Brown, 2004). The association of CaMKII, PP1, spinophilin, and neurotransmitter receptors with synapses and postsynaptic densities (PSDs) also is dynamically regulated

[Colbran, 2004a #140; Colbran, 2004b #274; Griffith, 2004 #278; Schulman, 2004 #277; Malinow, 2003 #128]. The morphological and functional effects of striatal dopamine depletion presumably result from changes in the expression and/or function of dendritic cytoskeletal and signaling proteins.

Acute dopamine signaling modulates both NMDA- and AMPA-type glutamate receptors (Svenningsson et al., 2004). Although the effects of chronic dopamine insufficiency associated with PD are poorly understood, studies in animal models have demonstrated modest changes in levels of some glutamate receptor subunits and of PSD-95 in synaptic membranes (Porter et al., 1994; Oh et al., 1999; Dunah et al., 2000; Betarbet et al., 2004; Picconi et al., 2004), as well as altered phosphorylation of the NMDA receptor NR2B, NR2A, and NR1 subunits (Menegoz et al., 1995; Oh et al., 1999; Dunah et al., 2000). However, most of these previous studies analyzed whole striatal samples from relatively young animals (3-6 months), typically within 6 weeks of dopamine depletion. Notably, PD is a progressive disease induced by chronic striatal dopamine depletion, most often in aging individuals. Normal aging modifies synaptic plasticity in the striatum and in other brain regions (Ou et al., 1997; Rosenzweig and Barnes, 2003), and also affects intracellular signaling proteins (Norris et al., 1998; Foster et al., 2001; Foster et al., 2003). Thus, the full manifestation of PD may result from the combined effects of dopamine depletion and normal aging.

Here, I report novel changes in signaling pathways of the dorsolateral striatum that occur in a graded manner following 6-OHDA lesion of the nigrostriatal pathway. Enduring elevations in the phosphorylation of CaMKII and DARPP-32 are seen within 3 weeks, whereas phosphorylation of GluR1 increases only after several months of

dopamine depletion.

Results

Short-term Dopamine depletion enhances Thr^{286} phosphorylation of CaMKIIα

Striatal TH levels were decreased by ~95% at 3-12 weeks after unilateral 6-OHDA injections into the substantia nigra (Fig. 9 A). No changes in total levels of several synaptic proteins were observed, including NMDA receptor NR2B subunit, synaptophysin, CaMKIIα, or CaMKIIβ (Fig. 9 A, B). In contrast, Thr^{286} phosphorylation of CaMKIIα was significantly increased by DA depletion relative to both the contralateral intact striatum, and to control striatum from sham-lesioned or non-lesioned rats (Fig. 9 B).

To test the effects of DA replacement on CaMKIIα phosphorylation, 6-OHDA lesioned rats were treated with either vehicle or L-DOPA for 9 days (see Chapter II). In rats that received vehicle injections, Thr^{286} phosphorylation was significantly elevated in the lesioned striatum relative to intact striatum. However, Thr^{286} phosphorylation in both hemispheres of L-DOPA injected rats was similar to that in the intact striatum from vehicle-injected rats; i.e., L-DOPA treatment completely reversed the increase in Thr^{286} phosphorylation without affecting CaMKII phosphorylation in the intact striatum (Fig. 9). However, L-DOPA did not significantly change the total levels of TH, CaMKIIα, CaMKIIβ, PP1γ1, and spinophilin (data not shown).

PSD-enriched cytoskeletal fractions from the striatum of 6-OHDA lesioned rats sacrificed 3 weeks after surgery were also analyzed by immunoblotting. As seen in whole striatal extracts, Thr^{286} phosphorylation of CaMKIIα in the PSD-enriched fractions was

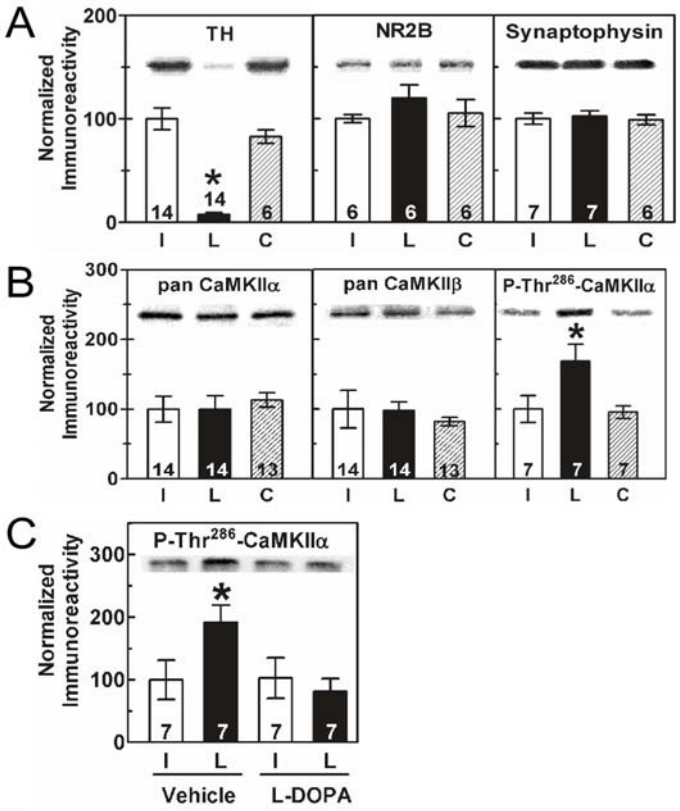

Figure 9. Short-term 6-OHDA lesion increases CaMKII phosphorylation at Thr^{286}.

A,B: Representative blots and summary graphs quantitating total striatal protein levels or phosphorylation (mean ± SEM), in samples harvested 3-12 weeks post-operatively. The number of rats analyzed is indicated within or above each bar. 'L' and 'I' indicate samples ipsilateral and contralateral to the lesion, respectively. 'C' indicates tissue from sham-lesioned rats. Only the decrease in TH ($F_{2,49}$=37.66, *p<0.0001) and the increase of P-Thr^{286}-CaMKII $F_{2,21}$=11.98, *p=0.0003) were significantly altered in dopamine depleted striatum. C: The increase in Thr^{286} phosphorylation of CaMKII is reversed by L-DOPA administration ($F_{1,27}$=5.61, *p=0.026).

significantly elevated. However, total levels of PSD-associated CaMKIIα (but not CaMKIIβ) were slightly but significantly decreased (Fig. 10).

Dopamine depletion does not globally affect PKA substrate phosphorylation

Using an antibody that recognizes phosphorylated serine/threonine PKA substrates containing the motif RRXpS/pT, no significant global change in immunoreactive signal was detected in comparing intact and lesioned hemispheres (Fig. 11). However, two unidentified proteins at ~81 kDa and at ~52 kDa may be more highly phosphorylated in the lesioned hemisphere.

Dopamine depletion does not alter the levels of striatal phosphatases and PP1 regulatory proteins

Increased Thr^{286} phosphorylation of CaMKIIα following DA depletion may result from altered regulation of striatal protein phosphatases, major targets of DA signaling (Svenningsson et al., 2004). We observed no significant changes in total levels of $PP1\gamma_1$ and protein phosphatase 2A (PP2A) catalytic subunits, or of the PP1 scaffolding proteins spinophilin and neurabin 3-12 weeks post-operatively (Fig. 12 A). Moreover, there were no significant changes in the amounts of $PP1\gamma_1$ or spinophilin associated with the PSD-enriched cytoskeletal fraction (Fig. 10). Interestingly, while levels of total DARPP-32 remained unchanged, the phosphorylation of DARPP-32 at Thr^{75} (but not Thr^{34}) was markedly increased in DA-depleted striatum (Fig. 12 B). The repeated L-DOPA injection paradigm (see Methods) completely reversed the increase in Thr^{75} phosphorylation (Fig. 12 C).

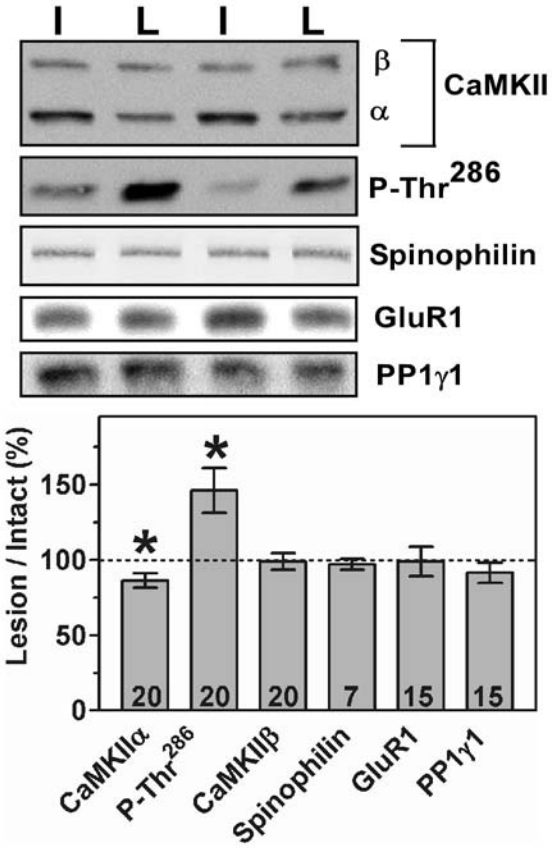

Figure 10. 6-OHDA lesions change PSD-associated CaMKII. PSD-enriched P2 fractions taken from rats 3 weeks after 6-OHDA lesion surgery were analyzed by immunoblotting. The top panel shows representative blots from contralateral (intact) and lesioned hemispheres of two representative animals. The levels in samples from 6-OHDA-lesioned striatum are expressed as a percentage of levels in samples from the contralateral (Intact) striatum (mean ± SEM). Total CaMKII was significantly decreased (t_{18}=2.92, *p=0.009) and Thr^{286} phosphorylated CaMKII was elevated (t_{19}=0.33, *p=0.006) following dopamine depletion, as determined by ANOVA followed by *post-hoc* t-test.

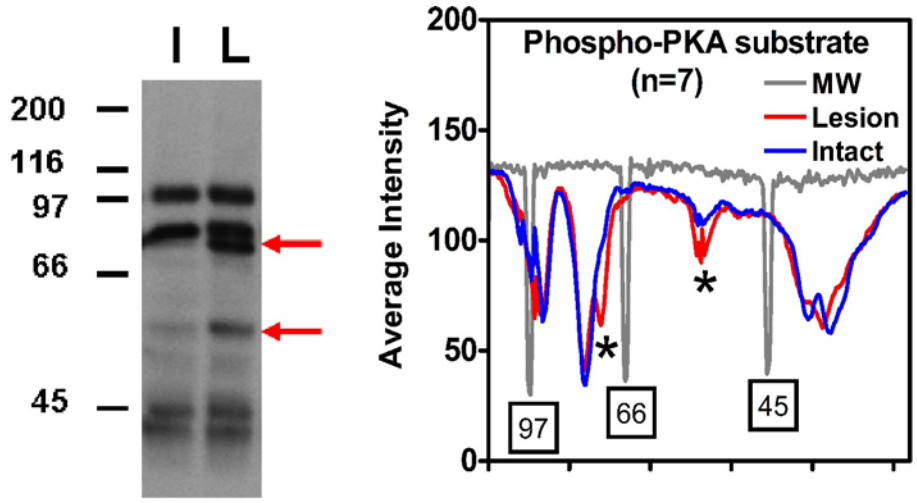

Figure 11. Lack of global increase in PKA substrate phosphorylation.
Whole striatal homogenates from unilaterally 6-OHDA lesioned rats (3 week lesion) were examined by immunoblot, using an antibody that recognizes phosphorylated serine/threonine PKA substrates containing the motif RRXpS/pT (Cell Signaling).
Left: Representative immunoblot from intact (I) or lesioned (L) hemispheres.
Right: Averaged line scans (Image J) from Intact (blue) and Lesioned(red) hemispheres. Relative location of molecular weight markers (97, 66, 45 kDa) is indicated by gray line. Intensity of bands is graphed on an inverted Y-axis scale, from 200(lowest intensity) to 0 (highest intensity). Initial results suggest that there are only two unidentified specific proteins at ~81 kDa and at ~52 kDa that are more highly phosphorylated in the lesioned hemisphere. Importantly, there does not appear to be a global increase in phosphorylation of PKA substrate proteins.

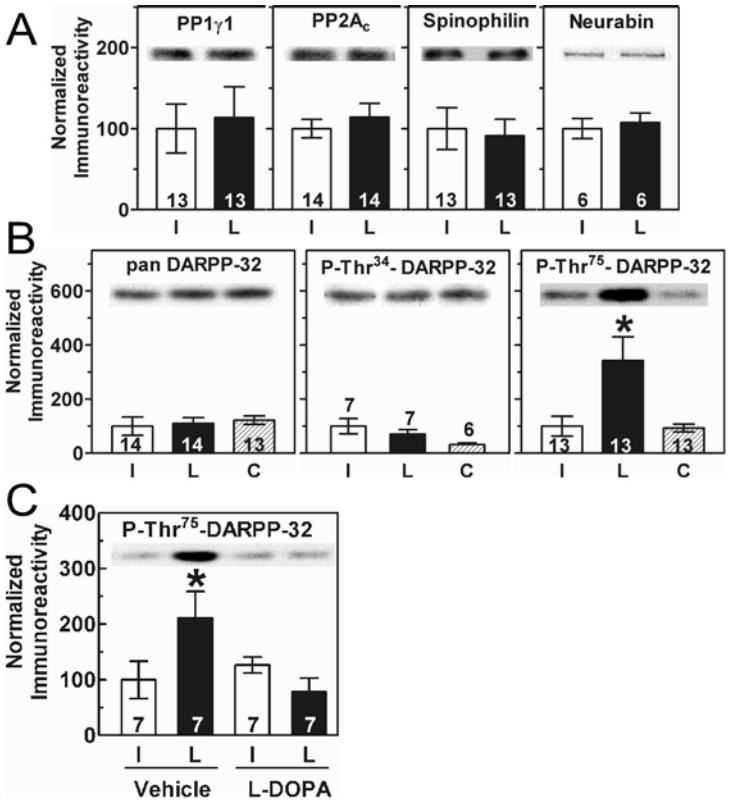

Figure 12. Effects of 6-OHDA lesion on striatal protein phosphatases.

A,B: Representative immunoblots and summary graphs quantitating total striatal levels of protein phosphatase catalytic subunits and PP1 targeting / regulatory proteins. Phosphorylation of DARPP-32 at Thr^{75} was significantly elevated in dopamine-depleted striatal samples ($F_{2,45}$=3.5, *p=0.038). C: The increase in Thr^{75} phosphorylation of DARPP-32 is reversed by L-DOPA administration ($F_{1,27}$=22.13, *p=0.0005).

Aging alters the expression of synapse-associated proteins

Since PD is associated with aging, the effects of aging on signaling proteins in normal and DA-depleted dorsolateral striatum were assessed. There were no significant differences in levels of CaMKIIα, phospho-Thr^{286}-CaMKIIα, phospho-Thr^{34}-DARPP-32, phospho-Thr^{75}-DARPP-32, PP2Ac, or PP2B in striatum from control rats at 4-6 months, 12-14 months, and 21-23 months of age (Fig. 13A, C, and E). However, there was a significant increase in PP1γ_1 and a significant decrease in spinophilin and neurabin in control rats at 21-23 months of age (Fig. 13 F). In addition, there was a trend for decreasing levels of DARPP-32 with normal aging, but this was only statistically significant at 12-14 months of age (Fig. 13 F).

Long-term effects of striatal dopamine depletion

Analysis of dorsolateral striatum from rats sacrificed 9-11 or 18-20 months after 6-OHDA injections (i.e., at 12-14 or 21-23 months of age) revealed results consistent with those in short-term lesioned rats. Total levels of multiple signaling proteins were unchanged after chronic DA depletion (Figs. 14, 15). However, the elevated phosphorylation of CaMKIIα at Thr^{286} and of DARPP-32 at Thr^{75} seen 3-12 weeks post-operatively was also detected at both later time points (Fig. 14).

Long-term dopamine depletion enhances phosphorylation of GluR1 at Ser^{831}

Since aging and chronic DA depletion yield complex changes in the levels and phosphorylation of CaMKIIα, PP1γ_1, and PP1 regulatory proteins, we examined a common downstream target of these enzymes, the GluR1 subunit of the AMPA-type

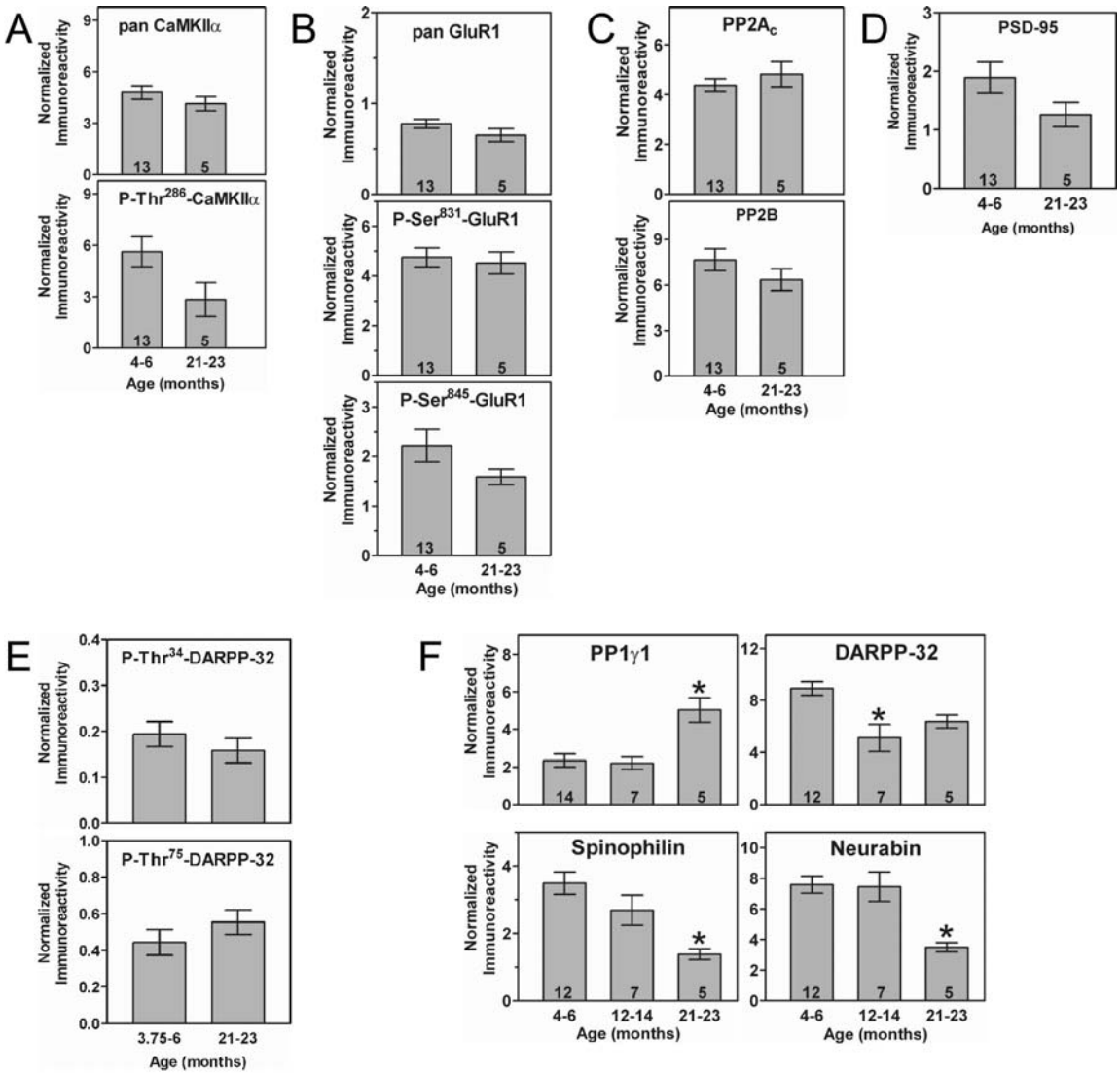

Figure 13. No significant changes in levels of CaMKII , GluR1, PP2Ac, PP2B or PSD95 or phosphorylation of CaMKII and DARPP-32 during normal aging.

Quantitation of proteins levels or protein phosphorylation in dorsolateral striatal homogenates from normal rats at 4-6 months and 21-23 months of age, normalized to total protein loading on the gel. No significant differences were observed, as determined by unpaired t-test. There were no significant changes in phosphorylation of CaMKII, DARPP-32 or GluR1 even when phospho-protein signals were normalized to the total levels of CaMKII, DARPP-32, or GluR1, respectively.

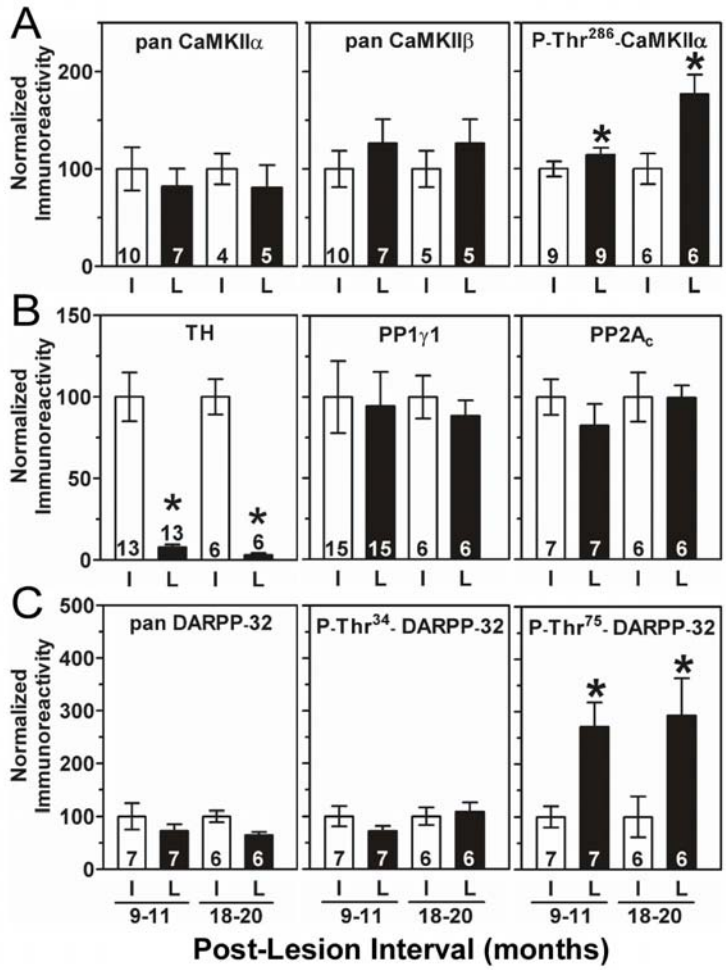

Figure 14. Effects of chronic dopamine depletion on striatal proteins.

A,B,C: Long-term dopamine depletion caused an enduring decrease in TH at 9-11 months (t_{12}=6.82, *p=0.0001) and at 18-20 months (t_5=7.99, *p=0.0005). This was paralleled by an enduring increase in phosphorylation of both CaMKII at Thr^{286} at 9-11 months (t_{11}=2.28, *p=0.043) and 18-20 months (t_5=3.50, *p=0.017) and of DARPP-32 at Thr^{75} at 9-11 months (t_6=5.03, *p=0.0024) and at 18-20 months (t_5=3.46, *p=0.018).

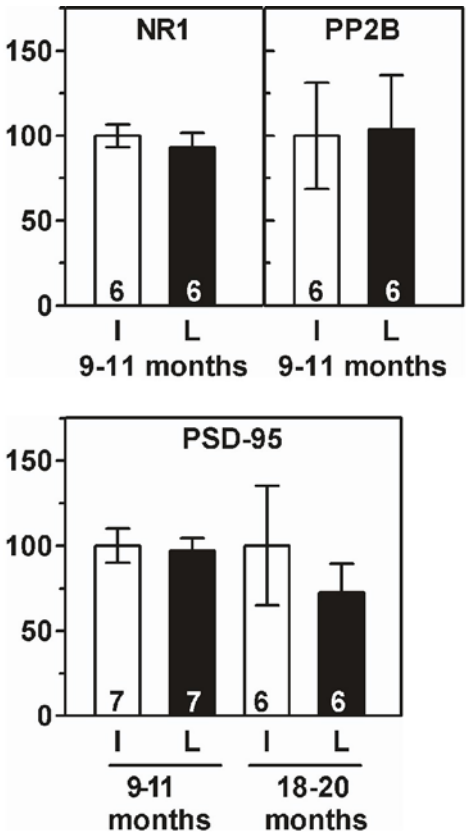

Figure 15. No significant changes in NR1, PP2B, or PSD-95 following chronic dopamine depletion.
Summary graphs of indicated total protein levels in samples harvested from rats 9-11 or 18-20 months after 6-OHDA lesion. Paired t-test (NR1, PP2B) or one-way ANOVA (PSD-95) revealed no significant differences as a result of dopamine depletion.

glutamate receptor. There were no changes in the levels of GluR1 or of the phosphorylation of GluR1 at Ser^{831} or Ser^{845} during normal aging (4-23 months of age; Fig. 13 B). DA depletion had no significant effect on the levels of total GluR1 in whole striatal extracts at any time point (Fig. 16), or in PSD-enriched fractions (Fig. 10). Phosphorylation of GluR1 at Ser^{831} was unaltered 3-12 weeks post-surgery, but was significantly elevated in DA-depleted striatum at both 9-11 and 18-20 months post-operatively (Fig. 16). In contrast, phosphorylation of GluR1 at Ser^{845} was not significantly different between the hemispheres at any time point after surgery (Fig. 16).

Discussion

Dopamine depletion increases Thr^{286} phosphorylation of CaMKIIα

Phosphorylation of CaMKIIα at Thr^{286} was significantly increased within 3 weeks of 6-OHDA lesion surgery in both total homogenates and PSD-enriched subcellular fractions of dorsolateral striatum (Figs. 9, 10). A recent study using whole striatum detected enhanced Thr^{286} phosphorylation of CaMKIIα in a PSD-enriched fraction, but not in the total homogenate (Picconi et al., 2004). Notably, dorsolateral striatum receives the densest nigrostriatal DA inputs (Nakano et al., 2000) perhaps suggesting that this region will be most severely affected by 6-OHDA lesion of the Substantia Nigra. Thus, differences between these studies may reflect the analysis of different parts of the striatum, or the choice of different rat strains.

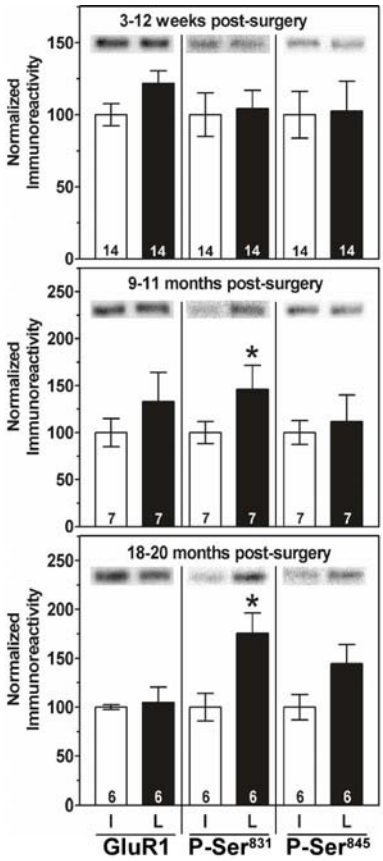

Figure 16. Chronic dopamine depletion selectively increases GluR1 phosphorylation at Ser^{831}.

Total GluR1 levels and levels of GluR1 phosphorylated at Ser^{831} or Ser^{845} at 3-12 weeks, 9-11 months, or 18-20 months following 6-OHDA lesion surgery. There was a significant effect of age ($F_{2,48}$ =4.10, *p=0.0322 for Ser^{831}); *post hoc* tests revealed that Ser^{831} phosphorylation was increased at both 9-11 months (t_6=2.495, *p=0.047) and at 18-20 months (t_4=4.738, *p=0.009) following dopamine depletion. In contrast, there was a trend for increased phosphorylation at Ser^{845} only 18-20 months following surgery (t_5=2.490, p=0.068).

Phosphorylation at Thr^{286} stabilizes CaMKII binding to PSD proteins and synapses in hippocampal neurons (Strack et al., 1997b; Yamauchi and Yoshimura, 1998; Shen and Meyer, 1999; Colbran, 2004a). Therefore, it was surprising that total CaMKIIα levels in the PSD-enriched fractions isolated from the dorsolateral striatum were decreased (Fig. 10). Significant changes in other PSD-associated protein levels were not detected, although quantitative ultrastructural studies may reveal more subtle changes in subcellular distribution. In addition, it should be noted that total CaMKIIα levels in PSD fractions from whole striatum were unaltered (Picconi et al., 2004). A similar decrease in hippocampal PSD-associated total CaMKIIα in the face of enhanced Thr^{286} phosphorylation was also observed in a mouse model of Angelman's Syndrome, which lacks an E6AP-ubiquitin ligase (Weeber et al., 2003). However, it is important to note that CaMKIIα association with PSDs is regulated by additional mechanisms, such as autophosphorylation at $Thr^{305/306}$ (Shen et al., 2000; Elgersma et al., 2002) and a protein kinase C-driven mechanism (Fong et al., 2002), likely involving dynamic interactions of CaMKII with multiple binding partners in the PSD (Colbran, 2004a). It would be interesting to examine the effect of DA depletion on CaMKII phosphorylation at $Thr^{305/306}$, but appropriately specific reagents were not available (see Chapter II). Thus, mechanisms of CaMKII targeting to PSDs in striatum and the role of DA modulation in regulating CaMKII phosphorylation and activity warrant further investigation.

Mechanisms for increased Thr^{286} phosphorylation following dopamine depletion

Thr^{286} autophosphorylation of CaMKII is acutely induced by Ca^{2+} influx via voltage-gated calcium channels or NMDA receptors or by the release of intracellular Ca^{2+}

stores (Hanson and Schulman, 1992). These effects of Ca^{2+} mobilization are opposed and reversed by multiple protein phosphatases (Colbran, 2004b). For example, PSD-associated CaMKII is selectively dephosphorylated by PP1, presumably the PP1γ_1 isoform that appears to be selectively targeted to dendritic spines by binding to spinophilin and/or neurabin (MacMillan et al., 1999; Strack et al., 1999; Terry-Lorenzo et al., 2002; Carmody et al., 2004; Bordelon et al., 2005). Thus, increased Thr^{286} phosphorylation following DA depletion might be due to increased Ca^{2+} mobilization resulting from lack of modulation of corticostriatal excitatory inputs to MSNs, or due to inhibition of protein phosphatases acting on CaMKII.

Many acute effects of DA in the striatum are mediated by modulation of protein phosphatases, especially via the modulation of PP1 by DARPP-32 and spinophilin (Svenningsson et al., 2004). However, we found no evidence for changes in the total levels of multiple phosphatase catalytic subunits or of PP1 regulatory proteins following DA depletion, in either whole dorsolateral striatal extracts or PSD-enriched subcellular fractions. DARPP-32 is phosphorylated at Thr^{34} by PKA following acute D1 receptor activation (presumably decreasing PP1 activity) and dephosphorylated by PP2B in response to activation of D2 receptors, as well as the NMDA-, AMPA-, and mGluR5-type glutamate receptors (Svenningsson et al., 2004; Nishi et al., 2005). Surprisingly, but consistent with prior reports (Picconi et al., 2003; Chergui et al., 2004), there were no significant changes in Thr^{34} phosphorylation at any time point following 6-OHDA lesion. In addition, no significant global change in immunoreactive signal was detected using an antibody that recognizes phosphorylated PKA substrates (Fig. 11). Although immunoreactive signal was elevated for two unidentified proteins, these data as a whole

suggest a lack of global change in PKA activity. These results are consistent with other reports indicating no change in striatal cAMP levels following 6-OHDA lesion (Nash and Brotchie, 2000). Thus, additional cellular mechanisms may normalize DARPP-32 phosphorylation at Thr^{34} after chronic DA depletion.

In contrast to the lack of changes in Thr^{34} phosphorylation, DARPP-32 phosphorylation at Thr^{75} was significantly elevated by DA depletion. Moreover, L-DOPA injections rescued this increase. Thr^{75} is phosphorylated by cyclin-dependent kinase 5 (cdk5) and dephosphorylated by PP2A. PP2A activity may be reduced following DA depletion due to the loss of a D1 receptor mediated, PKA-dependent activation of PP2A (Nishi et al., 2000), or due to CaMKII-dependent inhibition of PP2A (Fukunaga et al., 2000). It is possible that increased glutamatergic signaling following DA depletion also activates cdk5, contributing to the enhanced phosphorylation of DARPP-32 Thr^{75}.

In combination, these data provide no direct evidence for alterations in protein phosphatases following DA depletion, although the data cannot rule out alterations in specific subtypes or changes in activity due to other mechanisms. The elevated phosphorylation of specific residues in CaMKII, DARPP-32, GluR1, and two unidentified specific proteins at ~81 kDa and at ~52 kDa suggest an imbalance in the regulation of as yet unidentified cellular kinases and / or phosphatases. Alternatively, these changes reflect enhanced glutamatergic signaling due to the loss of DA modulation. Further studies will be required to determine the contributions of PP2A and cdk5 to the elevated phosphorylation of DARPP-32 following DA depletion, and whether these changes play a role in enhancing Thr^{286} phosphorylation of CaMKII.

Consequences of aging and chronic dopamine depletion

Increased Thr^{286} phosphorylation of CaMKIIα is evident within 3 weeks of 6-OHDA lesion surgery and is maintained for up to 18 months. However, increased phosphorylation of a well-established CaMKII substrate, Ser^{831} in the AMPA-type glutamate receptor GluR1 subunit, did not become evident until 9 -11 months and was maintained for up to 18 months. Thus, prolonged DA depletion can have biochemical consequences beyond those seen in the shorter-term studies that are typically performed.

GluR1 phosphorylation depends not only on CaMKII activity, but also on the activity of opposing phosphatases, such as PP1/ PP2A and PP2B (Lee et al., 2000; Snyder et al., 2000; Vinade and Dosemeci, 2000). Total PP1/PP2A and PP2B activities increase during aging even though PP2A and PP2B protein levels remain constant (Norris et al., 1998; Foster et al., 2001; Foster et al., 2003) (Fig. 13C). We found that while total levels of the $PP1\gamma_1$ isoform increased significantly with aging, the levels of spinophilin, neurabin and DARPP-32 decreased (Fig. 13 F). Thus, we hypothesize that in young rats, PSD-targeted PP1γ1 prevents the accumulation of Ser^{831} phosphorylated GluR1 following DA depletion; reduced levels of PSD-associated CaMKIIα may also contribute to the lack of increase in Ser^{831} phosphorylated GluR1. However, as levels of spinophilin and neurabin decrease with normal aging, levels of PSD-targeted PP1 may decrease (despite the overall increase in $PP1\gamma_1$ levels), allowing accumulation of Ser^{831} phosphorylated GluR1 in response to increased levels of Thr^{286} phosphorylated CaMKII following DA depletion. Further studies are clearly warranted to understand the interplay between changes in signaling pathways induced by DA depletion and by normal aging.

Relationship of observed changes to synaptic plasticity

DA acutely regulates both CaMKII (Gu and Yan, 2004; Picconi et al., 2004) and AMPA receptors (Snyder et al., 2000; Chao et al., 2002) and CaMKII regulates the unitary conductance and trafficking of AMPA-type glutamate receptors (Malinow and Malenka, 2002; Malinow, 2003; Allen, 2004). Moreover, normal synaptic plasticity requires phosphorylation of CaMKII at Thr^{286} (Lisman et al., 2002; Colbran and Brown, 2004) and of AMPA receptor GluR1 subunits (Lee et al., 2003). Although roles of CaMKII and GluR1 phosphorylation in the striatum are poorly understood, short-term striatal DA depletion disrupts multiple forms of striatal synaptic plasticity (Centonze et al., 1999; Partridge et al., 2000; Norman et al., 2005). Recently, CaMKII inhibitors were shown to rescue a defect in striatal synaptic plasticity following short-term DA depletion (Picconi et al., 2004). However, our data suggest that long-term DA depletion may enhance glutamate receptor-mediated transmission by increasing the levels of Ser^{831} phosphorylated GluR1.

The normal aging-related decrease in spine density of striatal MSNs in the rat (Ingham et al., 1993) likely contributes to an aging-related decline in motor behavior. Aging-related changes in corticostriatal synaptic function are indicated by decreased paired-pulse, post-tetanic, and NMDA receptor-dependent long-term potentiation (Akopian and Walsh, 2006). In addition, less stimulation is required to detect EPSPs in striatal slices from aged rats, suggesting that either more neurotransmitter is released from cortical terminals in response to presynaptic stimulation or that striatal neurons are more excitable (Akopian and Walsh, 2006). Together, these data are consistent with the idea that while the total number of synapses decreases with aging, a compensatory

mechanism allows the remaining synapses to become stronger, as suggested by the elevated levels of phospho-Thr^{286}-CaMKIIα and phospho-Ser^{831}-GluR1 after long-term DA depletion. It will be interesting to determine whether prolonged periods of DA depletion induce additional changes in striatal synaptic plasticity and whether CaMKII inhibitors are similarly effective after GluR1 phosphorylation has increased.

Relationship of observed changes to morphological changes in MSNs

Dendritic morphology of cortical and hippocampal neurons is sensitive to changes in expression of many proteins, including CaMKIIα, PSD-95, neurabin, and spinophilin (Feng et al., 2000; Oliver et al., 2002; Jourdain et al., 2003; Lee et al., 2004; Li et al., 2004; Tang et al., 2004). Given the morphological changes associated with striatal DA depletion in PD and in animal models (Ingham et al., 1998; Arbuthnott et al., 2000; Meshul et al., 2000; Zaja-Milatovic et al., 2005), it is somewhat surprising that total striatal levels of all proteins analyzed here were unchanged, even after prolonged DA depletion. However, our observations confirm and extend previous studies demonstrating a lack of changes in GluR2/3, α-actinin-2, and PSD-95 following 6-OHDA lesion (Dunah et al., 2000), although some studies report changes in levels of other glutamate receptor subunits (Porter et al., 1994; Oh et al., 1999; Dunah et al., 2000; Betarbet et al., 2004; Picconi et al., 2004). In contrast to the modest effects of DA depletion, decreased spinophilin levels (Fig. 13 F) may be associated with aging-related losses of dendritic spines (Ingham et al., 1989; Ou et al., 1997). Thus, morphological changes which occur following DA depletion and during normal aging are likely caused by different cellular mechanisms.

Relevance to Parkinson's Disease

L-DOPA administration completely reversed increases in the phosphorylation of CaMKII and DARPP-32 at Thr^{286} and Thr^{75}, respectively. In our studies, tissue was harvested 16 hours following the final L-DOPA injection and the half-life of L-DOPA is approximately 90 minutes. Thus, little (if any) L-DOPA would be present when animals were sacrificed, consistent with the fact that L-DOPA did not affect phosphorylation of CaMKII or DARPP-32 in the intact (normal) striatum (Figs. 9C, 12C). These data suggest that DA depletion induces significant, sustained alterations in striatal signaling mechanisms that can be reversed by L-DOPA within a few weeks of DA depletion. This mechanism may contribute to the sustained therapeutic benefits of L-DOPA administration during initial phases of PD (Thanvi and Lo, 2004). Additional studies that examine signaling in normal and DA-depleted striatum at various times after 6-OHDA lesion surgery may provide useful insights into the evolving responses to DA replacement therapy in PD.

Idiopathic PD is associated with aging. It will be important to determine whether changes in phosphorylation of CaMKII, DARPP-32, and GluR1 occur in PD, although such studies may be complicated by dephosphorylation of these proteins in postmortem human tissue. Nevertheless, to the best of my knowledge, the slow development of increased GluR1 phosphorylation at Ser^{831} following DA depletion represents the first report of unique biochemical effects of long-term (9-20 months) DA depletion in rodents. These data may be explained by a novel interaction between aging and DA depletion, indicating that short-term DA depletion in animal models of parkinsonism may not fully recapitulate the human disease. Moreover, the evolving responses of signaling proteins

following DA depletion may play a role in the progression of symptoms during PD, as well as in the changing efficacy and debilitating side effects associated with DA replacement therapy.

CHAPTER IV

PHOSPHORYLATION OF CAMKII IS ACUTELY REGULATED BY NMDA RECEPTOR ACTIVATION

Introduction

Parkinson's disease (PD) is a neurodegenerative disorder that arises from the loss of nigrostriatal dopamine (DA) input to the striatum. Terminals of corticostriatal glutamatergic neurons and nigrostriatal dopaminergic neurons converge on dendritic spine heads of striatal MSNs, suggesting that this region of the basal ganglia is in an ideal location for integration of dopamine and glutamate-mediated signaling pathways.

CaMKII is a well established modulator of excitatory transmission in the hippocampus. Activation of the NMDA receptor mediates Ca^{2+}-influx that enhances CaMKII autophosphorylation (Fukunaga et al., 1996). Phosphorylation of CaMKII at Thr^{286} enhances Ca^{2+}-independent CaMKII activity (Hudmon and Schulman, 2002, 2002; Colbran and Brown, 2004) and translocation to the PSD (Strack et al., 1997b). Active CaMKII is necessary for the induction of NMDA receptor-dependent LTP (Lisman and Zhabotinsky, 2001; Lisman et al., 2002).

Previous reports indicate a role for dopaminergic regulation of striatal CaMKII, as DA depletion results in elevated CaMKII phosphorylation at Thr^{286}, which is reversed by chronic L-DOPA administration (Chapter III, Fig. 9C); (Picconi et al., 2004; Brown et al., 2005). The most commonly used therapy for controlling motor symptoms of PD, L-DOPA treatment can cause unwanted motor complications, such as dyskinesias and a "wearing-off" phenomenon, and eventually becomes less effective with long-term use

(Schapira, 2005). The "wearing-off" phenomenon may be related to the pulsatile administration of L-DOPA, and describes the re-emergence or worsening of parkinsonian symptoms prior to taking the next scheduled dose. Acute administration of L-DOPA to rats with unilateral 6-OHDA lesions results in a contralateral turning behavior (Schwarting and Huston, 1996). With more chronic L-DOPA administration, rotation frequency increases (sensitization) and the effects of L-DOPA are maintained for a shorter period of time, reminiscent of the "wearing-off" observed after long-term L-DOPA therapy in human PD patients. This "wearing-off" effect is reversible by administration of the CaMKII inhibitor KN-93 (Oh et al., 1999), suggesting that active CaMKII plays a role in this phenomenon. In addition, CaMKII inhibition also normalizes DA-depletion induced deficits in LTP and behavior (Picconi et al., 2004). In combination, these studies suggest that CaMKII plays a critical role in the development of not only the parkinsonian phenotype, but also some of the side effects associated with chronic L-DOPA treatment.

While it is clear that chronic DA depletion modulates CaMKII autophosphorylation, the roles of glutamate and DA in the normal regulation of striatal CaMKII signaling have not been determined. Further examination of the acute regulation of striatal CaMKII may eventually provide insight into the evolving effectiveness and use of glutamatergic and dopaminergic receptor modulatory drugs as therapies for PD. Here, we show that CaMKII autophosphorylation is acutely regulated by NMDA receptor activation and extracellular Ca^{2+} influx in the normal striatum.

Results

Phosphorylation of CaMKII decreases with time after tissue collection.

It is critical to minimize the variability in CaMKII phosphorylation that may occur due to preparation of acute striatal slices. Therefore, freshly isolated striatal slices were incubated in aCSF over a time course of 0 to 120 minutes. Phospho-Thr^{286}-CaMKII levels in whole tissue extracts were then quantitated by immunoblot. A highly variable level of CaMKII phosphorylation was detected in individual slices immediately after slice preparation, which decreased to a relatively consistent and stable baseline after 60-90 min. incubation in aCSF (Fig. 17).

Striatal CaMKII phosphorylation at Thr^{286} is acutely regulated by NMDA receptors

Glutamate released from corticostriatal synapses is the major excitatory input to the striatum. Glutamate regulates CaMKII via multiple receptor pathways in other brain regions. To determine the acute regulation of CaMKII phosphorylation by NMDA receptor-mediated signaling pathways, increasing concentrations of NMDA were applied to striatal slices. NMDA (20 or 100 μM) increased CaMKII autophosphorylation by 60%, an effect blocked by prior application of APV (Fig. 18 A). In a separate experiment, NMDA again increased CaMKII autophosphorylation (Fig. 18 B), but incubation with APV alone did not significantly alter CaMKII phosphorylation, suggesting that there is a low basal NMDA receptor activity in acute striatal slices. The NMDA-induced increase in CaMKII autophosphorylation was compared with that of GluR1 phosphorylation. NMDA increased phosphorylation of GluR1 at Ser^{831}, but

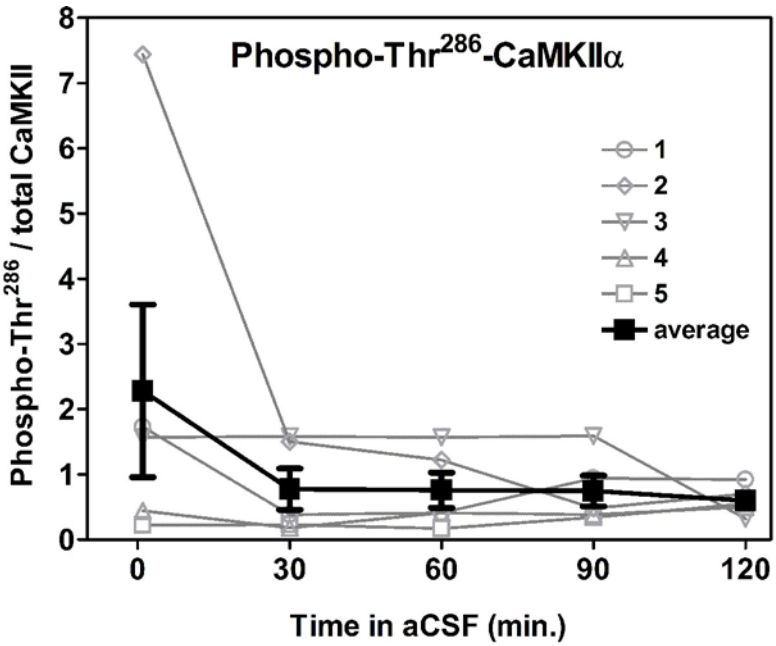

Figure 17. CaMKII autophosphorylation decreases with time in aCSF.

Coronal striatal slices (400 μM) were incubated in aCSF for 0 to 120 minutes. Immunoblot analysis of phospho-Thr^{286}-CaMKII levels is graphed. Data from individual slices (numbered 1-5) are graphed in gray, while the average values (+/- SEM) for each time point are in black.

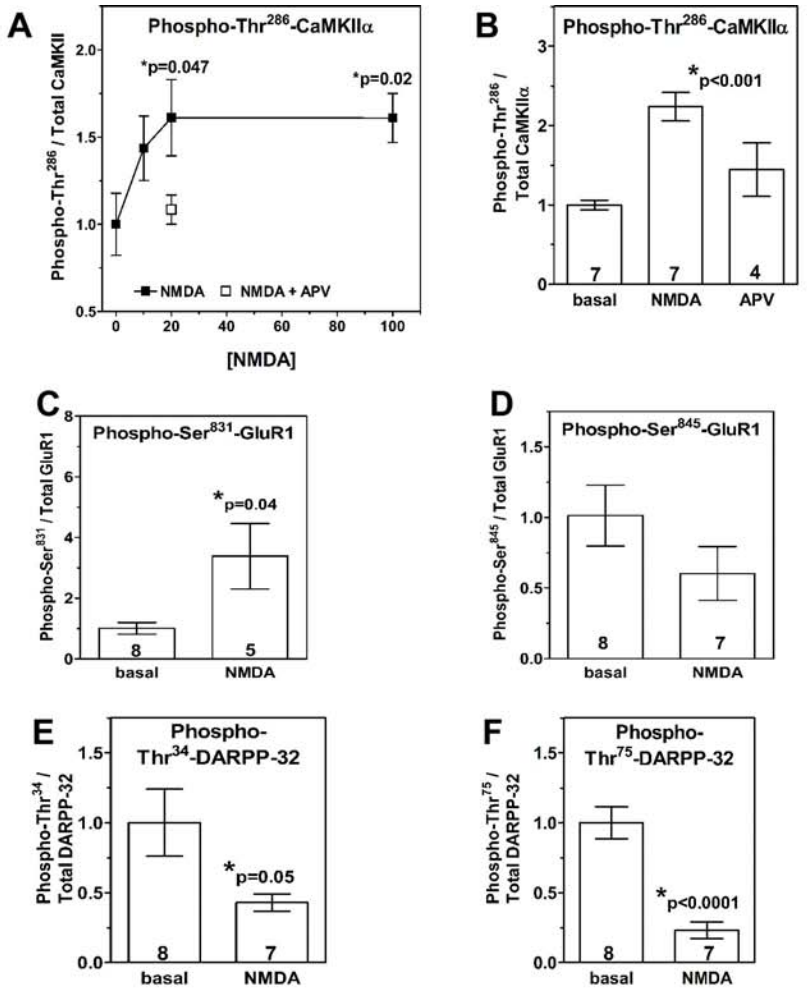

Figure 18. Striatal CaMKII is activated by NMDA receptors.
Acute striatal slices were preincubated in aCSF for 1 hour, and then incubated in 10, 20, or 100 μM NMDA for 5 minutes. One set of slices was preincubated in aCSF for 1 hour, then incubated in 200 μM APV for 10 minutes, followed by addition of 20 μM NMDA was added for 5 minutes (n=6-8 for each treatment). Phospho-protein levels were analyzed by immunoblot. (A) Phospho-Thr^{286}-CaMKII levels were significantly elevated by either 20 μM or 100 μM NMDA, when compared to incubation in 0 μM NMDA (1-way ANOVA, *p=0.045). (B) In a separate experiment, phosphorylation of CaMKII at Thr^{286} was increased by 100 μM NMDA (5 min), but was unchanged by 200 μM APV (20 min). (C,D) 100 μM NMDA incubation (5 min) both increased phospho-Ser^{831}-GluR1 levels and decreased phospho-Ser^{845}-GluR1 levels. (D, E) NMDA (100 μM, 5 min) decreased levels of both phospho-Thr^{34}-DARPP-32 and phospho-Thr^{75}-DARPP-32 in striatum. For all proteins examined, significant differences from basal are noted within each panel, as determined by *post-hoc* paired t-tests for each condition.

caused an insignificant decrease in phosphorylation at Ser^{845} (Fig. 18 C, D), consistent with previous reports in hippocampal tissue (Lee et al., 1998; Vanhoose and Winder, 2003). Furthermore, phosphorylation of striatal DARPP-32 at Thr^{34} and Thr^{75} was decreased by NMDA (Fig. 18 E, F), consistent with a previous report (Nishi et al., 2002). We also investigated the role of AMPA-type and metabotropic glutamate receptors in regulating autophosphorylated striatal CaMKII. Neither the AMPA receptor agonist AMPA (Fig. 19) nor the mGluR group I and II agonist ACPD had any significant effect on CaMKII phosphorylation (Fig. 20). However, phosphorylation of DARPP-32 at Thr^{34} and Thr^{75} was regulated by these agents. In combination, these results indicate that striatal glutamate elevates Thr^{286} phosphorylation of striatal CaMKII primarily via activation of NMDA receptor.

CaMKII is highly phosphorylated at Thr^{286} in striatal slices

We next compared the level of phospho-Thr^{286}-CaMKII immunoreactivity in acute striatal slices (incubated 1 hr. in aCSF) with acute hippocampal slices treated in an similar manner (Vanhoose and Winder, 2003). Inclusion of samples from both striatal and hippocampal slices on the same immunoblot enabled direct comparison of phospho-protein levels. The level of phospho-Thr^{286}-CaMKII in striatal slices was twofold higher than in hippocampal slices (Fig. 21). In comparison, levels of a CaMKII substrate, phospho-Ser^{831}-GluR1, were higher in the hippocampus, while phospho-Ser^{845}-GluR1 levels were similar in both tissues. These results indicate that under basal conditions CaMKII is more highly phosphorylated in the striatum than in the hippocampus.

We next sought to understand why CaMKII is so highly phosphorylated in the striatum

under basal incubation conditions. Release of endogenous neurotransmitters due to spontaneous activity may stimulate Ca^{2+} influx and / or inhibit phosphatases, resulting in high levels of phospho-Thr^{286}-CaMKII. However, blockade of spontaneous activity in the slice by incubation with the voltage-gated sodium channel blocker TTX (1 μM, 30 min) had no significant effect on phospho-Thr^{286}-CaMKII levels (Fig. 22).

We then explored the impact of removing extracellular Ca^{2+}, which would prevent entry of Ca^{2+} via multiple pathways. Addition of an extracellular Ca^{2+} chelator (BAPTA) to the aCSF resulted in a greater than 90% decrease in levels of phospho-Thr^{286}-CaMKII within 3 min (Fig. 23 A). This was accompanied by a more modest, but significant reduction in GluR1 phosphorylation at Ser^{831}, with no change in Ser^{845} phosphorylation (Figs. 23 B, C). In combination, these data suggest that Ca^{2+} influx via a pathway that does not require spontaneous activity is driving the high basal levels of phospho-Thr^{286}-CaMKII under basal conditions.

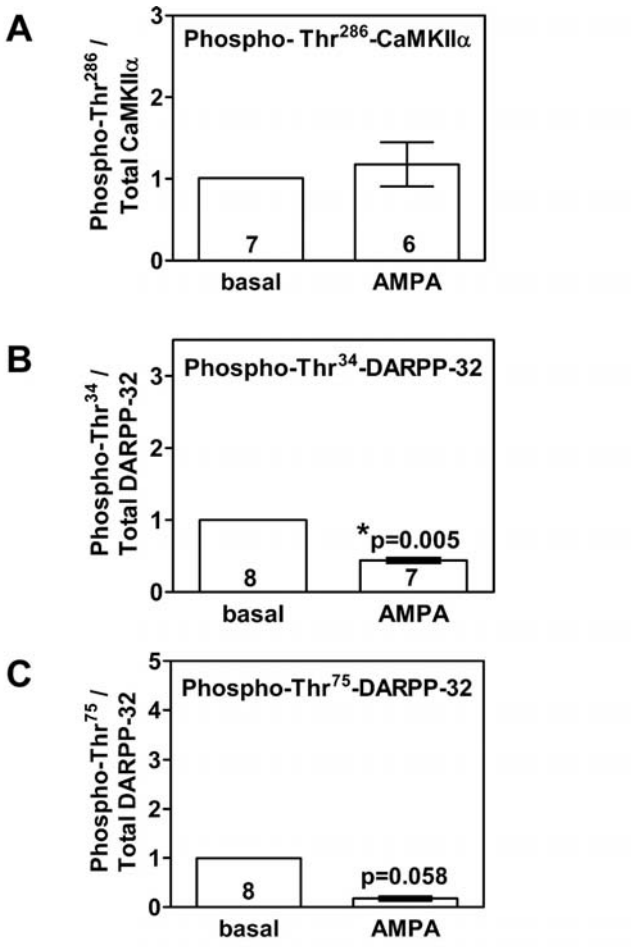

Figure 19. Striatal CaMKII is not modulated by AMPA receptors. Acute striatal slices were preincubated in aCSF for 1 hour prior to addition of 50 μM AMPA (5 min.). Phospho-protein levels were analyzed by immunoblot. (A) AMPA treatment did not alter phospho-Thr^{286}-CaMKII levels. (B, C) Phospho-Thr^{34}-DARPP-32 and phospho-Thr^{75}-DARPP-32 levels were both significantly decreased by AMPA. For all proteins examined, *post-hoc* paired t-tests for each condition significantly different from basal are noted within each figure.

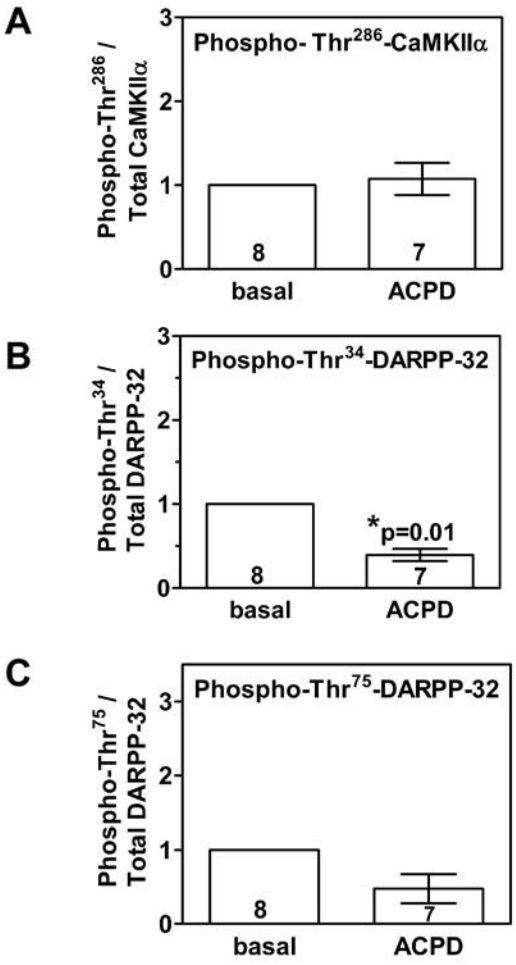

Figure 20. Striatal CaMKII is not significantly modulated by activation of metabotropic glutamate receptors.
Acute striatal or hippocampal slices were preincubated in aCSF for 1 hour prior to addition of 100 μM ACPD (20 min.). Phospho-protein levels were analyzed by immunoblot. (A, C) ACPD treatment did not significantly alter phospho-Thr^{286}-CaMKII or phospho-Thr^{75}-DARPP-32 levels. (B) ACPD treatment resulted in a significant decrease in phospho-Thr^{34}-DARPP-32 levels. For all proteins examined, paired t-tests for each condition significantly different from basal are noted within each figure.

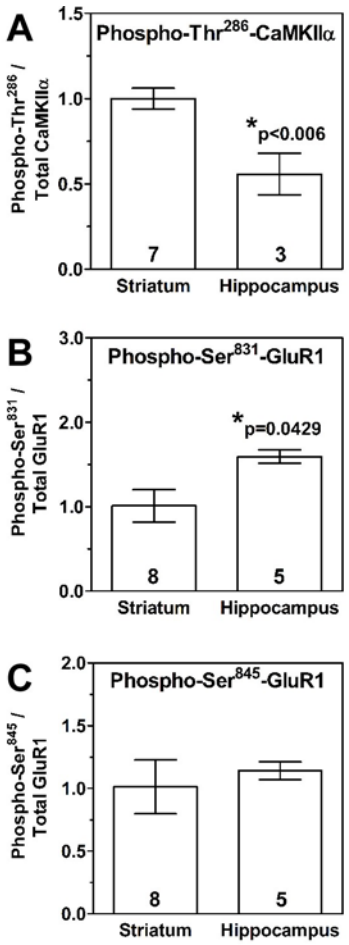

Figure 21. Basal levels of CaMKII autophosphorylation in striatum and hippocampus.

Acute slices from striatum were incubated for 1 hour in aCSF, and phospho-protein levels were analyzed by immunoblot. Tissue from hippocampal slices treated in a similar manner were provided by A. Vanhoose. (A) Phospho-Thr^{286}-CaMKII levels in striatal slices was twofold higher than in hippocampal slices (*p<0.01, unpaired t-test). (B) Phospho-Ser^{831}-GluR1 levels were significantly increased in hippocampal slices (*p=0.04, unpaired t-test). (C) Levels of phospho-Ser^{845}-GluR1 were not significantly different (p= 0.6, unpaired t-test). The number of slices is indicated within each bar.

Discussion

This chapter describes the first efforts to define the acute regulation of striatal CaMKII by glutamate. The regulation of CaMKII by glutamate has been studied extensively in other brain regions. While previous studies have examined the glutamatergic regulation of phosphatases and other proteins in the striatum, we are not aware of any reports on the acute impact of these signaling pathways on CaMKII.

Role of Glutamate in regulating CaMKII autophosphorylation

Glutamatergic regulation of CaMKII autophosphorylation has been previously defined in other brain regions. The contributions of NMDA and AMPA receptors to synaptic Ca^{2+} signaling depends on the activation state of the MSNs in acutely isolated slices. The majority of Ca^{2+} influx occurs through Ca^{2+}-permeable AMPA receptors in the downstate (Carter and Sabatini, 2004). In contrast, membrane depolarization to the upstate switches the source of Ca^{2+} influx to NMDA receptors (Carter and Sabatini, 2004). However, the contributions of NMDA or AMPA receptor-mediated Ca^{2+} influx on striatal CaMKII phosphorylation have not previously been characterized. Our data indicate that neither AMPAR nor mGluR regulation significantly alters CaMKII autophosphorylation. In contrast, activation of NMDAR significantly increased CaMKII autophosphorylation.

Despite the widespread effects of glutamate on other signaling proteins, we found that CaMKII autophosphorylation in the striatal slice was significantly enhanced by NMDA, and not by AMPA or mGlu receptor signaling pathways. I confirmed the

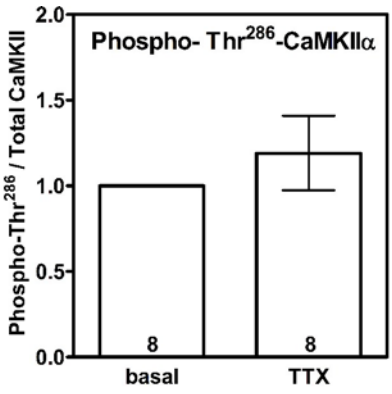

Figure 22. Spontaneous activity does not modulate CaMKII phosphorylation at Thr^{286}.
Striatal slices were preincubated in aCSF for 1 hour, followed by incubation with 1 μM TTX for 30 minutes. Immunoblot analysis revealed that TTX has no effect on phospho-Thr^{286}-CaMKII levels (n=8, p=0.4, paired t-test)

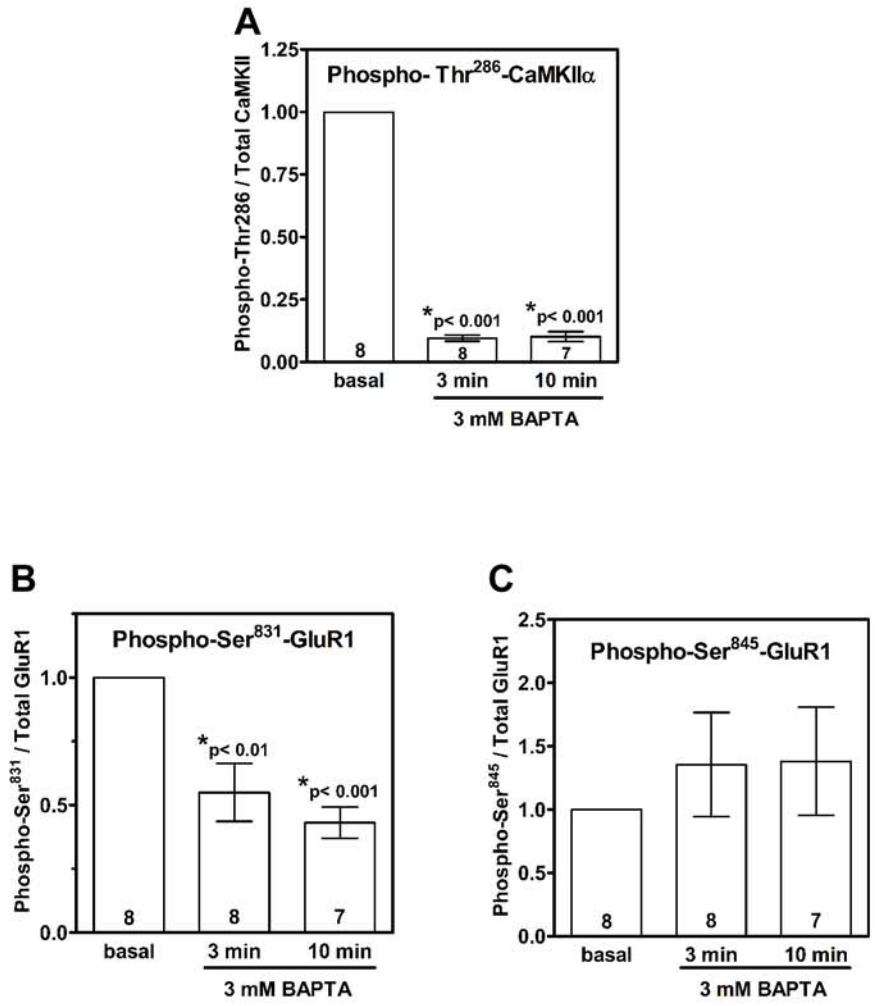

Figure 23. Chelation of extracellular Ca^{2+} rapidly reduces CaMKII autophosphorylation at Thr^{286}.

Acute striatal slices were preincubated in aCSF for 1 hour followed by incubation in aCSF alone (basal, n=8) or with 3mM BAPTA for either 3 or 10 minutes. Phospho-protein levels were analyzed by immunoblot. (A) Incubation in BAPTA for either 3 or 10 minutes decreased levels of phospho-Thr^{286}-CaMKII (1-way ANOVA: *p<0.0001). (B) Incubation with BAPTA also decreased levels of a CaMKII substrate, phospho-Ser^{831}-GluR1 (1-way ANOVA: *p<0.0001). (C) Levels of Phospho-Ser^{845}-GluR1 were not altered by incubation with BAPTA for either 3 or 10 minutes (1-way ANOVA: p=0.667). For all proteins examined, *post-hoc* paired t-tests for each condition significantly different from basal are noted within each figure. These experiments were performed by Salihah Dick.

reported response of different phospho-proteins to these various drugs, and despite the variations in experimental protocols, the responses of phospho-DARPP-32 and phospho-GluR1 were consistent with those found in previous literature.

CaMKII is highly phosphorylated at Thr^{286} in striatal slices

CaMKII autophosphorylation at Thr^{286} is twofold higher in striatal slices than in hippocampal slices. We considered several factors that may contribute to this difference. Spontaneous release of glutamate does not appear to be responsible since antagonists of NMDAR, AMPAR, or mGluR receptors have no significant effect on CaMKII autophosphorylation (data not shown). Moreover, spontaneous release of acetylcholine from cholinergic interneurons activates postsynaptic L-type Ca^{2+} channels on MSNs (Olson et al., 2005). However, blockade of action potentials in striatal slices using the voltage-gated Na^{+} channel blocker TTX had no effect on basal levels of CaMKII autophosphorylation. Finally, there may be a high basal Ca^{2+} efflux from intracellular stores. Any combination of these Ca^{2+} sources may mediate the high basal CaMKII autophosphorylation in striatal slices.

Basal CaMKII autophosphorylation is regulated by extracellular Ca^{2+}

Extracellular Ca^{2+} can enter the postsynaptic cell through voltage-gated ion channels, such as voltage-gated calcium channels (VGCC) or Ca^{2+}-permeable AMPA receptors. Basal influx of extracellular Ca^{2+} appears to promote CaMKII autophosphorylation in the striatum (Fig. 23 A), by an undetermined route of entry.

While postsynaptic Ca^{2+} influx plays a role in directly enhancing striatal CaMKII phosphorylation, presynaptic Ca^{2+} influx essential for neurotransmitter release may also play a role. It is possible that BAPTA-mediated inhibition of spontaneous neurotransmitter release or decrease in quantal size plays a role in lowering CaMKII autophosphorylation (Hardingham et al., 2006). This scenario appears unlikely, as inhibition of spontaneous neurotransmitter release by TTX had no effect on CaMKII autophosphorylation.

A high influx of extracellular Ca^{2+} in the absence of any external stimuli is surprising, although spontaneous Ca^{2+} transients have been observed in acute striatal slices (Osanai et al., 2006). Spontaneous, brief elevations in cytoplasmic Ca^{2+} may originate from the release of Ca^{2+} from intracellular stores, a possibility not addressed by experiments presented in this dissertation. Decreased extracellular Ca^{2+} results in a time-dependent dephosphorylation of CaMKII in cultured dorsal root ganglion neurons (Cohen and Fields, 2006). The decreased CaMKII autophosphorylation results from depletion of Ca^{2+} from intracellular stores without changing bulk intracellular Ca^{2+}. Although low extracellular Ca^{2+} decreases CaMKII autophosphorylation in both dorsal root ganglion cultures and hippocampal neuronal cultures (Scholz and Palfrey, 1998; Cohen and Fields, 2006), a rapid decrease is observed here in CaMKII autophosphorylation in striatal slices. This rapid CaMKII dephosphorylation in striatal slices suggests one or both of the following: (1) that striatal phosphatase activity is very high, and (2) a strong drive for enhanced CaMKII autophosphorylation may be present in striatal slices.

Implications for Parkinson's Disease

Previous reports indicate a role for DA in the regulation of striatal CaMKII, since DA depletion results in elevated CaMKII phosphorylation at Thr^{286} that can be reversed by repeated administration of L-DOPA (Chapter III, Fig. 9); (Brown et al., 2005). Treatment with L-DOPA is initially effective in controlling motor symptoms of PD, but commonly causes unwanted motor complications such as dyskinesias and a "wearing-off" phenomenon, and eventually becomes less effective with long-term use. Enhanced CaMKII activity has been previously implicated in the "wearing-off" phenomenon induced by chronic L-DOPA in the 6-OHDA lesioned rat. DA agonists are commonly used medications for PD patients, to help control motor symptoms early in the course of the disease. Unfortunately, side effects similar to those seen in animals treated with L-DOPA gradually develop after prolonged use. Therefore, a better understanding of the biochemical pathways modulated by DA and glutamate receptor signaling in the striatum may aid in future evaluation of aberrant signaling following DA depletion. Importantly, defining the normal DA and glutamate receptor regulation of CaMKII may help to determine how such signaling pathways are altered after DA depletion and long-term L-DOPA therapy.

Determining the acute regulation of CaMKII in the striatal slice by DA and glutamate will ultimately help in understanding the changes in biochemical signaling pathways that occur following long-term L-DOPA therapy. DA depletion alters NMDA receptor phosphorylation, which may modulate their subcellular distribution (Raman et al., 1996; Tingley et al., 1997; Dunah and Standaert, 2001; Dunah et al., 2004; Hallett et al., 2006). Similarly, DA depletion increases D_2 receptor number (Araki et al., 1998) and

B_{max} (Cai et al., 2002). Therefore, CaMKII regulation by NMDA receptor signaling is likely altered following DA depletion. It will be important for future studies to determine the effects of DA depletion and L-DOPA therapy in the acute regulation of CaMKII. In addition, it will be informative to investigate how DA treatment modulates the NMDA-induced elevation in CaMKII autophosphorylation. Targeting striatal Ca^{2+}-sensitive signaling pathways that are misregulated following DA depletion may be a useful strategy for treatment of PD.

CHAPTER V

DOPAMINE DEPLETION SELECTIVELY ALTERS THE BALANCE BETWEEN PP1 AND CAMKII IN THE DORSOLATERAL STRIATUM

Introduction

Calcium/calmodulin-dependent protein kinase II (CaMKII) and protein phosphatase 1 (PP1) are critical for synaptic plasticity. Autophosphorylation of CaMKII at Thr^{286} is required for normal long-term potentiation (LTP) and hippocampus-based learning and memory (reviewed in (Lisman et al., 2002)). PP1 selectively dephosphorylates CaMKII that is associated with PSDs, whereas soluble CaMKII is primarily dephosphorylated by PP2A (Strack et al., 1997a; Strack et al., 1997b), and inhibition of PP1 via cAMP-dependent pathways promotes autophosphorylation at Thr^{286} and LTP induction (Blitzer et al., 1998; Brown et al., 2000). Spinophilin and neurabin are similar F-actin-binding proteins that selectively target the $PP1\gamma_1$ isoform to dendritic spines, modulating spine morphology and synaptic plasticity (MacMillan et al., 1999; Strack et al., 1999; Feng et al., 2000; Oliver et al., 2002; Terry-Lorenzo et al., 2002; Carmody et al., 2004; Bordelon et al., 2005; Terry-Lorenzo et al., 2005; Allen et al., 2006). Thus, coordinated regulation of hippocampal CaMKII and PP1 is critical for normal physiological responses.

Loss of nigrostriatal dopamine inputs in Parkinson's Disease or in parkinsonian animal models results in morphological alterations in striatal medium spiny neurons (MSNs) (McNeill et al., 1988; Ingham et al., 1998; Meschul et al., 1999; Arbuthnott et al., 2000; Zaja-Milatovic et al., 2005; Day et al., 2006), which constitute >90% of the

total striatal neuron population, and impairment of multiple forms of corticostriatal synaptic plasticity (Centonze et al., 1999; Centonze et al., 2001; Picconi et al., 2003; Norman et al., 2005). Symptoms of Parkinson's Disease initially respond to dopamine replacement therapy, but as the disease progresses this approach generates debilitating side-effects and/or loses efficacy. The study of animal models (e.g., the 6-OHDA lesioned rat) has provided innumerable insights about terminal consequences of striatal dopamine depletion and the mechanisms underlying striatal deficits in Parkinson's Disease, resulting in improved therapeutic strategies (reviewed in (Cenci et al., 2002)).

Dopamine depletion increases Thr^{286} autophosphorylation of CaMKII and this increase is reversed by dopamine-replacement using L-DOPA (Picconi et al., 2004; Brown et al., 2005). Moreover, CaMKII inhibitors normalize dopamine depletion-induced alterations in both synaptic plasticity and behavior (Oh et al., 1999; Picconi et al., 2004), suggesting that altered regulation of CaMKII plays a critical role in the parkinsonian phenotype. The enhanced phosphorylation of CaMKII at Thr^{286} is sustained at similar levels for up to 18 months after inducing dopamine depletion (Brown et al., 2005). However, increased phosphorylation of a downstream CaMKII target, Ser^{831} in the AMPA-type glutamate receptor GluR1 subunit, is detected 9-18 months, but not 3-6 weeks, after dopamine depletion, (Brown et al., 2005). These data suggest complex interactions between the effects of long-term dopamine depletion and aging that may have additional effects on striatal function.

Striatal dopamine depletion could increase CaMKII autophosphorylation by at least two potentially linked mechanisms. First, increased corticostriatal glutamatergic drive (Meschul et al., 1999; Jonkers et al., 2002) might activate NMDA receptors or voltage-

gated Ca^{2+} channels, enhancing postsynaptic Ca^{2+} influx and CaMKII autophosphorylation. Second, reduced protein phosphatase activity(ies) might allow increased phosphorylation of CaMKII and other proteins. Many acute effects of dopamine are thought to require inhibition of striatal PP1 by the Thr^{34}-phosphorylated form of DARPP-32 (Svenningsson et al., 2004; Pisani et al., 2005), although reduced PP1 activity has not been directly demonstrated. Regulation of PP1 localization by spinophilin and neurabin also is critical for normal synaptic plasticity and dendritic spine morphology (Surmeier et al., 1995; Yan et al., 1999; Feng et al., 2000; Allen et al., 2006). Although total striatal levels of spinophilin, neurabin, DARPP-32 and the $PP1\gamma_1$isoform are not significantly affected when analyzed at times ranging from 3 weeks to 18 months following dopamine depletion, phosphorylation of DARPP-32 at Thr^{75}, but not at Thr^{34}, is increased at all time points (Brown et al., 2005). Here, we report that striatal DA depletion causes a selective decrease in the activity of a specific PP1 isoform, $PP1\gamma_1$. This chronic reduction of $PP1\gamma_1$ activity may play a key role in mediating changes in synaptic morphology and/or function by allowing enhanced phosphorylation of multiple synaptic proteins, including CaMKIIα.

Results

Protein phosphatase activities and/or localization can be regulated by direct phosphorylation of their catalytic subunits, or by phosphorylation of their regulatory subunits. Thus, we developed conditions for tissue homogenization that would limit dephosphorylation of putative regulatory sites following homogenization of striatal samples, but allowed for detection of phosphatase activities. Organic phosphatase

inhibitors such as microcystin LR are effective at preventing protein dephosphorylation following homogenization, but they are essentially irreversible. Indeed, no activity was detected in striatal homogenates when homogenates containing microcystin LR were diluted 10-fold immediately prior to the phosphatase assays (data not shown). Therefore, a mixture of inorganic phosphatase inhibitors was included in the tissue homogenization buffer (see Chapter II), allowing the effective detection of PP1 and PP2A activity if striatal homogenates were diluted 10-fold immediately prior to the assay.

Total phosphatase activity is decreased following DA depletion

We measured phosphatase activities in extracts of dorsolateral striatum ipsilateral or contralateral to 6-OHDA lesion of the substantia nigra. Extracts were prepared from different batches of animals 3-4 weeks or 10-11 months after lesion surgery in order to assess consequences of long-term dopamine depletion, mimicking Parkinson's Disease in humans. Activity in the DA-depleted striatum was significantly decreased by 18% using [^{32}P]-phosphorylase *a* as a model substrate when measured 3-4 weeks after lesion surgery (Fig. 24 A), or by 14% when measured 10-11 months after surgery (data not shown; n=13, p = 0.041). In contrast, there was no significant difference in total phosphatase activities at either time point when [^{32}P]-casein was used as the substrate (Fig. 24 B and data not shown).

DA depletion selectively decreases PP1 activity

The specific enzymes detected in phosphatase assays of whole tissue homogenates depends on the substrate used. Generally, [^{32}P]-phosphorylase *a* is considered a PP1-

selective substrate, but also detects PP2A activity, whereas [^{32}P]-casein is a selective PP2A substrate (Cohen, 1989). In order to more selectively measure PP1 and PP2A activities in striatal extracts from DA-depleted rats, I re-assessed phosphatase activites toward [^{32}P]-phosphorylase *a* and [^{32}P]-casein in the presence of either 0, 2.5 nM, or 2.5 μM okadaic acid. Okadaic acid is a selective PP2A inhibitor when used in low nanomolar concentrations, but will additionally inhibit PP1 at low micromolar levels (Cohen, 1991). Thus, I defined PP2A activity as the amount of activity inhibited by 2.5 nM okadaic acid, and PP1 activity as the difference in activities measured at 2.5 nM and 2.5 μM okadaic acid. PP1 activity detected using [^{32}P]-phosphorylase *a* was significantly decreased by 22% at 3-4 weeks following lesion surgery (Fig. 25 A) and by 16% at 10-11 months after surgery (data not shown; n=13, p= 0.014). However, PP2A activity detected using either [^{32}P]-phosphorylase *a* or [^{32}P]-casein substrate was not significantly different between the lesioned and intact striata when measured 3-4 weeks (Fig. 25 B) or 10-12 months (data not shown; n=13, p=0.153) after lesion surgery.

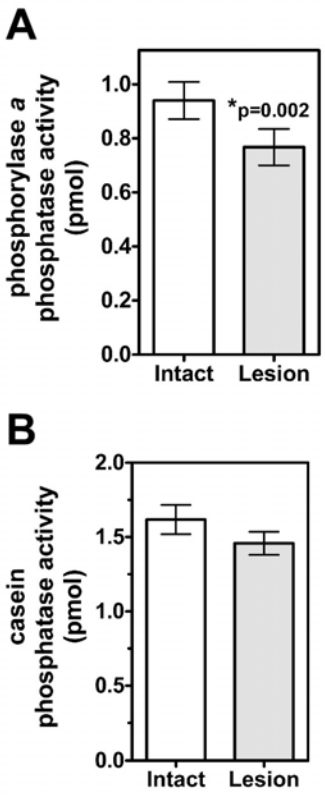

Figure 24. Total striatal phosphatase activity is decreased following DA depletion.
Dorsolateral striatal tissue ipsilateral (Lesion) or contralateral (Intact) to the 6-OHDA lesion was collected 3-4 weeks after surgery and whole extracts were assayed using [^{32}P]-phosphorylase *a* or [^{32}P]-casein as model substrates. (A) Total [^{32}P]-phosphorylase phosphatase activity was decreased by 18% in the lesioned hemisphere (n=8, paired t-test). (B) There was no significant difference in the total [^{32}P]-casein phosphatase activity between striatal hemispheres (n=8, p= 0.09, paired t-test).

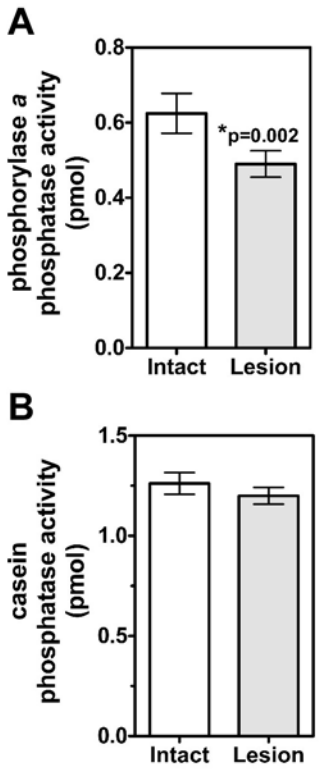

Figure 25. Dopamine depletion selectively decreases PP1 activity.

Phosphatase activities in extracts of dorsolateral striatum taken from rats 3-4 weeks after 6-OHDA lesion surgery were assayed using [^{32}P]-phosphorylase *a* or [^{32}P]-casein in the presence of either 0, 2.5 nM, or 2.5 μM okadaic acid. Selective contributions of PP1 and PP2A to these activities were calculated as described in Methods. (A) PP1 activity measured using [^{32}P]-phosphorylase *a* was significantly decreased by approximately 22% (n=8, paired t-test). (B) PP2A activity measured using [^{32}P]-casein was not significantly altered following DA depletion (n=8, p=0.280, paired t-test).

DA depletion inhibits PP1-mediated activity toward CaMKIIα

In order to ascertain whether DA depletion affects dephosphorylation of a physiologically relevant substrate, I assayed phosphatase activity toward exogenous Thr^{286}-autophosphorylated CaMKIIα in the presence of various concentrations of okadaic acid. The total phosphatase activity toward [^{32}P-Thr^{286}]-CaMKIIα was not significantly different between intact and DA depleted striatal extracts. As previously reported in whole forebrain extracts (Strack et al., 1997a), approximately 80% of this activity could be assigned to PP2A based on the sensitivity to 2.5 nM okadaic acid, but this PP2A activity was unaffected by DA depletion. In contrast, PP1 activity toward [^{32}P-Thr^{286}]-CaMKIIα (defined as the difference in activities at 2.5 nM and 2.5 μM okadaic acid) was significantly decreased by ~19% 3-4 weeks after DA-depletion surgery (Fig. 26).

Phosphatase isoform levels are unchanged by DA depletion

In order to examine the possibility that the decreased phosphatase activity in DA depleted striatum is due to changes in the levels of specific phosphatase catalytic subunit isoforms, aliquots of striatal extracts used in activity assays in Figs. 24-26 were immunoblotted for various isoforms of PP1 (α, β, γ_1) and PP2A. These proteins are highly expressed in MSNs, but are also present in presynaptic terminals. Despite substantial loss of dopaminergic terminals, as reflected by the loss of >90% of tyrosine hydroxylase, there were no significant differences in levels of PP1 and PP2A catalytic isoforms between intact and lesioned hemispheres (Fig. 27). This may reflect the relatively low expression of these proteins in dopaminergic terminals and/or the small contribution of these terminals to total striatal tissue.

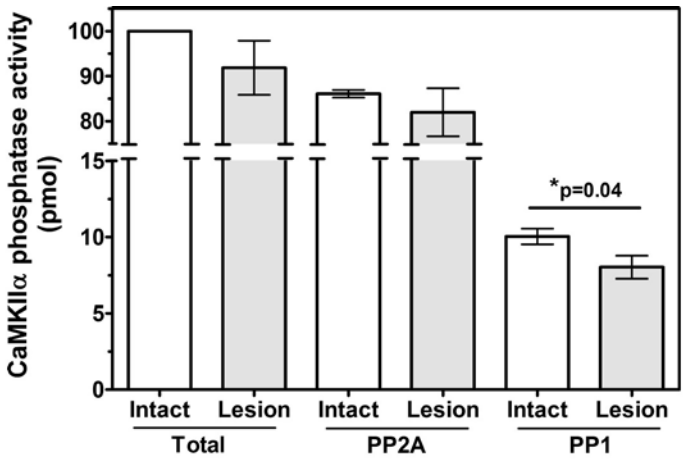

Figure 26. DA depletion decreases PP1-mediated dephosphorylation of [^{32}P-Thr^{286}]-CaMKII .
Phosphatase activities in dorsolateral striatum collected from from 6-OHDA lesioned rats 3-4 weeks after surgery were assayed using [^{32}P-Thr^{286}]-CaMKII in the presence of 0, 2.5 nM or 2.5 μM okadaic acid. Contributions of PP1 and PP2A were calculated as indicated in Methods and are plotted as the percentage of total activity in the intact hemisphere. Dopamine depletion had no significant effect on the PP2A-mediated dephosphorylation of [^{32}P-Thr^{286}]-CaMKII , but significantly decreased PP1-mediated dephosphorylation by approximately 19% (n=8).

Nb(146-493) is a novel, selective inhibitor of PP1γ1 in striatal extracts

PP1 isoforms are differentially targeted in neurons. We developed an assay to determine contributions of PP1 isoforms toward total striatal PP1 activity. Nb(146-493) is a GST-fusion protein that inhibits purified brain $PP1\gamma_1$ catalytic subunit ≈20-fold more potently that it inhibits purified brain PP1β catalytic subunit (Carmody et al., 2004). In contrast, Nb(146-453) is a GST fusion protein that lacks the canonical PP1 binding domain, and is therefore not an effective PP1 inhibitor (Carmody et al., 2004). The sensitivity of PP1 holoenzymes in normal striatal extracts to Nb(146-493) and other phosphatase inhibitors was determined using [^{32}P]-phosphorylase *a* as a model substrate. Approximately 15-20% of the activity was inhibited by nM concentrations of okadaic acid, and the remaining activity was almost completely inhibited by μM concentrations of okadaic acid, suggesting that total PP1 activity accounted for about 80% of the [^{32}P]-phosphorylase phosphatase activity. Consistent with this value, Inhibitor-2, a PP1-specific inhibitor, blocked approximately 75% of the total activity with an apparent $EC_{50\%}$ of ~10 nM. Nb(146-493) inhibited a maximum of about 45% of the total activity at the highest concentration tested (1 μM), with an apparent EC_{50} of about 100 nM. However, Nb(146-453), a GST-Nb fusion protein lacking the PP1-binding domain, had no significant effect on striatal phosphatase activity. Thus, Nb(146-493) only partially inhibits striatal PP1 activity at a concentration of 1 μM (Fig. 28 A).

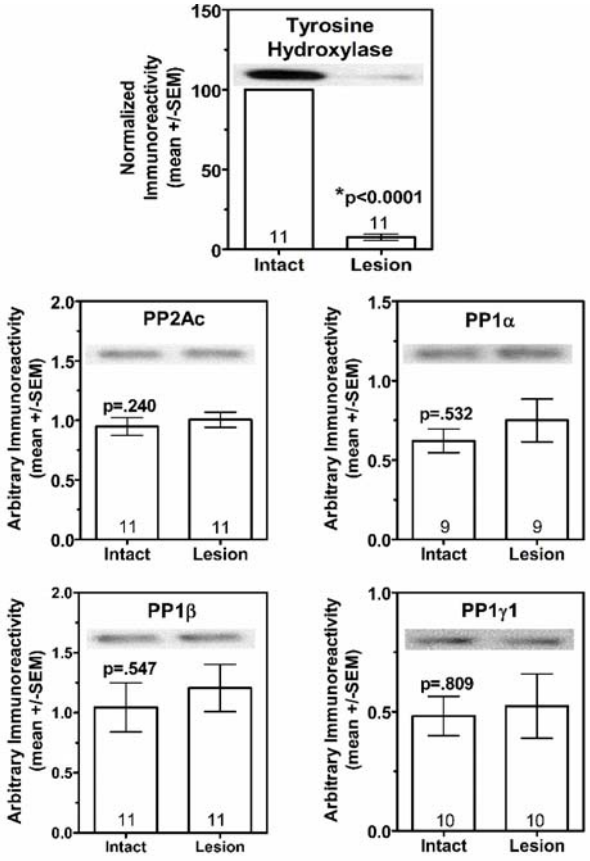

Figure 27. Dopamine depletion does not affect total levels of protein phosphatase catalytic subunits.

Whole dorsolateral striatal extracts collected 10-11 months after 6-OHDA lesion surgery were immunoblotted for tyrosine hydroxylase, PP2Ac and various isoforms of PP1 (α, β, γ_1). The efficacy of the 6-OHDA lesion was confirmed by the approximately 90% decrease of tyrosine hydroxylase immunoreactivity (n=11, paired t-test). However, these was no significant difference in the levels of PP2Ac (n=11, p=0.240), PP1α (n= 9, p=0.532), PP1β (n= 11, p=0.547), or PP1γ_1 (n= 10, p=0.809) between intact and lesioned hemispheres.

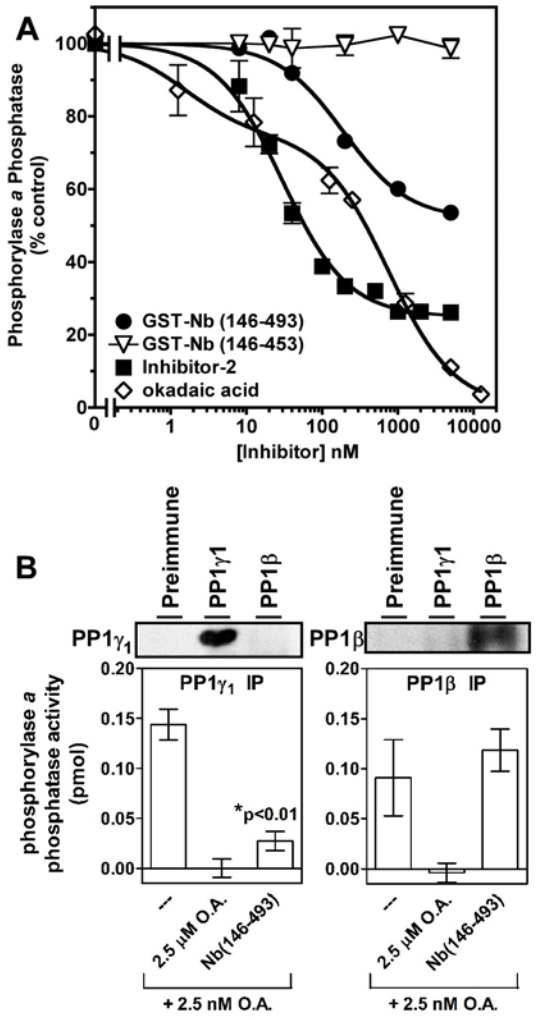

Figure 28. Nb(146-493) is a selective inhibitor of striatal PP1γ_1. (A) Phosphatase activity in a control whole striatal extract was assayed using [^{32}P]-phosphorylase *a* in the presence of indicated concentrations of okadaic acid (open squares), Inhibitor-2 (solid squares), Nb(146-493) (solid circles), or Nb(146-453) (inverted triangles). Each data point represents mean± S.E.M. of 2-6 observations, plotted as a percentage of the total activity (without inhibitor). (B) PP1γ_1 or PP1β were immunoprecipitated from whole striatal extracts. The upper panels show representative immunoblots confirming the specificity of the immunoprecipitations. The lower panel shows [^{32}P]-phosphorylase phosphatase activities in immunoprecipitated samples in the presence of 2.5 nM okadaic acid alone or with the additional presence of 2.5 μM okadaic acid or 1 μM Nb(146-493). Nb(146-493) inhibited immunoprecipitated PP1γ_1, but not immunoprecipitated PP1β.

The partial inhibition of total PP1 activity by Nb(146-493) may be due to the differential inhibition of PP1 isoforms. In order to assess the effect of Nb(146-493) on native PP1 isoform complexes, I assayed striatal PP1γ_1 or PP1β complexes isolated by immunoprecipitation. Analysis of the immunoprecipitates by immunoblotting for PP1 isoforms confirmed the specificity of the immunoprecipitations (Fig. 28 B), as documented previously. Both immunoprecipitated isoforms displayed activity toward phosphorylase *a* in the presence of 2.5 nM okadaic acid and their activities were completely blocked by 2.5 μM okadaic acid, consistent with the specific immunoprecipitation of PP1. Nb(146-493) almost completely blocked activity in the PP1γ_1 immunoprecipitate, but had no significant effect on activity in the PP1β immunoprecipitate. These data establish that 1 μM Nb(146-493) functions as a novel, selective inhibitor of native striatal PP1γ_1 complexes over PP1β complexes.

DA depletion selectively decreases striatal PP1γ_1 activity

To examine the possibility that DA depletion alters PP1γ_1 activity, dorsolateral striatal extracts from 6-OHDA-lesioned rats were assayed for [^{32}P]-phosphorylase phosphatase activity in the absence and presence of Nb(146-493) (Fig. 29). Nb(146-493) inhibited approximately 50% of the activity in extracts prepared from the intact, non-lesioned hemisphere, but only approximately 35% of the activity in DA-depleted samples. Significantly, DA depletion reduced the total PP1 activity by approximately 22%, but there was no significant difference in the Nb(146-493)-insensitive activities in

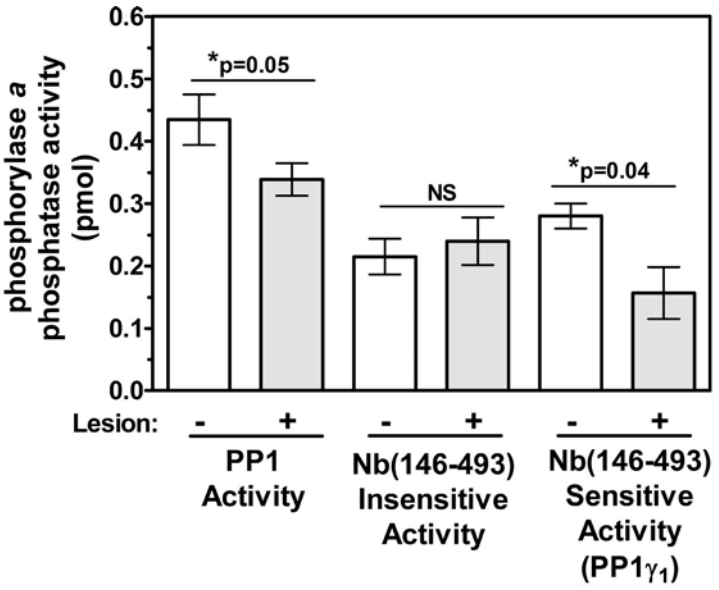

Figure 29. Dopamine depletion selectively decreases striatal PP1γ_1 activity.

Extracts of dorsolateral striatum collected 10-11 months following 6-OHDA lesion surgery were assayed using [^{32}P]-phosphorylase a in the absence or presence of 1 μM Nb(146-493). Total PP1 activity was significantly reduced by approximately 17% following dopamine depletion (n=8), but the activity that is insensitive to Nb(146-493) was not significantly different between the two hemispheres. Thus, dopamine depletion significantly reduced the Nb(146-493)-sensitive PP1 activity (i.e., PP1γ_1) by approximately 44% (n=8).

samples from the two hemispheres. Similar data were obtained in samples collected 3-4 weeks and 10-11 months after the 6-OHDA lesion surgery. Thus, DA depletion significantly reduced the Nb(146-493)-sensitive activity at both 3-4 weeks (17%, p= 0.03, data not shown) and 10-11 months (Fig. 29) after 6-OHDA-lesion surgery. These data suggest that DA depletion results in a significant, selective and sustained reduction of the activity of striatal PP1γ_1.

Discussion

Striatal protein phosphatases play a key role in mediating many of the acute effects of DA on the striatum. In particular, it is widely accepted that the activity and localization of PP1 are acutely modulated by DA via the phosphorylation/ dephosphorylation of regulatory and targeting proteins such as DARPP-32 and spinophilin, even though corresponding changes in PP1 activity have not been directly demonstrated. The α and γ_1 PP1 isoforms are found within both the presynaptic corticostriatal terminals and are more abundant in the postsynaptic MSN dendritic spines (Bordelon et al., 2005), indicating possible postsynaptic and presynaptic contribution to the changes in phosphatase activity reported here. Here, I show that PP1 activity is significantly reduced following striatal DA depletion and I present evidence that this may be achieved by the selective regulation of one of the three PP1 isoforms. The decreased activity of a specific PP1 isoform may be important in mediating the chronic effects of DA depletion.

DA depletion selectively decreases PP1 activity

My initial studies using model substrates showed that there was a significant reduction in phosphatase activity toward [^{32}P]-phosphorylase *a*, but not toward [^{32}P]-casein (Fig. 24). Although both PP1 and PP2A can dephosphorylate [^{32}P]-phosphorylase *a*, PP1 accounts for approximately 80% of the activity toward this substrate in brain extracts (Strack et al., 1997a). Various concentrations of okadaic acid were used to more selectively isolate PP1 activity from PP2A, confirming that PP1 activity was significantly decreased following DA depletion, and that PP2A activity was unaffected. Similar reductions in the total PP1 activity were detected 3-4 weeks and 10-11 months after the 6-OHDA lesion surgery. Moreover, PP1 activity toward the Thr^{286} autophosphorylation site in CaMKIIα (a physiologically relevant substrate in postsynaptic densities) was significantly decreased by DA depletion. This reduction of PP1 activity may contribute to the enhanced autophosphorylation of CaMKIIα at Thr^{286} and other synaptic proteins that has been previously documented following DA depletion (Oh et al., 1999; Dunah et al., 2000; Picconi et al., 2004; Brown et al., 2005).

PP1γ_1 is selectively modulated following dopamine depletion

Three major PP1 isoforms are present in the striatum (α, β, and γ_1). Although the activities of PP1 isoforms are not known to differ, they are enriched in distinct subcellular compartments (da Cruz E Silva et al., 1995; Ouimet et al., 1995; Strack et al., 1999; Bordelon et al., 2005), presumably due to isoform-selective interactions with targeting subunits. In particular, PP1γ_1 is enriched in dendritic spines and at the PSD, where it colocalizes with spinophilin/neurabin (MacMillan et al., 1999; Terry-Lorenzo et

al., 2002; Bordelon et al., 2005). Thus, PP1γ_1 is poised to efficiently dephosphorylate synaptic substrates, such as CaMKII and GluR1, and also to respond to activation of D_1 and D_2 DA receptors that are localized on dendritic spine heads and necks.

In the present studies, I developed a new approach to selectively assess the contribution of PP1γ_1 to total PP1 activity. Previously, our lab has shown that Nb(146-493) is a 20-fold more potent inhibitor of purified PP1γ_1 catalytic subunits ($EC_{50} \approx 1$ nM) than of purified PP1β catalytic subunits (Carmody et al., 2004). In assays of whole striatal homogenates, I show here that Nb(146-493) only partially inhibits the total PP1 activity. The isoform-independent inhibitors okadaic acid and inhibitor-2 show that 75-80% of the total phosphorylase phosphatase activity is due to PP1, but 1 μM Nb(146-493) surprisingly inhibits only about 45% of the total activity (Fig. 28 A). In addition, 1 μM Nb(146-493) is unable to inhibit native PP1β holoenzymes isolated by immunoprecipitation, but completely inhibits native PP1γ_1 holoenzymes (Fig. 28 B). The reduced inhibitory potency of Nb(146-493) in whole tissue extracts ($EC_{50} \approx 200$ nM) presumably reflects the fact that the GST-fusion protein has to competitively displace endogenous regulatory subunits from the catalytic subunits.

Assays of striatal extracts from 6-OHDA lesioned rats showed that Nb(146-493)-insensitive PP1 activity was unaltered, but that Nb(146-493)-sensitive activity was substantially reduced following DA depletion. Since Nb(146-493) can be effectively used to differentiate contributions of PP1 isoforms, these data suggest that DA depletion significantly decreases the activity of striatal PP1γ_1, but does not affect the activity of striatal PP1β. These observations represent the first evidence that the activities of PP1 isoforms can be differentially modulated *in situ*.

Mechanisms for regulating striatal PP1 activity

The simplest mechanism to account for isoform-selective changes in PP1 activity following DA depletion would be alterations in the expression levels of the catalytic subunits. However, no significant differences in the total tissue levels of any PP1 catalytic subunit isoform were detected (Fig. 27). Multiple mechanisms can acutely modulate striatal PP1 activity in response to activation of various DA receptors. Most prominently, DARPP-32 inhibits PP1 following phosphorylation at Thr^{34} by PKA in response to D1 receptor activation. Phosphorylation of DARPP-32 at Thr^{75} in response to activation of D2 receptors interferes with Thr^{34} phosphorylation (Svenningsson et al., 2004). However, DARPP-32 does not appear to play a role in the reduction in PP1γ_1 activity following DA depletion. DARPP-32 interactions are not known to be isoform-selective, and DA depletion does not alter the total levels or phosphorylation of DARPP-32 at Thr^{34} (Picconi et al., 2003; Chergui et al., 2004; Brown et al., 2005). However, it should be noted that phosphorylation at Thr^{75} of DARPP-32 is substantially increased following DA depletion (Brown et al., 2005).

Other mechanisms that have been implicated in regulating PP1 activity include the actions of inhibitor-2 (Cohen, 1989) and direct phosphorylation of the catalytic subunit by cdc2 (Dohadwala et al., 1994). However, these mechanisms are not known to be PP1 isoform-selective. Thus, it seems unlikely that these mechanisms account for the selective reduction of PP1γ_1 activity following DA depletion.

In contrast to these isoform-independent regulatory mechanisms, PP1γ_1 selectively interacts with spinophilin and neurabin (MacMillan et al., 1999; Carmody et al., 2004), F-actin binding proteins that colocalize with PP1γ_1 in dendritic spines (Allen,

2004). Spinophilin and neurabin play critical roles in the acute DA signaling and in modulating corticostriatal synaptic plasticity (Feng et al., 2000; Allen et al., 2006). However, DA depletion does not change the total levels of spinophilin/neurabin, or alter the amount of these proteins present in PSD-enriched cytoskeletal fractions (Brown et al., 2005). Moreover, normal DA signaling does not appear to modify the interaction between spinophilin and PP1 (Hsieh-Wilson et al., 2003). Nevertheless, I suggest that the isoform-selective effects of DA depletion may reflect the fact that PP1γ_1 co-localizes with spinophilin/neurabin and DA receptors in dendritic spines, whereas PP1β is preferentially localized to dendritic shafts and the soma.

Impact of reduced PP1γ_1 activity following dopamine depletion

Decreased PP1γ_1 activity following DA depletion may play a critical role in the increased phosphorylation of numerous synaptic PP1 substrates, including subunits of NMDA- and AMPA-type glutamate receptors and CaMKIIα (Oh et al., 1999; Dunah et al., 2000; Picconi et al., 2004; Brown et al., 2005). Reduced PP1 activity and/or the hyperphosphorylation of these PP1 substrates is presumably important in the disruptions of bidirectional striatal synaptic plasticity and behavior following DA depletion (Centonze et al., 1999; Oh et al., 1999; Centonze et al., 2001; Picconi et al., 2003; Picconi et al., 2004; Norman et al., 2005). Many of the effects of DA depletion can be rescued by "treatments" that normalize these processes. For example, an L-DOPA injection paradigm rescues the hyperphosphorylation of CaMKII at Thr^{286}, and administration of a CaMKII inhibitor normalizes defects in synaptic plasticity and behavior that result from striatal DA depletion (Oh et al., 1999; Picconi et al., 2004). Moreover, disruptions in the

balance between kinase and phosphatase activities may play a role in several other neurodegenerative diseases or disease models. For example, increased phosphorylation of several proteins has been reported in Angelman's mental retardation syndrome (Weeber et al., 2003), amyotrophic lateral sclerosis (Strong et al., 2005), multiple sclerosis (Schneider et al., 2004), Charcot-Marie-Tooth disease (Begley and Dixon, 2005), and Alzheimer's disease (Liu et al., 2005). In particular, studies in a mouse model of Angelman's syndrome suggest that decreased protein phosphatase activity results in increased phosphorylation of hippocampal CaMKII at Thr^{286} and Thr^{305} and disruptions of synaptic plasticity, learning and memory. Thus, the development of strategies to increase the activity of critical protein phosphatases might be a fruitful therapeutic strategy for treatment of Parkinson's Disease, Angelman's syndrome and possibly other neurodegenerative diseases.

CHAPTER VI

SUMMARY AND FUTURE DIRECTIONS

Summary

Protein phosphorylation and dephosphorylation acutely modulate protein function, protein-protein interactions, and neuronal plasticity. DA depletion in the 6-OHDA lesioned rat model of PD results in both the enhanced phosphorylation of numerous serine / threonine substrates and alterations in synaptic plasticity and behavior (Girault et al., 1992; Oh et al., 1998; Centonze et al., 1999; Oh et al., 1999; Dunah et al., 2000; Centonze et al., 2001; Picconi et al., 2003; Picconi et al., 2004; Brown et al., 2005; Norman et al., 2005). In combination, the data reported in this dissertation extend previous reports in the literature by demonstrating that striatal DA depletion induces an imbalance in the activity of specific serine / threonine phosphatases and kinases.

CaMKII is a critical neuronal signaling enzyme, and phosphorylation at Thr^{286} correlates with an increase in enzyme activity (Hudmon and Schulman, 2002, 2002; Colbran and Brown, 2004). Increased Thr^{286} phosphorylation of CaMKIIα is evident within 3 weeks of 6-OHDA lesion surgery and is maintained for up to at least 18 months (see Chapter III). In addition, slow development of the phosphorylation of a well-established CaMKII substrate, Ser^{831}-GluR1, represents the first report of unique biochemical effects of long-term (9-20 months) DA depletion in rodents. Thus, prolonged DA depletion results in unique biochemical consequences beyond those seen in the shorter-term studies typically performed.

In the normal striatum, PP1 is a key phosphatase that may 'gate' the activity of CaMKII, preventing prolonged hyperphosphorylation and hyperactivation of CaMKII. Decreased striatal PP1 activity measured in these studies is consistent with the elevated phosphorylation of numerous PP1 substrates following DA depletion (Chapters III and V). Furthermore, this dissertation includes the first direct measurement of a specific decrease in the activity of the PP1γ_1 isoform following DA depletion. Since PP1γ_1 is enriched at the PSD, these results suggest that PSD-localized PP1 is critical for normal corticostriatal synaptic plasticity.

As levels of the PP1γ_1 scaffolding proteins spinophilin and neurabin decrease with normal aging, levels of PSD-targeted PP1 may also decrease, despite the aging-related increase in PP1γ_1 levels. This might result in a synaptic mislocalization of PP1γ_1 away from synapses, disrupting the balance between CaMKII and PP1 activities at the synapse. This imbalance would allow the DA depletion-induced accumulation of phospho-Ser^{831}-GluR1 in response to increased CaMKII autophosphorylation. Thus, it is possible that the combination of aging-related and DA depletion mechanisms result in the novel findings associated with the long-term DA depletion.

Misregulated CaMKII has been implicated in the DA depletion-induced changes in synaptic plasticity, and the "wearing-off" phenomenon associated with chronic L-DOPA therapy. By defining how glutamate acutely regulates striatal CaMKII, my findings provide the basis for future studies. Investigation of the evolving changes in biochemical signaling pathways following DA depletion and during DA replacement strategies may be key in understanding the changing efficacy of chronic L-DOPA therapy. These findings indicate a high level of basal CaMKII autophosphorylation in the

striatum, which is coupled to Ca^{2+} influx (Chapter IV). NMDA receptors acutely regulate striatal CaMKII autophosphorylation (Chapter IV). In light of the known DA depletion-induced changes in both NMDA and D_2 receptors, it is possible that glutamatergic and dopaminergic regulation of CaMKII is altered.

Outstanding Questions

What mediates the selective decrease in activity of PP1γ_1?

It is unclear how DA depletion causes a decrease in the activity of a specific PP1 isoform, especially with no evidence for a decrease in the total levels or the subcellular localization of PP1γ_1. There are no known endogenous isoform-specific inhibitors of PP1γ_1. Two synapse-associated proteins, spinophilin and neurabin, show a preference for binding the PP1γ_1 isoform over the PP1β isoform. These isoform-specific interactions with spinophilin and neurabin are believed to target PP1γ_1 to the synapse. Therefore, it is possible DA depletion somehow alters the interactions between spinophilin/neurabin and PP1γ_1 in a way that renders PP1γ_1 less active. To examine this possibility, striatal spinophilin/neurabin/ PP1γ_1 complexes can be immunoprecipitated from DA-depleted striata, and examined not only for the amount of each protein, but also the activity of PP1γ_1 bound to either scaffolding protein. In addition, it is also possible that an as yet unknown endogenous isoform-specific inhibitor is recruited to the spinophilin/neurabin/ PP1γ_1 complexes. To examine this possibility, the immunoprecipitated spinophilin/neurabin/ PP1γ_1 complexes can be examined by mass spectrometry to detect unknown associated proteins.

Does a short-term 6-OHDA lesion in aged rats result in elevated phospho-Ser^{831}-GluR1?

In comparing the effects of 6-OHDA lesion at different survival times after lesion surgery, I found that only long-term lesion (9-20 months) and not short-term lesion (3-12 weeks) resulted in elevated levels of phospho-Ser^{831}-GluR1 in the lesioned hemisphere (Chapter III). From these data alone, it is unclear whether the elevated phospho-Ser^{831}-GluR1 observed in long-term lesioned rats was a result of either chronic, long-term DA depletion alone or in combination with the aging-related changes in PP1, spinophilin, and neurabin levels (Chapter III). It is possible that the decrease in spinophilin and neurabin with age mislocalizes PP1 away from synaptic substrates, such as GluR1. Examination of the 6-OHDA lesion-induced changes in *aged* rats allowed to survive 3 weeks after unilateral 6-OHDA lesion surgery may help to dissect out the reasons for the enhanced GluR1 phosphorylation.

Is striatal synaptic plasticity or responsiveness to L-DOPA altered in long-term lesioned rats (minimum 9-11 month 6-OHDA lesion)?

The data presented here suggest that long-term dopamine depletion may enhance glutamate receptor-mediated transmission by increasing the levels of Ser^{831} phosphorylated GluR1. It will be interesting to determine whether prolonged periods of DA depletion induce additional changes in striatal synaptic plasticity beyond those previously seen after short periods of DA depletion. It is currently unknown whether CaMKII inhibitors or L-DOPA are similarly effective in reversing the deficits in behavior and synaptic plasticity after GluR1 phosphorylation has increased. Traditionally, it is difficult to study synaptic plasticity in aged rats, which may impede this line of investigation.

Does L-DOPA rescue other 6-OHDA lesion-induced abnormalities?

While it is clear that chronic L-DOPA rescues the DA depletion-induced elevation in phospho-Thr^{286}-CaMKII and phospho-Thr^{75}-DARPP-32 (Chapter III), it is unknown whether changes in other proteins noted here are similarly rescued by L-DOPA treatment. It is possible that L-DOPA may rescue the decreased PP1γ_1 activity reported in Chapter V. To examine this possibility, after receiving at least 9 days of L-DOPA treatment, striatal tissue from 6-OHDA lesioned rats can be examined for changes in PP1γ_1 activity. It will also be important to assess whether L-DOPA can rescue the elevated phosphorylation of GluR1 at Ser^{831} seen only after long-term 6-OHDA lesion. A failure of L-DOPA to rescue increased phosphorylation of GluR1 may be a factor in the loss of efficacy and development of side effects associated with long-term DA replacement therapy.

How long does the L-DOPA-mediated rescue of lesion-induced abnormalities last?

Data reported here indicate that chronic L-DOPA treatment for 9 days reverses the lesion-induced enhancement in phospho-Thr^{286}-CaMKII levels. While the half-life of L-DOPA is ~1.5 hours, the decrease in CaMKII autophosphorylation persists until at least 16 hours after the last L-DOPA injection (Chapter III). Surprisingly, *in vivo* L-DOPA treatment for only 1 day does not rescue any of the DA-depletion induced changes in phospho-Thr^{286}-CaMKII levels, CaMKIIα activity, limb-use asymmetry, and synaptic plasticity (Picconi et al., 2004). Comparison of these two studies raises the following questions: (1) how long does the L-DOPA-induced rescue in phospho-Thr^{286}-CaMKIIα in the lesioned hemisphere last, and (2) how many L-DOPA injections are needed for

sustained rescue? To address these questions, lesioned rats injected with L-DOPA for up to 9 days can be evaluated for phospho-Thr^{286}-CaMKII levels at 30 min, 2hrs, and 32 hrs after final L-DOPA injection.

CaMKII activity has been linked with the enhanced "wearing-off" phenomenon associated with only chronic (21 days) L-DOPA use (Oh et al., 1999). As treatment with a CaMKII inhibitor rescues the "wearing-off" phenomenon, this raises the possibility that CaMKII activity has become elevated despite prolonged L-DOPA use. It is possible that chronic *in vivo* L-DOPA treatment may enable a different mechanism of phospho-CaMKII regulation by NMDA / D1 / D2 signaling. To examine this possibility, experiments using acute striatal slices from 6-OHDA lesioned rats chronically treated with L-DOPA may yield some answers. It is possible that chronic (21 days) L-DOPA modulates the NMDA/D1/D2 receptor regulation of phospho-CaMKII, possibly in both the lesioned and intact hemispheres. These experiments may provide insight into the biochemical mechanisms that evolve with prolonged L-DOPA therapy and may underlie some of the problems associated with long-term use of this therapeutic strategy in PD.

Does DA depletion alter the acute regulation of striatal CaMKII?

Chronic DA depletion results in enhanced levels of striatal phospho-Thr^{286}-CaMKII, which is reversed by *in vivo* L-DOPA (Brown et al., 2005). Striatal DA depletion results in alterations in the subcellular localization as well as phosphorylation of NMDA receptors (Oh et al., 1998; Oh et al., 1999; Dunah et al., 2000; Dunah and Standaert, 2003), possibly modulating receptor activity. Interestingly, the changes in induced by DA depletion in MSN morphology and DA receptors appear restricted to D_2-

expressing MSNs (Gerfen et al., 1990; Araki et al., 1998; Cai et al., 2002; Day et al., 2006), suggesting that DA depletion modulates D_2 receptor signaling, to disrupt normal regulation of CaMKII. In light of the high basal CaMKII autophosphorylation, it is critical to determine if the DA depletion-induced increase in CaMKII autophosphorylation is maintained in slices from 6-OHDA lesioned rats. In addition, it would be informative to learn whether the NMDA or DA regulation of CaMKII is modified following 6-OHDA lesion. Data from an initial experiment provide tantalizing clues to suggest that D2 and NMDA receptor signaling pathways together control CaMKII autophosphorylation in the lesioned striatum (Figs. 30 and 31). Although the interpretation of these data is compromised by the relatively low number of samples in some treatment groups due to technical problems.

The results presented here are consistent with the finding that MSNs spend less time in the downstate and more time in the upstate following 6-OHDA lesion (Pang et al., 2001; Tseng et al., 2001). In combination, increased time in the upstate (Pang et al., 2001; Tseng et al., 2001) and increased Ca^{2+} influx through NMDA receptors in the upstate (Carter and Sabatini, 2004) suggest that influx of extracellular Ca^{2+} via NMDA receptors may contribute to the increase in CaMKII autophosphorylation in the DA-depleted striatum. The idea that influx of extracellular Ca^{2+} via NMDA receptors contributes to the increase in CaMKII autophosphorylation in the DA-depleted striatum is supported by the apparently complete reversal of CaMKII autophosphorylation by APV (Fig. 30).

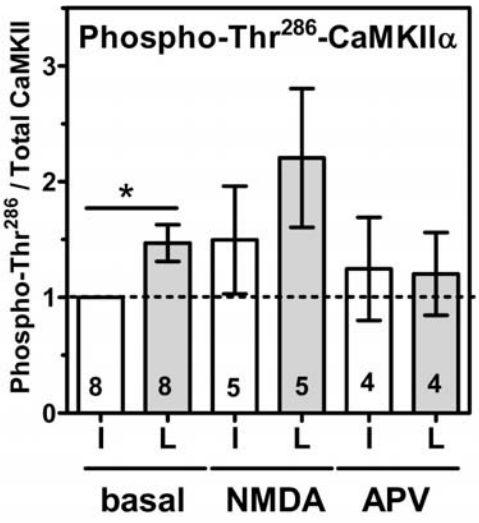

Figure 30. Acute NMDA receptor regulation of striatal CaMKII autophosphorylation following DA depletion

Striatal slices were incubated with NMDA receptor agonists or antagonists, as in chapter IV. Two-way ANOVA analysis does not indicate any significant differences, possibly due to the low n# for some groups. Paired t-tests were used to compare values in opposite hemispheres. The lesion-induced increase in phospho-Thr^{286}-CaMKII levels is maintained in acute striatal slices (47% increase; n=8, *p=0.0216). Initial data suggest that NMDA enhances the level of phospho-Thr^{286}-CaMKII in both the intact and lesioned hemispheres, consistent with data presented in control slices (Chapter IV). In addition, the elevated phospho-CaMKII in the lesioned vs. intact hemisphere was maintained in response to NMDA treatment (47% increase; n=5, p=0.1899). Thus, it appears that the impact of DA depletion and NMDA receptor activation may be additive. In contrast, the NMDA receptor antagonist APV appeared to abolish the lesion-induced increase in CaMKII autophosphorylation, suggesting that DA depletion enhances CaMKII autophosphorylation via an elevated basal NMDA receptor activity (n=4, p=0.7054). The idea that NMDA receptor activity is elevated in the lesioned hemisphere is consistent with reports suggesting an enhanced corticostriatal glutamatergic drive following DA depletion (Meschul et al., 1999; Jonkers et al., 2002).

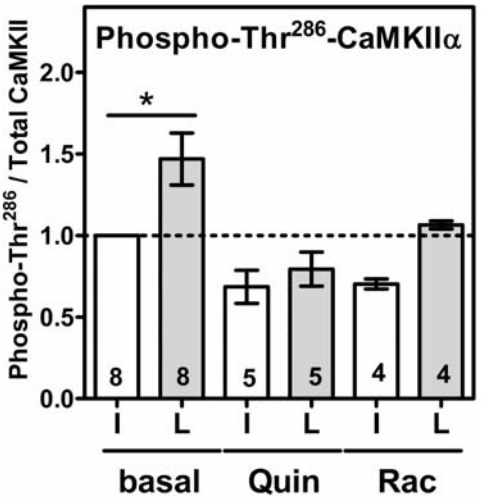

Figure 31. Acute regulation of striatal CaMKII autophosphorylation following DA depletion

Striatal slices were incubated with D2-like receptor agonists or antagonists, as in chapter IV. Two-way ANOVA analysis does not indicate any significant differences, possibly due to the low n# for some groups. Paired t-tests were used to compare values in opposite hemispheres. Initial data suggest an apparent quinpirole-mediated decrease in CaMKII autophosphorylation in both hemispheres. Incubation with quinpirole (n=5, p=0.1184), but not raclopride (51% increase, n=4, *p=0.0028) abolished the lesion-induced elevation in CaMKII phosphorylation. In combination, these findings are consistent with previous data showing that L-DOPA reverses the DA-depletion-induced elevation in CaMKII autophosphorylation (Chapter III). Ablation of the lesion-induced elevation in CaMKII autophosphorylation by either quinpirole or APV raises the possibility that the D2 and NMDA receptor pathways may be linked (see Fig. 33). Activation of D2DR on presynaptic corticostriatal terminals inhibits neurotransmitter release from a subset (~80%) of these terminals in acute striatal slices (Bamford et al., 2004a; Bamford et al., 2004b). Thus, either D2DR-mediated decrease in glutamate release or direct inhibition of NMDA receptors with APV decreases NMDAR signaling, resulting in a reduction in CaMKII autophosphorylation.

Is the cellular and subcellular distribution of spine-associated proteins altered following the DA-depletion induced decrease in spine density?

Although there is a distinct decrease in the MSN spine density of striatopallidal neurons (D_2, indirect pathway) and in the axospinous asymmetric synapses, it is unclear whether the PSDs are maintained on the dendritic shaft (Day et al., 2006). Moreover, the total levels of multiple synapse-associated proteins remains unchanged in total striatal homogenates following DA depletion (Dunah et al., 2000; Picconi et al., 2004; Brown et al., 2005). In fact, it is surprising how few proteins change after either 6-OHDA lesion, or throughout aging, or a combination of both. Therefore, it is probable that these synaptic proteins are not simply degraded.

In light of the data presented in Chapter III, it is probable that synaptic proteins are not simply degraded following the DA depletion-induced decrease in MSN spine density. Instead, synaptic proteins may be maintained at dendritic shaft synapses or redistributed elsewhere in the cell. Phosphorylation can modulate the subcellular localization of several proteins, and phosphorylation of many synaptic proteins is elevated in the DA depleted striatum. The subcellular redistribution of hyperphosphorylated proteins, such as CaMKII, GluR1, and DARPP-32 can be examined by either immunofluorescent confocal microscopy or immunoelectron microscopy.

It would be very informative to determine how DA depletion might alter the cellular localization of hyperphosphorylated proteins, such as CaMKII, GluR1, and DARPP-32. Immunostaining striatal tissue from the recently developed BAC-D1 or BAC-D2 transgenic mice would to help identify the cellular localization of these changes. Alternatively, striatal slices from rats could be immunostained for both the phospho-proteins noted above, and specific markers of the direct or indirect pathway

MSNs, such as substance P and dynorphin (direct pathway MSNs), or enkephalin (indirect pathway MSNs). The determination of the cellular localization for the phosphorylation changes noted in CaMKII and other proteins may enable more precise targeting of PD therapies.

Does elevated phospho-Thr^{286}-CaMKII cause decreased MSN spine density?

MSN spine density decreases and phospho-Thr^{286}-CaMKII levels increase following DA depletion, but it is unclear whether these changes are functionally linked. Recently, Day et al. (2006) reported that nimodipine, an L-type Ca^{2+} channel antagonist, prevents the DA depletion-induced decreased spine density. In an initial experiment, examination of dorsolateral striatal homogenates from these same animals suggested that nimodipine lowers the overall levels of autophosphorylated CaMKII in both intact and lesioned hemispheres, and abolishes the difference between the hemispheres (Fig.32). These findings might indicate that the elevated CaMKII autophosphorylation does not play a role in the decrease in MSN spine density, but these experiments need to be repeated to increase statistical power. In addition, it will be exciting to quantitate MSN spine density in wild-type, T286A-CaMKII, or T286D-CaMKII transgenic mice with and without prior exposure to MPTP to induce DA depletion. If CaMKII autophosphorylation does not cause the spine decrease, all groups should display a similar degree of spine loss after MPTP. One assumption in these experiments is that both the decrease in MSN spines and the elevated CaMKII autophosphorylation will be observed in wild-type mice after MPTP exposure.

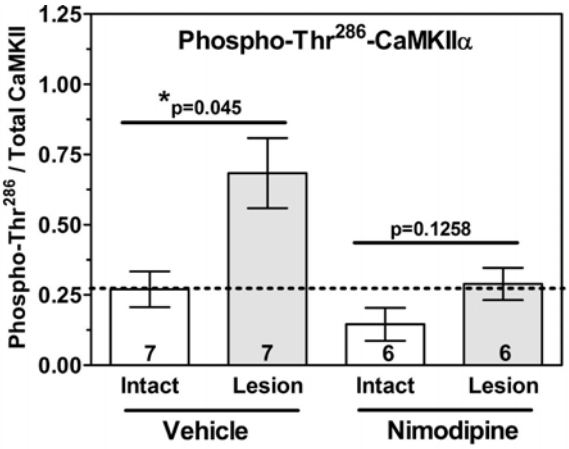

Figure 32. Effect of chronic *in vivo* nimodipine on phospho-Thr^{286}-CaMKII levels in normal and DA-depleted striatum.
Rats received chronic nimodipine for 3 weeks, as in (Day et al., 2006), followed by immunoblot analysis of phosphoThr^{286}-CaMKII levels in dorsolateral striatum homogenates. Vehicle treated animals displayed a 68% increase in phosphoThr^{286}-CaMKII levels in the lesioned hemisphere (n=7, *p=0.005, paired t-test). Nimodipine treatment decreases phosphoThr^{286}-CaMKII levels in both hemispheres.

If CaMKII autophosphorylation does play a role in spine loss, T286A-CaMKII mice should be protected against MPTP-induced spine loss.

Are the DA-depletion-induced behavioral abnormalities mediated by elevated phospho-Thr^{286}-CaMKII?

I have shown that chronic L-DOPA normalizes 6-OHDA lesion-induced increases in phospho-Thr^{286}-CaMKIIα levels (Chapter III). Similar studies have reported that L-DOPA rescues DA depletion-induced changes in CaMKIIα activity, limb-use asymmetry, and synaptic plasticity in rats (Picconi et al., 2004). The duration of contralateral turning behavior in response to an acute injection of L-DOPA decreases with more chronic L-DOPA administration. This alteration in behavioral response to L-DOPA is rescued by treatment with a CaMKII inhibitor, KN-93 (Oh et al., 1999). In combination, these studies raise the possibility that DA depletion induces behavioral effects that are mediated by CaMKII. To address this question, locomotor / rearing measurements could be taken from wild-type or T286A-CaMKIIα transgenic mice +/- MPTP treatment. Wild-type adult C57BL/6 male mice given MPTP injections resulting in ~90% decrease in striatal dopamine display decreased locomotion and rearing (Sedelis et al., 2001). Treatment of these mice with L-DOPA increases the locomotion and rearing behaviors. The hypothesis here is that if elevated phospho-Thr^{286}-CaMKIIα plays a role in mediating behavioral abnormalities, T286A-CaMKIIα mice will be either completely or partially resistant to MPTP-mediated disruptions in behavior.

How does L-DOPA regulate CaMKII in vivo?

In light of the increase in D_2 receptor density, it is possible that the D2 receptor signaling pathways are more responsive to L-DOPA following DA depletion. One hypothesis that arises from these findings is that L-DOPA, acting primarily at the D2 receptors, rescues the elevated CaMKII autophosphorylation. To investigate this possibility further, CaMKII phosphorylation may be monitored in 6-OHDA lesioned rats treated with either L-DOPA, a D2 agonist, or a combination of a D2 antagonist and L-DOPA. If L-DOPA does primarily act via stimulation of D2 receptors, treatment with either a D2 agonist or L-DOPA are both expected to reverse the lesion-induced increase in CaMKII autophosphorylation. The D2 antagonist is expected to prevent the L-DOPA reversal of phospho-Thr^{286}-CaMKII levels. L-DOPA likely acts at both D1 and D2 receptors. Therefore, simultaneous administration of D1 and D2 receptor antagonists to unlesioned rats may recapitulate elevated phospho-Thr^{286}-CaMKII observed after 6-OHDA lesion.

It is possible that L-DOPA acts via presynaptic D_2 to inhibit glutamate release from corticostriatal glutamatergic terminals. Based on the results reported from the acute slice experiments in Chapter IV and Fig. 30 (this chapter), administration of an NMDAR antagonist to rats with a unilateral 6-OHDA lesion is expected to reverse the lesion-induced elevation in CaMKII autophosphorylation. This is an exciting hypothesis, as future studies could further examine the impact of NMDA receptor subunit-specific antagonists to treat the motor symptoms of some patients with PD.

Final Summary

This dissertation presents evidence of dopaminergic regulation of two key synaptic signaling enzymes, CaMKII and PP1. It will be important to determine whether changes in phosphorylation of CaMKII, PP1, DARPP-32, and GluR1 also occur in PD, although such studies may be complicated by protein dephosphorylation in postmortem human tissue. Moreover, the evolving responses of signaling proteins following DA depletion may play a role in the progression of symptoms during Parkinson's Disease, as well as in the changing efficacy and debilitating side effects associated with dopamine replacement therapy. Although it is unclear what causes PD in most human patients, the possibility that such striatal Ca^{2+}-sensitive signaling pathways are misregulated implies that targeting these pathways may be a useful strategy in developing future treatments for PD.

Disruptions in the balance between kinase and phosphatase activities may be a more global phenomenon in neurodegenerative disease than previously realized. Numerous reports indicate protein hyperphosphorylation in neurological disorders and neurodegenerative diseases or disease models, such as PD (Girault et al., 1992; Oh et al., 1998; Oh et al., 1999; Dunah et al., 2000; Picconi et al., 2004; Brown et al., 2005), Angelman mental retardation syndrome (Weeber et al., 2003), amyotrophic lateral sclerosis (Strong et al., 2005), multiple sclerosis (Schneider et al., 2004), Charcot-Marie-Tooth disease (Begley and Dixon, 2005), and Alzheimer's disease (Liu et al., 2005). Additional studies which examine the regulation of kinase/phosphatase signaling pathways may provide useful insights for development of future therapies for Parkinson's disease and many other neurological diseases.

APPENDIX

PHOSPHORYLATION OF CAMKII MAY BE ACUTELY REGULATED BY DOPAMINE

Nigrostriatal dopaminergic input is ideally positioned to modulate the activity of striatal MSNs, and thus possibly alter the output of the entire basal ganglia. Striatal MSNs contain dopamine receptors of the D1 class (D_1, D_5) and the D2 class (D_2, D_3, and D_4). CaMKII autophosphorylation may be modulated via DARPP-32/PP1 signaling. Alternatively, these receptor pathways may modulate CaMKII phosphorylation via their effects on Ca^{2+} channels, glutamate receptors, or mobilization of Ca^{2+} from intracellular stores (Nishi et al., 1997; Hernandez-Lopez et al., 2000).

Dopamine minimally regulates CaMKII phosphorylation at Thr^{286}

In preliminary efforts to determine whether D1 receptors acutely regulate CaMKII phosphorylation, striatal slices were incubated with SKF81297, a D1 receptor agonist (5 min). SKF81297 did not significantly alter the levels of phospho-Thr^{286}-CaMKII (Fig. 33 A). To confirm that SKF81297 was effectively penetrating the slice and acting at D1 receptors, the phosphorylation of GluR1 was evaluated. SKF81297 increased GluR1 phosphorylation at Ser^{845} by 68%, but not at Ser^{831} (Fig. 33 B ,C), consistent with previous reports (Snyder et al., 2000; Chao et al., 2002; Swayze et al., 2004).

Next, to determine whether D2 receptor activation modulates striatal CaMKII autophosphorylation, slices were incubated with quinpirole, a D2 receptor agonist (5 min.). Quinpirole application resulted in a statistically insignificant increase in phospho-

Thr^{286}-CaMKII by 54% (n=6, p=0.06) but a significant increase of phospho-Ser^{831}-GluR1 by 101% (n=6, p=0.01) (Fig. 34 A, B).

In combination, our data do not provide convincing evidence for regulation of CaMKII autophosphorylation by either D1 or D2 receptor pathways in normal striatum, at least at the time points tested. However, this interpretation is limited by the variability within and between individual batches of slices. Although these results may suggest a minimal D1/D2 regulation of CaMKII autophosphorylation, a small, but significant effect might be revealed either by increasing the sample size or by examination of different time points.

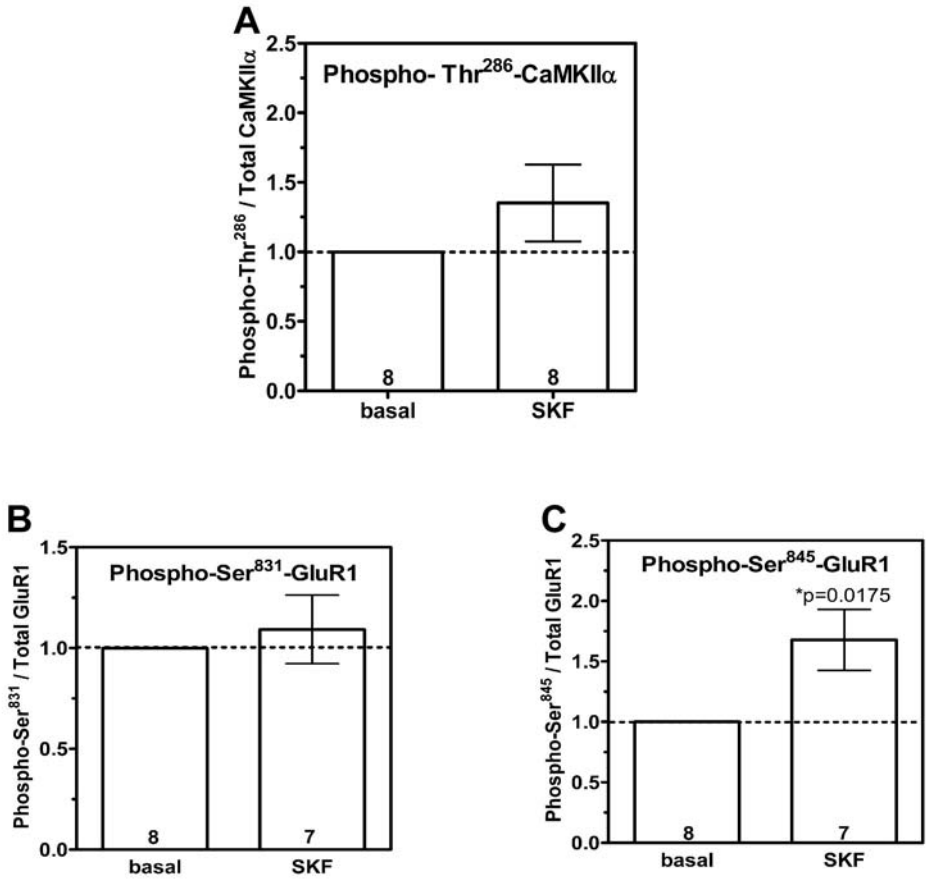

Figure 33. Striatal CaMKII is not acutely modulated by D1-like dopamine receptors.

Acute striatal slices were preincubated in aCSF for 1 hour prior to addition of 1 μM SKF81297 (5 min.). Phospho-protein levels were analyzed by immunoblot, and plotted at mean +/- SEM. (A, B) Levels of phospho-Thr^{286}-CaMKII or phospho-Ser^{831}-GluR1 were not altered by any drug treatment (p=0.738). (C) SKF81297 elevated levels of phospho-Ser^{845}-GluR1 (*p=0.006). For all proteins examined, only significant *post hoc* paired t-test results for each drug vs. basal are noted within each figure.

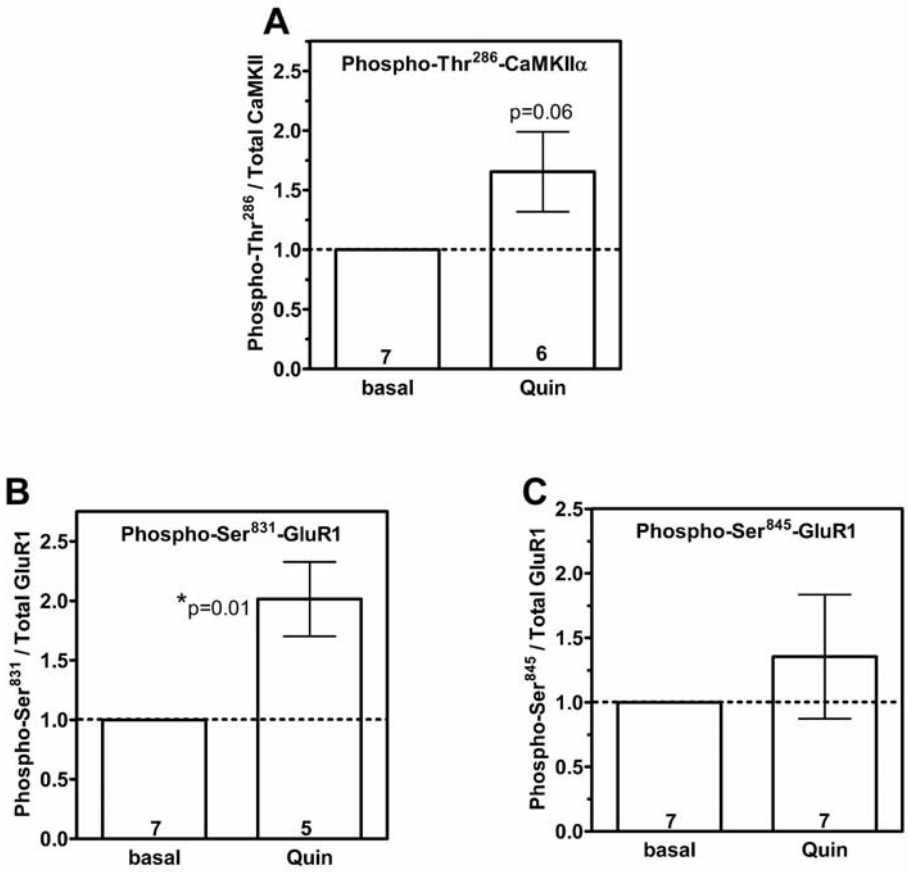

Figure 34. Striatal CaMKII may be acutely modulated by D2-like dopamine receptors.

Acute striatal slices were preincubated in aCSF for 1 hour prior to addition of 1 μM quinpirole (5 min.). Phospho-protein levels were analyzed by immunoblot. (A,C) Levels of phospho-Thr^{286}-CaMKII and phospho-Ser^{845}-GluR1 were not significantly changed by treatment with either quinpirole or raclopride. (B) Quinpirole significantly increased phosphorylation of GluR1 at Ser^{831}. For all proteins examined, paired t-tests for each condition significantly different from basal are noted within each figure.

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