ROLE OF CAMKII AND THE GLUN2B SUBUNIT OF THE NMDAR IN DORSAL STRIATAL GLUTAMATERGIC SYNAPTIC TRANSMISSION, MSN MORPHOLOGY AND STRIATAL-BASED BEHAVIORS

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To my supportive family: Mom, Dad, Mikey, Aunt "B", Bim, Bear and Mom 2.

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Acetylcholine...Ach

Adenosine 2a Receptor..A2aR

Adenylate Cyclase ....AC

Autocamtide-2 Related Inhibitory Peptide ....AIP

AMPA Receptor Subunit 1 ....GluA1

AMPA Receptor Subunit 2/3 ....GluA2/3

AMPA Receptor Subunit 4 ....GluA4

Artificial Cerebro-spinal Fluid .... ACSF

AMPA Receptor .... AMPAR

Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid ....AMPA

Bacterial Artificial Chromosome .... BAC

Brain-derived Neurotrophic Factor ....BDNF

Big conductance calcium-activated potassium channel...................................BK

DL-2-Amino-4-phosphonovaleric Acid...DL-APV

cAMP-Responsive Elements... CRE

Calcium/Calmodulin-Dependent Protein Kinase I...CaMKI

Calcium/Calmodulin-Dependent Protein Kinase II..CaMKII

Calcium/Calmodulin-Dependent Protein Kinase IV .... CaMKIV

Cannabinoid Receptor 1 ....CB_1R

Catechol-O-methyl transferase .... COMT

Central Amygdala ....CeA

Choline acetyltransferase...ChaT

Cyclin-dependent Kinase 5 ....CDK5

Guanine nucleotide exchange factor .... GEF

Glutamic Acid Decarboxylase .... GAD

Globus Pallidus .... GP

Guanosine 5'-triphosphate .... GTP

High-Frequency Stimulation .... HFS

Inhibitory Postsynaptic Currents .... IPSCs

Intraperitoneal .... i.p.

Inositol triphosphate IP3

Inwardly rectifying potassium channel .... Kir

Long-term Depression .... LTD

Long-term Potentiation .... LTP

Low frequency stimulation LTD .... LFS-LTD

Low-threshold-spiking GABAergic Interneuron .... LTS

Medium Spiny Neuron .... MSN

Metabotropic Glutamate Receptor .... mGluR

Metabotropic Glutamate Receptor Long-term Depression .... mGluR-LTD

Miniature Excitatory Postsynaptic Currents .... mEPSCs

Mitogen-activated Protein Kinase .... MAPK

Monoacyl-glycerol 2- arachidonoylglycerol 2-AG

Muscarinic Acetylcholine Receptor 1-5 .... M_1R, M_2R, M_3R, M_4R, M_5R

(5S,10R)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]
cyclohepten-5,10-imine maleate MK801

Nicotinic Acetylcholine Receptor .... nAChR

2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]
quinoxaline -7-sulfonamide .... NBQX

NMDA Receptor .... NMDAR

CHAPTER I

INTRODUCTION

In this introductory chapter, I primarily focus on striatal excitatory glutamatergic synaptic transmission as well as the anatomy and molecular mechanisms underlying motor learning and the formation of goal-directed and habit based learning. I further consider the role of dopamine in the modulation of glutamatergic transmission, including alterations in glutamatergic synaptic transmission, plasticity, and morphology following dopamine depletion and subsequent levodopa administration. I further compare and contrast long lasting synaptic plasticity and its mechanisms in hippocampus with striatal plasticity. Furthermore, I introduce an important kinase, CaMKII, as well as the GluN2B subunit of the NMDAR and their role in synaptic plasticity. Finally, I review medium spiny neuron excitability and its modulation by numerous players including a novel role for CaMKII. The work in this area will not only improve the basic understanding of excitatory transmission in the striatum and the role of CaMKII in these processes including the formation of normal motor repertoires, but also will lend understanding to neurodegenerative disorders, addiction and other striatal-based disorders.

The basal ganglia are a collection of subcortical nuclei that subserve a variety of behaviors, including motor planning, procedural learning, and motivation state   Although there is no universal agreement on which nuclei

comprise the basal ganglia (early anatomists used basal ganglia to refer to all large subcortical masses, including the thalamus), the term today generally refers to the neostriatum (caudate nucleus and putamen) and its projection targets.

As knowledge has accrued concerning the anatomical organization of the striatum, including its characteristic cell types and efferent projections, the term neostriatum has reduced to the simpler term striatum. The striatum encompasses a dorsal portion (with its pallidal and nigral targets) as well as the ventral striatum, including the nucleus accumbens and its targets in the ventral pallidum. While anatomical studies have revealed an overarching theme for the dorsal and ventral striatum, subdivisions within each of these structures have become apparent, with corresponding functional specialization. For example, within the dorsal striatum, the dorsomedial aspect appears to be associated with goal-directed learning in rodents, while the dorsolateral striatum is associated with habit learning (Yin and Knowlton, 2006). The ventral striatum includes the multi-compartment nucleus accumbens (shell, core, and septal pole) and the olfactory tubercle, and plays a critical role in translating motivational states into goal-directed behavior (Robbins et al., 2008).

Given the diverse behavioral conditions in which striatal function appears critical, it is not surprising that there are a number of disorders that are associated with basal ganglia pathology. Among these are Parkinson's and Huntington's diseases, dystonia, and Tourette's syndrome. Several psychiatric conditions, including obsessive-compulsive disorder, substance abuse, and

schizophrenia, also appear in part to be reflecting changes in basal ganglia function.

Basal Ganglia Circuitry

As noted above, a formal definition of the basal ganglia is lacking. Our current informal use of the term has evolved over the past century as advances in our understanding of the anatomy of the brain have grown. Today most consider the basal ganglia to be a collection of subcortical structures with interconnected neurons. Such a loose definition encompasses the striatum, containing both interneurons and medium spiny neurons, the latter type of cell projecting to several downstream sites. Targets of the rodent dorsal striatum include the globus pallidus, subthalamic nucleus, and substantia nigra. From these targets, projections are directed to thalamic relay nuclei, which in turn innervate various neocortical regions. Finally, the cortex projects in a topographically-defined manner back onto the striatum. Differences in the topographical organization of these projection systems are thought to relate to different functional attributes of the striatum (Figure 1).
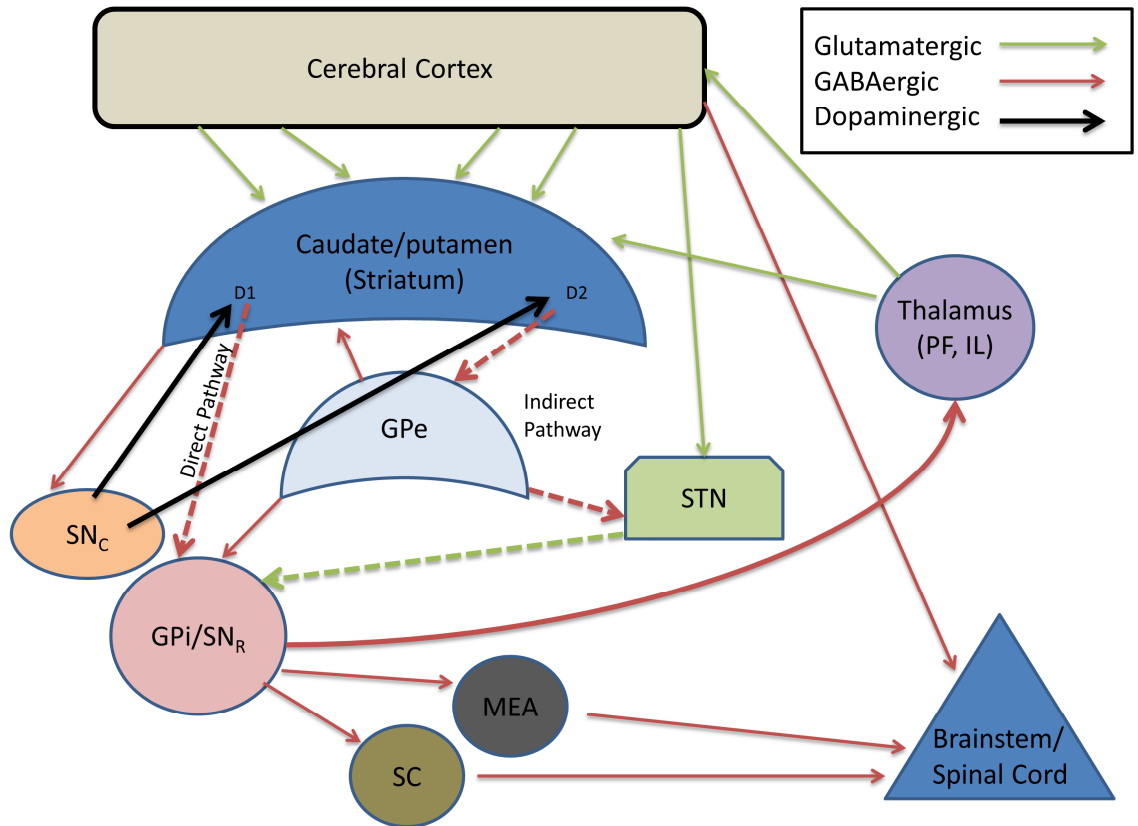
Figure 1. Simplified diagram of basal ganglia circuitry. Excitatory glutamatergic connections are indicated by green arrows, inhibitory GABAergic connections by red arrows, and modulatory dopaminergic connections by black arrows. Dotted arrows from the striatum to the GP_i/SN_R mark the direct and indirect pathways. GP_e, globus pallidus external segment; GP_i, globus pallidus internal segment; SN_R, substantia nigra pars reticulata; SN_C, substantia nigra pars compacta; STN, subthalamic nucleus; PF, parafascicular nucleus; IL, intralaminar thalamic nucleus; MEA, midbrain extrapyramidal area; SC, superior colliculus

The afferents that innervate the striatum are not typically included in the definition of basal ganglia. This is a somewhat surprising omission, particularly because all parts of the cortex innervate the striatum, thus rendering the striatum a funnel through which cortical information is relayed back to the cortex to hone subsequent motor and cognitive acts. Another major input to the dorsal striatum of the rodent originates in the thalamic central medial complex (including the central medial, centrolateral, and paracentral nuclei) and the parafascicular nucleus. These thalamostriatal neurons, like their corticostriatal counterparts, are glutamatergic (Smith et al., 2004). The third and final major input to the striatum originates in the ventral midbrain dopamine cell groups of the substantia nigra, retrorubral field, and ventral tegmental area. There are a restricted number of other "minor" afferents, including those from the globus pallidus, ventral pallidum and contiguous basal forebrain, as well as the serotonin neurons of the dorsal and median raphe in the upper brainstem. The ventral striatum, including the nucleus accumbens, which abuts both the septum and the striatum, receives inputs from sites adjacent to those that innervate the dorsal striatum, thus defining a parallel set of ventral basal ganglia structures. Although one might guess that the connections of the various parts of the basal ganglia are fully known, it is becoming clear even after the advent of the modern era of neuroanatomical tract-tracing methods, that there are gaps in our knowledge.

The major type of striatal neuron is the medium spiny neuron (MSN). Approximately 95% of striatal neurons are MSNs, with a cell body of medium size (~9-17 µm in diameter) and radially extending dendrites that are densely studded

with dendritic spines.   These GABAergic MSNs are the projection neurons of the striatum (Tepper et al., 2007).

Different portions of the relatively long dendrites of MSNs, with their high density of dendritic spines, are targeted by various inputs to the neuron (Bolam et al., 2000). Recurrent collaterals of one MSN often terminate on the soma or proximal-most dendrite of another MSN (Taverna et al., 2008; Chuhma et al., 2011). Striatal interneurons similarly synapse onto the soma or most proximal dendritic segment of MSNs (Kita et al., 1990; Bennett and Bolam, 1994).   In contrast, cortical afferents synapse onto spines located more distally on the dendrite, as do the terminals of dopamine neurons in the substantia nigra (Kemp and Powell, 1971; Hersch et al., 1995). A single cortical terminal generally synapses with a single dendritic spine (Kincaid et al., 1998). Thus, even modest changes in the numbers of MSN dendritic spines, particularly those on distal portions of the dendrite, can have major effects on ability of afferent volleys to be propagated to the cell body and thence to projection targets of MSNs (Plotkin et al., 2011).

MSNs, despite their morphological similarities, are not a homogeneous population of cells. Indeed, subtle variations in the dendritic tree of MSNs have led one group to characterize five different types of MSNs on structural grounds, although the great majority of MSNs fall into one class (Chang et al., 1982). Medium spiny neurons can also be differentiated on the basis of their projection targets, the receptors they express, and the co-transmitters in addition to GABA that they possess; these three factors co-segregate to yield two major

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populations of MSNs.   One type of MSN sends a dense projection to the globus

pallidus (GP), with the other type densely innervating the substantia nigra (SN).

Retrograde tract tracing studies from these sites have consistently revealed two

non-overlapping populations of MSNs. The MSNs that innervate the SN form the

so-called direct pathway, while those that project to the GP, neurons of which in

turn send their projections to the SN, form the indirect pathway. These two

populations of MSNs differ on the basis of the dopamine receptor they express,

with the direct pathway cells expressing the D_1 receptor and the striatopallidal

indirect pathway cells expressing the D_2 receptor. Expression of other dopamine

receptors, including the D_3 receptor of the D2 class of dopamine receptors

follows the D_1/D_2 segregation to different MSNs under basal conditions but not in

pathophysiological states. Finally, MSNs can be differentiated on the basis of

their co-transmitter. Direct pathway D_1-expressing MSNs uses tachykinin

peptides such as substance P as a co-transmitter, while D_2-expressing

striatopallidal MSNs contain enkephalin.

The direct and indirect pathway MSNs have figured prominently in models

of basal ganglia function, with these two striatofugal pathways contributing to

opposing effects on thalamic output to the cortex. Activation of the direct

pathway disinhibits the thalamus, increasing output to the cortex and promoting

intended movement, while activation of the indirect pathway further inhibits

thalamocortical neurons preventing unintended movements. These ideas have

figured prominently in our approach to diseases of the basal ganglia. For

example, it is hypothesized that there is an imbalance of these two pathways in

Parkinson's disease, tipping the balance in favor of the indirect pathway and therefore inhibiting voluntary movement.

While this model has been invaluable in guiding research into the pathophysiology of parkinsonism, over the years it has become clear that various aspects of the model are incorrect and that the model often lacks predictive validity (DeLong and Wichmann, 2009). Moreover, the structural basis for the differences in MSNs now appears to reflect a limitation of the retrograde-tract tracing methods originally used to label the two types of cells. Thus, more recent single cell labeling studies have revealed that essentially all MSNs project to both the GP and SN, but with markedly different axonal arbors (Wu et al., 2000). The direct MSNs have a large axonal plexus in the SN, with a very small axon terminal array in the GP–so small that it is insufficient to accumulate significant amounts of the retrograde transporter. Conversely, the axons of indirect pathway MSNs collateralize extensively in the GP but have a very small axonal arbor in the SN. While strictly speaking these data belie the concept of the direct and indirect pathways, functional data indicate that striatal efferent projections do indeed segregate into two classes, with one type of MSN primarily modulating the SN and the other the GP (Squire, 2003). The idea of the direct and indirect pathways having opposing effects on behavior has undergone a renaissance recently with the use of optogenetic approaches to exclusively stimulate the direct or indirect pathway *in vivo* (Kravitz et al., 2010).

A very different type of segregation of MSNs from the direct and indirect pathway distinction can be seen when examining the intra-striatal spatial

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localization of MSNs. The striatum which seems uniform at first glance under a light microscope can be further divided into unique compartments based of immunohistochemical staining patterns and connectivity. The most commonly used marker to define these regions is the mu opioid receptor. Mu opioid receptor-expressing clusters of cells have been called striosomes or patches, and occupy about 15% of the volume of the striatum, with the much larger diffuse compartment named the matrix (Gerfen, 1992a). MSNs that contribute to the direct and indirect pathways are found in both compartments, yet direct pathway MSNs in the patch compartment project to the substantia nigra pars compacta (SNc), instead of the substantia nigra pars reticulata (SNr) (Gerfen, 1984).   In contrast to MSNs that contribute to the direct and indirect pathways, which are intermingled throughout the striatum, distinct compartments of MSNs that express the mu opioid receptor (Herkenham and Pert, 1981), substance P (Bolam et al., 1988), but lack cholinergic markers (Graybiel and Ragsdale, 1978) can be seen as patches throughout the striatum. Superimposed and surrounding these patches is the larger matrix area containing MSNs that do not express the mu opioid receptor and is instead rich in acetylcholinesterase, choline acetyltransferase (ChAT), somatostatin and calbindin (Graybiel and Ragsdale, 1978; Gerfen et al., 1985; Graybiel et al., 1986). These neurochemical localizations suggest that ChAT and somatostatin positive low threshold spiking interneurons reside predominantly in the matrix. Fast spiking interneurons axons however have been observed in both regions (Cowan et al., 1990). The patch/matrix organization is particularly important during development (Johnston

et al., 1990; Gerfen, 1992b). Although these two compartments were at one time

thought to receive inputs from spatially segregated cortical cells, more recent

data have suggested that one cannot define these compartments based strictly

on the origin of their cortical innervations. Nonetheless, important functional

differences have emerged, with relative activity in MSNs across the two

compartments suggested to be disturbed in several basal ganglia disorders,

including those involving disturbances of habit, such as obsessive-compulsive

disorder and Tourette's syndrome (Graybiel, 2008).

Dorsal Striatal Subregions: Dorsal Lateral Versus Dorsal Medial Striatum

In primates the dorsal striatum is divided by the internal capsule

separating the medially located caudate nucleus from the laterally positioned

putamen. Yet in rodents there is no real clear division between dorsal medial

(caudate-like) and dorsal lateral (putamen-like) striatum. Instead the internal

capsule which is made up of descending motor axon bundles honeycombs the

striatum (Voorn et al., 2004). While these rodent striatal regions lack defined

boundaries, there are distinct anatomical and functional differences. First, inputs

to the dorsal medial striatum are primarily from association cortices while the

dorsal lateral striatum receives inputs from the sensorimotor cortex (McGeorge

and Faull, 1989). The ventral striatum receives inputs from limbic and frontal

cortex (Brog et al., 1993). Other differences between regions include cell type

preference and gene expression differences. Parvalbumin positive fast spiking

interneurons are more readily seen in lateral versus medial striatum, while

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somatostatin-positive low threshold spiking neurons are more prevalent in medial

striatum (Gerfen et al., 1985; Kita et al., 1990). Cannabinoid CB1R are highly

expressed in the dorsal lateral and nucleus accumbens but show little expression

in the dorsal medial striatum (Herkenham et al., 1991). In contrast, calbindin is

highly expressed in dorsal medial striatum with very minimal expression in the

dorsal lateral region (Gerfen et al., 1985). Expression of the dopamine

transporter is also highly enriched in the dorsal lateral striatum and less so in

medial and ventral regions where catechol-O-methyltransferase (COMT) instead

dominates (Matsumoto et al., 2003; Arbuthnott and Wickens, 2007). Additional

differences in subregion plasticity and behavior control will be reviewed later.

The Dendritic Spine: Gateway to the Medium Spiny Neuron

Dendritic spines on striatal MSNs receive excitatory drive from the cortex

and thalamus, the former relaying the consequences of higher order processing

and the latter of ascending reticular drive to the MSN.   The cortical influence

over MSNs has been intensively studied over the past generation. The heads of

MSN dendritic spines receive a single excitatory input from a cortical neuron, with

the neck of the spine often being the site at which dopamine axons from the

substantia nigra synapse, forming a synaptic triad. At the individual spine level,

this close spatial arrangement allows dopamine to modulate incoming excitatory

glutamatergic drive (Kemp and Powell, 1971; Freund et al., 1984; Smith and

Kieval, 2000). While reports suggest that the frequency of dopaminergic

synapses onto MSN spines is lower than originally proposed (Freund et al., 1984;

Groves et al., 1994), even in those cases where dopamine axons do not synapse

onto the spine one finds that dopamine terminals are located within 1.0 μm of a

spine, allowing volume (paracrine) transmission and emphasizing temporal as

well as spatial regulation of MSNs (Arbuthnott and Wickens, 2007).

Dopaminergic boutons represent nearly 10% of all the synapses in the striatum

(Groves et al., 1994) and there is evidence that the dopamine released from this

dense matrix of *en passant* synapses is not able to be adequately limited by

reuptake or metabolic mechanisms suggesting an important role of dopamine in

volume transmission (Cragg and Rice, 2004). The primarily extrasynaptic

localization of dopamine receptors at glutamatergic synapses (Hersch et al.,

1995; Yung et al., 1995) further fits with this model where they are linked to

modulation of dendritic conductances and integration of synaptic depolarization

(Nicola et al., 2000).

In addition to dopaminergic modulation of cortical drive onto MSNs at the

level of the MSN spine, D_2 heteroceptors on glutamatergic corticostriatal

terminals can also tonically inhibit glutamate release from these terminals

(Bamford et al., 2004). The cortical influence over striatal cells is critical because

MSNs have spontaneous fluctuations in their membrane potential, ranging from a

relatively hyperpolarized "downstate" around -80mV to a more depolarized

"upstate" around -50mV (Wilson and Groves, 1981). While the downstate is

maintained by a rapidly activating inwardly rectifying potassium (Kir) current

which limits membrane depolarization, transition to the upstate appears to be

determined largely by strong, correlated release of glutamate from corticostriatal

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glutamatergic terminals leading to sufficient depolarization to inactivate Kir (Wilson and Kawaguchi, 1996; Plenz and Kitai, 1998). The closure of dendritic Kir channels increases the input resistance of the neuron and reduces the overall electrotonic length. Spiking is only observed in the upstate, but not all upstate transitions lead to the firing of an action potential. The upstate transition persists as long as there is sufficient excitatory drive to maintain depolarization, often for hundreds of milliseconds, and evidence suggests that the transitions are more prominent under anesthesia but far noisier in the waking state (Mahon et al., 2006). These different states of the MSN membrane dictate if an incoming volley to the MSN will depolarize the cell. Simultaneous activation of many cortical afferents at various points on the MSN dendritic tree is needed to depolarize MSNs from a hyperpolarized potential to action potential firing (Wilson and Kawaguchi, 1996; Carter and Sabatini, 2004; Carter et al., 2007). Upstate transition magnitude is determined by voltage-sensitive potassium conductances which are activated by depolarization and subsequently limit its extent (Wilson and Kawaguchi, 1996). Upstates correlate amongst neighboring MSNs, consistent with the idea that convergent cortical inputs drive these transitions (Stern et al., 1998). The different states determine properties underlying synaptic conductance. In the down state, excitatory postsynaptic potentials are primarily AMPAR-mediated, while upstates recruit NMDARs. This leads to prolonged excitatory potentials in the upstate which lead to a greater likelihood of temporal summation. Additionally, in the upstate the main source of calcium entry is through NMDARs and L-type calcium channels that have been shown to be very

important in the induction of long-lasting synaptic plasticity (Choi and Lovinger, 1997a; Carter and Sabatini, 2004; Adermark and Lovinger, 2007a). Recently, it was shown that uncaging of glutamate on multiple dendritic spines on distal dendrites evoked somatic upstates lasting hundreds of milliseconds, while uncaging on more proximal sites only produced modest potential changes (Plotkin et al., 2011). These state transitions were dependent on NMDAR and T- and R-type calcium channels. This study shows that distally regenerative upstate transition can occur with the concerted stimulation of only tens of synapses.

Role of Dopamine in Basal Ganglia Function

A unique feature of the striatum is the extraordinarily dense dopaminergic innervation that it receives. A remarkable study employing a viral vector to target green fluorescent protein to the membranes of neurons has found that single dopamine neurons in the substantia nigra give rise to remarkably long intra-striatal axons (up to 780,000 μm, i.e., 780 cm in length), and that the dense portion of the striatal axonal plexus derived from one nigral dopamine neuron can cover up to 5.7% of the total striatal volume (Matsuda et al., 2009). As such, even one dopamine neuron can influence a very large number of striatal MSNs, and it is therefore not surprising that striatal dopamine plays a critical role in modulating motor behavior and learning.

While the timing and magnitude of dopamine release is important for normal voluntary movement, the firing patterns of dopamine neurons do not

correlate with voluntary movements. Instead dopamine neurons of the ventral

tegmental area and substantia nigra play a central role in positive reinforcement

learning (Mirenowicz and Schultz, 1994, 1996). Midbrain DA neurons are

spontaneously active, firing at low frequencies (1-8Hz) at rest *in vivo* (Schultz,

2007). This firing maintains a basal level of tonic dopamine that binds to high

affinity D2Rs, maintaining normal striatal function (Richfield et al., 1989).

Alternatively, dopamine neurons show phasic or burst-like firing patterns

following unexpected reward. These bursts of action potentials transiently

elevate dopamine levels at the synapse activating lower affinity D1Rs. However

after repeated conditioning sessions with an environmental cue (e.g., a light or

tone) preceding the reward, phasic firing of dopamine neurons can be induced by

the cue alone, as a predictor of a reward (Schultz, 1998). In the case of a

predicted reward the actual delivery of reward does not lead to an increase firing.

If reward is delayed a depression in firing rates occurs at the original time and

instead activation occurs at the new time (Fiorillo et al., 2008; Zaghloul et al.,

2009). Also the delay itself decreases the dopamine response to the same

reward (Kobayashi and Schultz, 2008). Aversive events such as air puffs,

hypertonic saline and electric shock actually enhance firing in awake animals in a

small proportion of dopamine neurons, but the majority of dopamine neurons

show reductions or no change in firing (Mirenowicz and Schultz, 1996; Joshua et

al., 2008; Matsumoto and Hikosaka, 2009). Whether the small percentage of

dopamine neurons activated by aversive events causes a functionally relevant

release of dopamine is still controversial (Mirenowicz and Schultz, 1996; Young,

2004). Overall, these findings have led to the hypothesis that dopamine acts as a mean reward prediction error signal, acting to gauge between subjective expected and actual reward (Schultz, 1997). Natural rewards, as well as many drugs of abuse (Wise, 2004), elevate dopamine concentrations in the nucleus accumbens (Ahn and Phillips, 2007) and striatum (Nakazato, 2005). Long lasting neuroadaptations in dopamine tone can be observed in response to behavioral states including stress, uncertainty, and protracted drug use (Schultz, 2007). Recent studies utilizing channelrhodopsin to stimulate firing of dopamine neurons induces Pavlovian place preference conditioning in mice (Tsai et al., 2009).

Canonically, D1 and D5 receptors normally enhance cAMP levels while D2, D3, and D4 receptors inhibit the production of cAMP. Phasic bursts of dopamine are hypothesized to preferentially activate low affinity D1 receptors, while tonic release of dopamine would favor high affinity D2 receptor binding (Lovinger et al., 2003). In addition to D1 and D2 receptor expressing cells in the dorsal striatum, expression of D3, D4 and D5 receptors are present at varying extents. Striatal GABAergic interneurons express primarily D5 receptors, while cholinergic interneurons express both D2 and D5 receptors (Yan et al., 1997; Yan and Surmeier, 1997). While the expression levels of these dopamine receptors are lower than D1 and D2 receptors they most likely have important functional roles. While there is little expression of D4 and D5 receptor in MSNs (Bergson et al., 1995), the D3 receptor is expressed at significant levels in a subpopulation of D1 receptor-expressing cells. In fact many drugs that are intended to be specific for D2 receptors actually bind to D3 receptors as well.

Interestingly, following dopamine depletion and subsequent levodopa administration D3 receptors are upregulated in the dorsal striatum (Bordet et al., 1997). Additionally, in dyskinetic monkeys D3 receptor binding is higher than in non-dyskinetic or control monkeys following dopamine depletion and levodopa administration. Also D3 receptor binding levels in the striatum correlated with the severity of levodopa-induced dyskinesias (Guigoni et al., 2005). Brain-derived neurotrophic factor (BDNF) is important in D3 receptor expression and the maintenance of its expression. Moreover, the administration of a D3 receptor partial agonist strongly attenuates levodopa-induced dyskinesias (Guillin et al., 2003). These data exemplify the importance of examining other less characterized dopamine receptors in the striatum and their possible connection to basal ganglia related disorders.

Role of Acetylcholine in Striatal Function

Acetylcholine (ACh) represents another important neuromodulator class in the striatum. Acetylcholine is released by cholinergic interneurons which are tonically active, releasing Ach locally and possibly via a volume transmission mechanism (Izzo and Bolam, 1988; Contant et al., 1996). Even though cholinergic interneurons represent a small proportion of the total striatal neuron population, there dense and widespread release of Ach plays an important role modulating glutamatergic transmission, synaptic plasticity and ultimately action selection and decision-making. *In vivo*, cholinergic neurons are tonically active (<10Hz) during rest, but during behaviorally salient stimuli the neurons show

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bursting followed by a pause in firing lasting for up to a second (Aosaki et al., 1995; Ding et al., 2010). The pause seems to require coordinated inputs from SNc and intralaminar thalamic nuclei (Matsumoto et al., 2001). When the burst/pause was recreated in a brain slice the pattern of stimulation initiates a brief presynaptic M2R modulation followed by a slow M1R modulation which may serve to reset the corticostriatal circuit allowing for possible reassessment and suppression of action selection (Ding et al., 2010).

Both nicotinic and muscarinic receptors transduce ACh signals in the striatum. Nicotinic receptors (nAChR) are ligand-gated ion channels that are expressed on DA terminals and on fast spiking interneurons (Koos and Tepper, 2002; Zhou et al., 2002). Muscarinic receptors are GPCRs widely expressed on axon terminals to the striatum and by all striatal neurons that have been examined. The muscarinic receptors can be divided into the M1-class (M1, M3, M5), which is coupled to Gq/11 and activates PLC and M2-class (M2, M4), which couples to Gi/o and inhibits adenylyl cyclase (AC). The M1 and M4 are the most abundant in the striatum at the tissue level. M1Rs are found in both direct and indirect MSNs, while M4R are more highly expressed in direct-pathway MSNs (Bernard et al., 1992; Ince et al., 1997; Yan et al., 2001). M2R are exclusively located in cholinergic interneurons where along with M4R they function as autoreceptors limiting ACh release (Bernard et al., 1992; Alcantara et al., 2001). Glutamatergic afferents to MSNs contain M2-class mAChRs and their activation reduces glutamatergic EPSCs (Pakhotin and Bracci, 2007; Higley et al., 2009). Yet postsynaptic activation of M1Rs potentiates the postsynaptic response to

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glutamate without altering glutamate receptor number and/or function (Higley et al., 2009). At first glance, M2R-mediated inhibition seems to oppose the M1R-mediated potentiation, but under bursts of action potentials the M2-mediated presynaptic inhibition can be overcome tuning synapses to repetitive stimulation.

Synaptic Triad

Glutamatergic synapses on MSN dendritic spines are a major site of long-lasting neuroadaptations and likely a key site underlying the neural correlates of motor learning. Dopamine plays an important role in regulating plasticity in the striatum. In addition to dopamine the striatum receives highly topographic glutamatergic thalamostriatal projections from intralaminar nuclei, such as the centromedian and parafascicular nuclei, and non-intralaminar nuclei (Smith et al., 2004). Thalamic inputs, unlike cortical inputs, target a higher percentage of dendritic shafts in both rats and monkeys (Smith and Bolam, 1990; Sadikot et al., 1992; Smith et al., 1994; Sidibe and Smith, 1996). In monkeys, centromedian projections preferentially synapse onto "direct" pathway neurons versus "indirect" pathway neurons (Sidibe and Smith, 1996). In addition to innervating distinct targets, there is evidence that synaptic transmission is not similar between these inputs. For example, the probability of release at cortical and thalamic glutamatergic synapses on MSNs appears to be different (Smeal et al., 2007; Ding et al., 2008). Further studies utilizing techniques to better separate these distinct inputs are necessary to characterize their properties.

While electron microscopy analysis of D2R immunohistochemical localization on glutamatergic synaptic terminals is controversial, revealing exceedingly rare or low levels of D2R (Fisher et al., 1994; Sesack et al., 1994; Hersch et al., 1995; Wang and Pickel, 2002), an elegant study by Sulzer's group showed functional evidence of presynaptic dopamine acting to reduce glutamate release (Bamford et al., 2004). Utilizing a dye (FM1-43) to load glutamatergic synaptic vesicles they were able to monitor release via destaining coupled with electrochemical recordings to directly measure the effects of dopamine at the level of individual presynaptic terminals. Interestingly, another study showed that the inhibition of glutamate release by D2R stimulation was frequency dependent; resulting in inhibition of glutamate release at higher frequency stimulation (20Hz), but not at lower frequencies (1Hz) (Yin and Lovinger, 2006). This inhibition was dependent on CB1R, mGluRs, and rises in internal calcium levels. These data suggest a postsynaptic mechanism for D2R-mediated control of glutamatergic synaptic transmission, but they leave open the question of exactly where the D2Rs are localized that mediate this inhibition.

GABAergic MSN Synapses

Another component of the corticostriatal circuitry that needs consideration in order to understand synaptic transmission and MSNs firing is the influence of local inhibitory collaterals. MSNs have local GABAergic connections to other neighboring MSNs. This feedback may underlie lateral inhibition (Beiser et al., 1997), but its functional significance has been controversial. One reason for this

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is that many of the recurrent inhibitory synapses are on distal dendrites (Wilson

and Groves, 1980; Bolam et al., 1993) leading to space clamp issues and

difficultly measuring these distal inhibitory currents with a somatic patch

electrode. In randomly selected MSN neighbors using dual-patch techniques the

percentage of synaptically connected neighbors is small (10-15%) (Czubayko

and Plenz, 2002; Guzman et al., 2003; Koos et al., 2004), yet optogenetic

approaches suggest a much higher degree of connectivity (Chuhma et al., 2011).

Using D1R and D2R BAC transgenic mice it was found that indirect pathway

MSNs connect to both direct and other indirect pathway MSNs, but direct

pathway MSNs only connect to other direct pathway MSNs (Taverna et al.,

2008). Additional study will be needed to understand the functional significance

of GABAergic recurrent connectivity.

Other sources of GABAergic inhibition come from intrinsic GABAergic

interneurons, which make up a very small percentage of the total striatal

neuronal pool. Fast spiking (FS) interneurons receive excitatory inputs from the

cortex and thalamus and synapse on or near the soma of direct and indirect

pathway MSNs (Koos and Tepper, 1999; Sidibe and Smith, 1999; Planert et al.,

2010). A report suggested preferential connectivity of FS interneurons with direct

pathway MSNs (Gittis et al., 2010). Individual MSNs are estimated to receive 4-

27 FS interneuron synapses and a single FS interneuron can connect to 135-541

MSNs allowing for bursts from single FS interneurons to significantly delay AP

firing in numerous MSNs (Koos and Tepper, 1999). FS interneuron activation is

the primary mechanism for feed-forward inhibition, contributing to action selection

by inhibiting MSNs in circuits with competing inappropriate actions (Parthasarathy and Graybiel, 1997; Gage et al., 2010). They are enriched in the dorsal lateral striatum, suggesting an important role in sensorimotor integration (Kita et al., 1990; Bennett and Bolam, 1994). Indeed, mice with decreased numbers of striatal fast spiking interneurons exhibit procedural learning deficits (Marrone et al., 2006). Another GABAergic interneuron subtype includes the somatostatin/ neuropeptide Y (SOM/NPY) expressing interneuron that forms another feed-forward circuit in the striatum (Tepper et al., 2010). These interneurons are also referred to as low-threshold spiking (LTS) interneurons due to their firing properties. LTS interneurons exhibit high input resistance, depolarized resting potentials, plateau potentials, and low threshold spiking (Kawaguchi, 1993). LTS interneurons receive glutamatergic inputs from the cortex and the thalamus as well as a dopaminergic innervation (Vuillet et al., 1989; Sidibe and Smith, 1999; Hidaka and Totterdell, 2001). Little is known of their functional importance, but their innervation of distal MSN dendrites hampers their understanding (Gittis et al., 2010). Even less is known about another LTS striatal interneuron that is immunopositive for calretinin (Tepper and Bolam, 2004).

The Glutamate Synapse

Glutamate synapses in the striatum contains a presynaptic terminal that releases glutamate and a postsynaptic dendritic spine filled with receptors that bind glutamate. These receptors come in two varieties: ionotropic and

metabotropic glutamate receptors. Two major ionotropic glutamate receptors are the AMPA and NMDA receptor subtypes, which are defined by their specific pharmacological sensitivity to the respective agonists and are tethered to the postsynaptic density (PSD) by associated anchoring and scaffolding proteins. AMPA receptors (AMPARs), which mediate the majority of fast excitatory transmission in the brain, are tetramers that are made up of some combination of GluA1-GluA4 AMPAR subunits. Most AMPARs respond to synaptic glutamate release by gating a monovalent cation current that helps depolarize the postsynaptic terminal. Tetrameric NMDA receptors (NMDARs) are thought to contain two obligatory GluN1 subunits and some combination of GluN2A-2D subunit and GluN3A or 3B. Binding of glutamate and glycine to GluN2 and GluN1 subunits, respectively, gates a cation channel that a) fluxes calcium and b) is blocked at hyperpolarized membrane potentials by a magnesium ion. The magnesium block of NMDARs is relieved by a strong depolarization greater than what is achieved via upstate transitions. Thus, NMDARs act as coincidence detectors allowing calcium influx following coincident presynaptic release of neurotransmitters coupled with AMPA-mediated postsynaptic depolarization. The consequential rise in intracellular calcium is a critical trigger for long-term changes in the efficacy of transmission at these synapses.

NMDAR-dependent Synaptic Plasticity: Hippocampal Long-term

Potentiation and Depression

Glutamatergic synapses have a unique ability to undergo long lasting changes in synaptic efficacy in response to very transient signals. The pioneering work of Bliss and Lomo in the perforant-pathway dentate gyrus synapse using high frequency stimulation to induce a long lasting potentiation of excitatory transmission set the stage for much of the subsequent work published on neural plasticity (Bliss and Lomo, 1973). Two classic forms of synaptic plasticity, long-term potentiation (LTP) and long-term depression (LTD) have been characterized initially in the hippocampus and involve long-lasting changes in glutamatergic transmission. NMDAR -dependent LTP and LTD in the CA1 region of the hippocampus has been the most widely studied form of plasticity. These activity dependent, long-lasting synaptic adaptations are hypothesized to play key roles in learning and memory. High frequency stimulation or stimulation paired with postsynaptic depolarization leads to the induction of an NMDAR-dependent form of LTP in the CA1 region of the hippocampus. Additionally, LTP has been shown to persist for at least hours *in vitro* and for months *in vivo* (Abraham et al., 2002). While late phases of LTP, like learning and memory, are dependent on gene transcription and new protein synthesis (Madison et al., 1991), a transient (2-3 second) elevation of calcium via NMDARs seems to be sufficient for induction of this form of LTP (Lynch et al., 1983; Malenka et al., 1988; Malenka et al., 1992). One important mediator of NMDAR-dependent LTP in the hippocampus, no matter the induction mechanism, is the postsynaptic calcium-activated kinase,

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CaMKII (Lisman et al., 2002). A major locus of potentiation is postsynaptic via an activity-dependent increase in function and/or number of AMPARs at the synapse (Malenka and Nicoll, 1999; Malinow and Malenka, 2002; Song and Huganir, 2002; Bredt and Nicoll, 2003). Potentiation can proceed by the addition of new AMPARs at the synapse or by the recruitment of AMPARs to synapses that initially only contain NMDARs. So-called "silent" synapses (synapses that only contain NMDAR) are thought to be key sites for NMDAR-dependent LTP. The unsilencing of synapses in adulthood is thought to be similar to LTP in that it shares its NMDAR- and CaMKII-dependence, and data suggest that LTP is induced in part by the unsilencing of synapses (Liao et al., 1992; Manabe et al., 1992; Liao et al., 1995). A decrease in synaptic failure rates following LTP induction supports the idea that CaMKII activation converts silent synapses into functional contacts (Lledo et al., 1995). Lledo *et al.* showed that the addition of constitutively active CaMKII in the recording pipette leads to a decrease in the number of synaptic failures, an increase in the amplitude of the evoked responses and an increase in the spontaneous EPSC frequency and amplitude (Lledo et al., 1995). These data in part can be interpreted as an increase in the number of silent synapses following the activation of CaMKII. Wu *et al.* also presented data implicating CaMKII in the unsilencing of synapses: early during development retinotectal synapses of the frog are silent, lacking AMPARs, but upon transfection with a constitutively active CaMKII fragment (tCaMKII) the strength of AMPAR mediated transmission increases and the fraction of silent synapses decreases (Wu et al., 1996). Additionally, LTP at hippocampal

synapses is also thought to involve enlargement of the postsynaptic dendritic spine (Lisman and Harris, 1993; Lisman and Zhabotinsky, 2001). Indeed, induction of LTP at single dendritic spines by local uncaging of glutamate results in actin-dependent structural enlargement (Fukazawa et al., 2003; Matsuzaki et al., 2004). Previous studies have shown that introduction of constitutively active CaMKII into hippocampal neurons is sufficient to induce spine growth (Jourdain et al., 2003).

While NMDAR-dependent LTP in the CA1 region of the hippocampus is the most studied form of plasticity, another major form of potentiation is NMDAR-independent LTP as found at the mossy fiber-CA3 synapses of the hippocampus (Harris and Cotman, 1986). This form of LTP, dubbed mossy fiber LTP, is thought to be modulated by kainate receptors (Bortolotto et al., 1999; Contractor et al., 2001; More et al., 2004). Moreover, in contrast to NMDAR-LTD, mossy fiber LTP is thought to be maintained presynaptically, involving an increase in the probability of synaptic vesicle release. Other studies suggest that R-type calcium channels (Breustedt et al., 2003; Dietrich et al., 2003), calcium activated adenylyl cyclases (AC1/8)(Xia et al., 1991; Glatt and Snyder, 1993), cAMP-PKA signaling (Huang et al., 1994; Weisskopf et al., 1994; Huang et al., 1995; Lopez-Garcia et al., 1996; Tong et al., 1996), the synaptic vesicle protein Rab3a (Castillo et al., 1997), and the active zone protein RIM1α are all important in mossy fiber LTP (Castillo et al., 2002).

In addition to LTP, NMDAR activation can also produce long-term depression (LTD) at glutamate synapses in the hippocampus. Typically, this LTD

is induced in the CA1 region of the hippocampus via prolonged low frequency stimulation (0.5-3Hz) (Dudek and Bear, 1992; Mulkey and Malenka, 1992). This LTD is dependent on NMDARs, increases in postsynaptic calcium, and activation of serine-threonine protein phosphatases (Mulkey and Malenka, 1992; Mulkey et al., 1993; Mulkey et al., 1994), which are thought to drive internalization of AMPARs (Carroll et al., 1999; Beattie et al., 2000). Dephosphorylation at GluA1 Ser845, a site that seems to be constitutively phosphorylated under basal conditions, accompanies LTD induction (Lee et al., 1998).

In addition to NMDA receptor-dependent LTD, an mGluR-dependent form of LTD (Bolshakov and Siegelbaum, 1994; Oliet et al., 1997) can be elicited in several brain regions by paired-pulse low frequency stimulation (PP-LFS) or bath application of the group 1 selective agonist (R,S)-3,5-dihydroxyphenylglycine (DHPG) (Ito et al., 1982; Kano and Kato, 1987; Huber et al., 2000). Both forms of mGluR-LTD via PP-LFS or DHPG occlude each other suggesting similar expression mechanisms (Huber et al., 2001), but there are notable mechanistic differences from NMDAR-mediated LTD. mGluR-LTD was first characterized at parallel fiber-Purkinje cell synapses of the cerebellum and is primarily dependent on mGluR1 activation and the resulting activation of PKC. Within the cerebellum, PKC phosphorylates GluR2 at Ser880 leading to the clathrin-dependent removal of GluR2/3 containing AMPA receptors from the synapse (Wang and Linden, 2000; Chung et al., 2003). Induction of hippocampal mGluR-LTD is independent of NMDARs and does not occlude the induction of NMDAR-dependent LTD, suggesting it utilizes different mechanisms (Oliet et al., 1997). Hippocampal

mGluR-LTD has been shown to be dependent on intracellular calcium concentration in certain experiments (Oliet et al., 1997) yet independent of intracellular calcium concentration in others (Fitzjohn et al., 1999). In the hippocampus, mGluR-LTD seems to depend on both mGluR5 and mGluR1 receptors: a combination of mGluR1 and mGluR5 antagonists is required to block its induction (Huber et al., 2001), but hippocampal mGluR-LTD is totally blocked in mGluR5-KO mice and only partially blocked in mGluR1KO mice (Volk et al., 2006). This suggests that activation of these two receptors produces synergistic responses to induce LTD. Group 1 mGluRs, like mGluR1 or 5, couple to effectors via Gαq G proteins. The addition of GDPβS to the patch pipette or recordings in Gαq (-,-) mice inhibited the induction of mGluR-LTD. Activation of group 1 mGluRs leads to the activation of phospholipase C, increasing IP3 and DAG levels. Activation of these two signaling molecules leads to the liberation of calcium from internal stores and in combination with DAG activates PKC. In experiments where a PKC inhibitor was added to the patch pipette, the induction of mGluR-LTD in both the hippocampus and cerebellum was blocked (Linden and Connor, 1991; Wang et al., 2007). Group I mGluR-LTD also involves the mitogen-activated protein kinase/extracellular signal regulated kinase (MAPK/ERK) pathway. Indeed ERK inhibitors block the induction of mGluR-LTD in the hippocampus (Huber et al., 2000; Huber et al., 2001; Gallagher et al., 2004; Volk et al., 2006; Ronesi and Huber, 2008) and cerebellum (Ahn et al., 1999; Kawasaki et al., 1999).

ERK signaling can regulate translation machinery, which suggests that mGluR-LTD may regulate translation of new proteins. There is much evidence suggesting that mGluR-LTD requires new protein synthesis (Huber et al., 2001), yet some reports suggest it may be independent of new protein synthesis (Huber et al., 2000; Moult et al., 2008). The putative new protein synthesized and needed during mGluR-LTD is possibly the AMPA receptor GluA2 subunit, due to the fact that mGluR-LTD is blocked following pretreatment with siRNA and oligonucleotides blocking GluA2 translation (Mameli et al., 2007). Group I mGluR-LTD induction is also thought to be dependent on activation of postsynaptic protein tyrosine phosphatases, specifically striatal-enriched tyrosine phosphatase (STEP), which dephosphorylates the GluA2 subunit of AMPARs triggering lateral diffusion and subsequent endocytosis (Moult et al., 2002; Huang and Hsu, 2006; Moult et al., 2006; Gladding et al., 2009). However, conflicting results do not allow a conclusive statement about the primary locus for induction of mGluR-LTD (presynaptic, postsynaptic, or both). Inconsistencies could be possibly explained by the use of different experimental bath temperatures and/or animal ages. Additional work will be needed to delineate complex mechanisms underlying the induction and expression of mGluR-LTD.

CaMKII and Plasticity

CaMKII is a calcium/calmodulin-activated kinase that is highly expressed throughout the brain and is enriched in the postsynaptic density (PSD) of glutamatergic synapses (Lisman et al., 2002). Recent, estimates of the

concentration of CaMKII in the dendritic spine is around 100µM (Feng et al.,

2011). The predominant neuronal CaMKIIα and CaMKIIβ isoforms are enriched

in the forebrain and cerebellum, respectively (Yamagata et al., 2009). One or

more CaMKII isoforms assemble to form a dodecameric holoenzyme and it has

been estimated that around 80 CaMKII holoenzymes reside in the average

dendritic spine (Chen et al., 2005; Shinohara et al., 2008). Many lines of

evidence show that CaMKII responds to changes in intracellular calcium levels to

promote biochemical signaling cascades that lead to potentiated synaptic

transmission (Figure 2). While signaling via CaMKII is an important mechanism

underlying synaptic potentiation, alternate signaling pathways like cAMP-PKA

and MAPK signaling pathways may also be engaged depending the induction

parameters, but this extensive literature will not be reviewed here (Thomas and

Huganir, 2004). CaMKII is activated during hippocampal LTP induction and its

activation is necessary and sufficient for LTP (Malinow et al., 1989; Tokumitsu et

al., 1990; Otmakhov et al., 1997; Lisman et al., 2002; Colbran, 2004; Sanhueza

et al., 2007). A recent study found that a knock-in mutation of CaMKIIα to

inactivate the kinase disrupts LTP induction, the associated enlargement of

spines, and spatial learning (Yamagata et al., 2009). Following robust calcium

entry into the postsynaptic neuron, CaMKII is rapidly autophosphorylated at

threonine 286, converting the enzyme from a calcium-dependent into a calcium-

independent form (Miller and Kennedy, 1986; Fukunaga et al., 1993; Rich and

Schulman, 1998; Lee et al., 2009). Activation of CaMKII drives the translocation

of the enzyme from the cytoplasm to the PSD, where it can bind to important

plasticity-associated proteins like NMDARs (Strack and Colbran, 1998; Leonard et al., 1999; Shen and Meyer, 1999). CaMKII's potential to remain active in the PSD/spine long after the postsynaptic calcium transient has subsided is why it is often thought of as a molecular switch capable of long-term memory storage. Indeed, increased threonine 286 phosphorylation of CaMKII can persist for at least sixty minutes (Barria et al., 1997b). However, one study reported that autonomous CaMKII activity returned to basal levels within minutes of LTP induction (Lengyel et al., 2004), suggesting that mechanisms other than threonine 286 phosphorylation control autonomous CaMKII activity. Although the Thr286 autophosphorylated CaMKII may have roles independent of autonomous kinase activity during LTP maintenance, this study also highlights an uncertain role for CaMKII activity during LTP maintenance. Indeed, labs have reported that intracellular perfusion of CaMKII inhibitor peptides after LTP induction does not disrupt maintenance (Malenka et al., 1989). In contrast, one recent study found that LTP maintenance can be disrupted by bath application of newly developed membrane-permeant CaMKII inhibitor following LTP induction (Sanhueza et al., 2007). It is possible that membrane permeant peptides have better access to block CaMKII activity in the appropriate subcellular compartment (e.g., in the spines/PSD). Alternatively, the requirement for CaMKII activity may depend on the induction mechanism, or be evident only during certain phases of LTP maintenance. Interestingly, behavioral studies suggest that CaMKII activity in restricted time windows is required for memory consolidation (Wang et al., 2003; Wan et al., 2010)
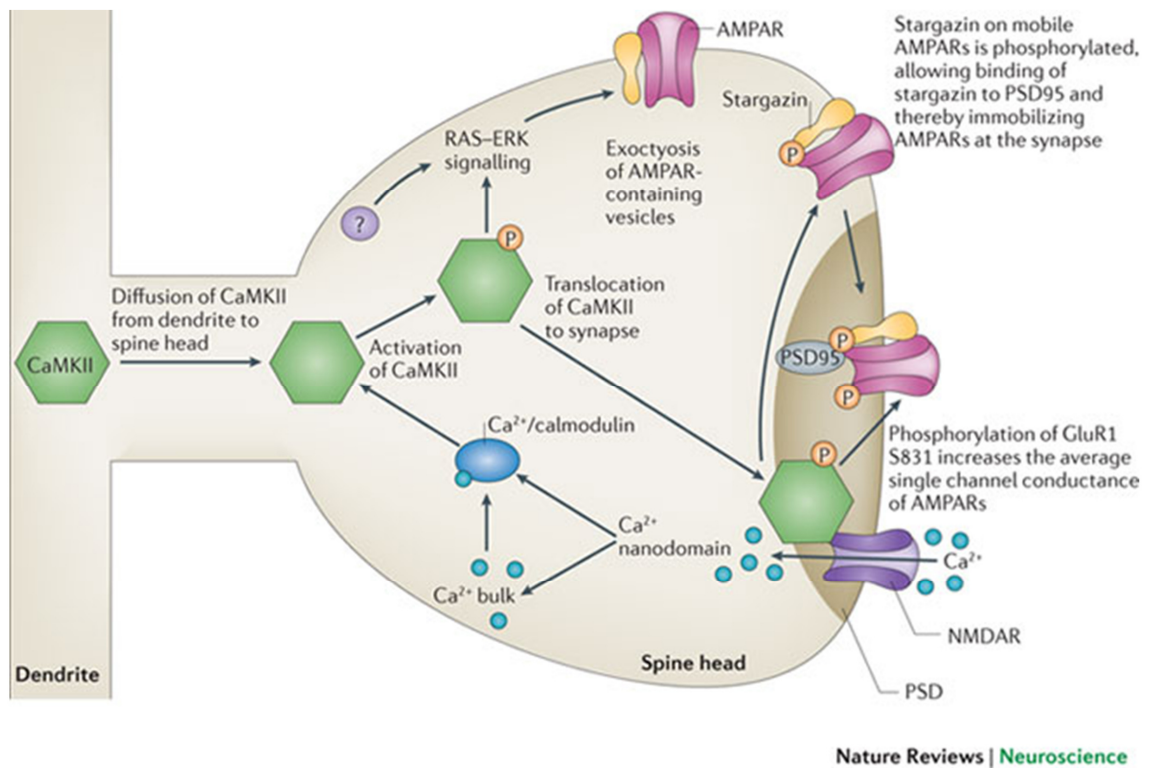
Figure 2. Role of CaMKII in Synaptic Plasticity and LTP in Dendritic Spines.
Calcium influx via NMDAR leads to the activation of CaMKII and the potentiation of synaptic transmission. Activated CaMKII translocates to the PSD where it can bind the GluN2B subunit of the NMDAR prolonging its activation. Active CaMKII can potentiate excitatory synaptic transmission by driving new AMPAR to the synapse or phosphorylating existing AMPARs (at the GluR1 S831 site) enhancing the average conductance. CaMKII's phosphorylation of stargazin allows for the binding of AMPARs to PSD-95 increasing the dwell time of AMPAR in the synapse. Active CaMKII also plays structural roles in dendritic spine and synapse enlargement. Image from (Lisman et al., 2012)

Despite uncertainties about the role of CaMKII activity during LTP maintenance, a critical role of Thr286 phosphorylation was elegantly demonstrated by the creation of knock-in transgenic mice with a non-phosphorylated alanine residue replacing Thr286 in CaMKIIα. The resulting disruption of CaMKII phosphorylation at Thr286 leads to profound disruption of hippocampal LTP (Giese et al., 1998). Conversely, transgenic mice overexpressing CaMKIIα with a threonine 286 to aspartate mutation (CaMKIIα^{T286D}), which mimics autophosphorylation, leads to a loss in LTP induction at lower frequencies and shifts the size and direction of synaptic change in favor of LTD induction (Mayford et al., 1995; Mayford et al., 1996). These data suggest that CaMKII serves as a sensor of the level of neuronal activity controlling the frequency-response function (Bienenstock et al., 1982). Additionally, overexpression of CaMKIIα^{T286D} in the adult forebrain disrupts spatial memory and fear conditioned memory (Mayford et al., 1996).

Another important phosphorylation site on CaMKII is the Thr305/306 site, in the calmodulin-binding domain. Following T286 phosphorylation, T305/306 sites are phosphorylated, strongly reducing calcium/calmodulin-dependent kinase activity (Hanson and Schulman, 1992; Jama et al., 2009; Coultrap et al., 2010). Phospho-T305/306 inhibits CaMKII from binding and/or prevents dissociation from the PSD which is associated with learning deficits (Elgersma et al., 2002). Indeed, enhanced phosphorylation of T305/306 sites is linked to disrupted plasticity in a mouse model of Angelman's disease (Weeber et al., 2003).

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CaMKII activation shares many similarities with the induction of LTP. To determine if two forms of plasticity use a similar mechanism an occlusion test can be performed. For example, LTP induction by HFS is prevented or occluded following potentiation induced by activated CaMKII, and vice-versa, suggesting a common downstream mechanism (Pettit et al., 1994; Lledo et al., 1995). Addition of activated CaMKII in the patch pipette can mimic LTP by enhancing the amplitude and frequency of spontaneous excitatory postsynaptic currents (sEPSCs, action potential dependent and independent currents that are recorded in a voltage clamped neuron in response to the release of synaptic glutamate) and decreasing the failure rate which is attributed with an increase in the probability of release (Lledo et al., 1995). These results suggest that CaMKII alone is sufficient to enhance synaptic transmission and that this enhancement shares common features underlying the mechanism seen following LTP induction.

The activation of CaMKII can strengthen synaptic transmission by multiple mechanisms. Active CaMKII can phosphorylate AMPAR GluA1 subunits at serine 831 and increase the conductance state of AMPARs (Barria et al., 1997a; Mammen et al., 1997; Derkach et al., 1999; Lee et al., 2000). The mechanism of the increase in conductance state was recently revealed. A knock-in mutation encoding a S831 pseudo-phosphorylated GluR1 does not alter conductance state magnitude. Instead, it increases the likelihood of high conductance state transitions (Kristensen et al., 2011). At least for heteromeric GluR1/GluR2-containing AMPARs this increase in conductance only occurred in the presence

of stargazin. Phosphorylation of GluR1^{Ser831} is increased following LTP induction and inhibiting CaMKII blocks phosphorylation at this site (Barria et al., 1997b). However, LTP can still occur when phosphorylation at GluR1^{Ser831} is blocked. Only when GluR1^{Ser845} is also inhibited is LTP disrupted, suggesting that additional mechanisms as well as distinct interactions between these phosphorylation sites underlie synaptic potentiation (Hayashi et al., 2000; Lee et al., 2010).

Another important postsynaptic mechanism is the activity-dependent, CaMKII-dependent trafficking of AMPARs to the synapse (Shi et al., 1999; Opazo and Choquet, 2011). Hayashi *et al.* showed that constitutively activated CaMKII leads to the insertion of new AMPARs in the synapse; one potential mechanism driving the potentiation seen following LTP induction (Hayashi et al., 2000). A more recent study showed by tagging AMPARs with a quantum dot that the molecular dynamics and trafficking of AMPARs could be observed (Opazo et al., 2010). Extrasynaptic AMPARs were trapped at the synapses by phosphorylation of an auxiliary protein stargazin by CaMKII. Phosphorylation of stargazin allows AMPARs to bind to PSD-95 and increases the overall dwell time of AMPARs at the synapse. Mutation of the putative CaMKII phosphorylation sites on stargazin did not affect surface expression, but prevented the enhancement in synaptic transmission seen following LTP induction (Tomita et al., 2005). While CaMKII is involved in the trafficking of AMPARs from extrasynaptic sites to synapses by likely phosphorylating stargazin during LTP, the initial exocytosis of AMPARs is thought to depend on RAS-ERK pathway, RAB-GTPase proteins, kalirin-7,

SNARE proteins, certain syntaxin isoforms and myosin V, a molecular motor protein (Zhu et al., 2002; Park et al., 2004; Xie et al., 2007; Wang et al., 2008; Kennedy et al., 2010; Patterson et al., 2010). Indeed, CaMKII inhibition only partially inhibits exocytosis, leaving room for additional calcium sensors to be identified (Patterson et al., 2010). Inhibition of exocytosis or any of the above mentioned proteins does not affect the first 20 minutes of LTP, yet has a strong effect on subsequent LTP. The initial potentiation is thought to be maintained by trafficking from extrasynaptic stores (Makino and Malinow, 2009). However, despite the preponderance of data linking CaMKIIα to LTP induction in cortex and hippocampus, it is worth noting that CaMKIIα can also play a key role in the induction of LTD at parallel fiber-Purkinje cell synapses in the cerebellum (Hansel et al., 2006).

More recent optical methods utilizing glutamate uncaging on single dendritic spines combined with high-resolution imaging of CaMKII activation with the Camui activity sensor has given more insight into the role CaMKII plays during the induction of LTP. The Camui sensor is a modified CaMKII subunit with an enhanced monomeric green-fluorescent protein (mEGFP) and a resonance energy-accepting chromoprotein (REAch) attached to opposite ends (Takao et al., 2005). During activation of CaMKII a conformational change occurs that moves the fluorescence resonance energy transfer (FRET) acceptor and donors farther apart thus reducing the FRET signal. This FRET sensor with allows for monitoring of CaMKII at the single spine level with real-time two-photon fluorescence lifetime imaging (2pFLIM). In hippocampal pyramidal

neuron spines, glutamate uncaging activated CaMKII quickly and it remained

localized exclusively to the activated spine. The activation of wildtype CaMKII

decayed within 1-2 minutes to baseline, but the T286 to alanine mutant reduced

the duration of CaMKII activation to seconds (Lee et al., 2009). The short lived

activation of CaMKII combined with slow diffusion due binding to PSD partners

along with a narrow dendritic spine neck limits the spread of plasticity to

neighboring spines.

Structural plasticity often is linked to synaptic plasticity. LTP is often

accompanied by a rapid and persistent increase in dendritic spine volume. This

increase is linked to CaMKII activation and can be mimicked by overexpression

of active CaMKII (Lee et al., 2009; Pi et al., 2010b). A slower process of synapse

enlargement, dependent on protein synthesis, increases the pre and

postsynaptic compartments size (Ostroff et al., 2002; Tanaka et al., 2008). A

study suggested that CaMKII phosphorylation of the guanine-nucleotide

exchange factor (GEF) kalirin-7 may mediate activity-dependent spine

enlargement and enhanced AMPAR-mediated synaptic transmission under some

conditions (Xie et al., 2007).

While an overwhelming majority of research on CaMKII is on its role in

excitatory transmission, some recent work has begun to look at the role of

CaMKII in inhibitory transmission. Moderate increases in calcium levels lead to

the translocation of CaMKII to GABAR synapses and the insertion of new

$GABA_A R$ at the inhibitory synapse (Marsden et al., 2010). Deeper investigation

of the role of CaMKII in inhibitory synaptic transmission is needed.

GluN2B Subunit of the NMDAR and Plasticity

The N-methyl-D-aspartate (NMDA) receptor is a heteromeric ligand-gated ion channel that passes sodium, potassium and calcium as well as interacts with multiple intracellular proteins. It is comprised of a combination of four subunits from seven known subunits including obligatory GluN1 subunits (formerly NR1, ζ1), and some combination of GluN2A-2D (formerly NR2A-2D or ε1-4), and/or GluN3A-3B (formerly NR3A-3B). The precise combination of subunits determines the functional properties of the NMDAR channels.   The NR1 subunit undergoes alternative splicing yielding eight unique functional splice forms. GluN2A-2D subunits arise from multiple, related genes whose specific expression profiles vary based on developmental and regional regulation (Cull-Candy and Leszkiewicz, 2004). GluN2A and GluN2B subunits exist in a di-heteromer (GluN1/GluN2A or GluN1/GluN2B) or in a tri-heteromer (GluN1/GluN2A/GluN2B), with around one-third of the subunits in the tri-heteromer form in the adult hippocampus - yielding unique functional properties (Al-Hallaq et al., 2007).   Importantly, the GluN2B subunit has received a lot of attention being implicated in learning and memory, pain perception, feeding behaviors and numerous human neurological disorders.

Developmentally, GluN2B protein is expressed in the entire embryonic brain, yet is restricted to the forebrain in the adult (Watanabe et al., 1992). Expression levels of GluN2B in the forebrain are highest in the early postnatal brain and then declines while GluN2A levels increase as the animal ages (Hoffmann et al., 2000; Yashiro and Philpot, 2008; Stoneham et al., 2010).

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Regionally, GluN2B and GluN2A are most abundant in the cortex, hippocampus, striatum, olfactory bulb and thalamus. GluN2B-containing NMDAR have lower peak current densities and lower peak channel open probabilities than GluN2A-containing receptors. Additionally, deactivation is significantly slower for GluN2B-containing versus GluN2A-containing NMDARs. These single channel kinetic differences lead to macroscopic kinetic differences including a slower rise and decay time of GluN2B - containing NMDAR versus GluN2A - containing NMDARs (Monyer et al., 1994; Vicini et al., 1998; Chen et al., 1999). These differences allow GluN2B-containing receptors larger charge transfer and an increased time window for detecting synaptic coincidence which plays a role in the induction of synaptic plasticity (Erreger et al., 2005). This suggests that GluN2B-containing NMDAR carry more calcium per unit of current than GluN2A-containing NMDARs (Sobczyk et al., 2005). Additionally, GluN2B is thought to be located more peri-synaptically, while GluN2A is thought to be centrally located at the synapse (Dalby and Mody, 2003; Townsend et al., 2003; Zhao and Constantine-Paton, 2007), yet these findings still remain controversial (Mohrmann et al., 2000; Harris and Pettit, 2007). While reports have connected increases in synapse size with potentiated synaptic transmission, the density of synaptic GluN2B subunits in the CA1 region of the hippocampus is constant regardless of spine size (Shinohara et al., 2008).

The affinity of activated CaMKII binding to GluN2B is far greater than for activated CaMKII binding GluN2A (Strack and Colbran, 1998; Mayadevi et al., 2002) and this CaMKII-GluN2B complex has been shown to be important the

induction of LTP (Bayer et al., 2001; Barria and Malinow, 2005). Indeed deletion of the GluN2B c-terminal tail disrupts LTP induction in hippocampal neurons, but does not affect NMDAR-mediated currents (Foster et al., 2010). Overexpression of GluN2B in the forebrain of transgenic mice leads to the enhanced activation of NMDAR receptors and enhanced LTP. Additionally, GluN2B overexpressing mice showed enhanced long term memory for a novel object in a retention test, enhanced fear memory (contextual and cued), and enhanced spatial memory in the Morris water maze (Tang et al., 1999). Alternatively, overexpression of GluN2B c-terminal tails actually show reductions in LTP magnitude and exhibit learning deficits (Zhou et al., 2007). While these deficits were suggested to arise from the disruption of the CaMKII-GluN2B complex, it seems likely that several other intracellular interactions would also be affected.

Traditional GluN2B knock out animals die soon after birth (Kutsuwada et al., 1996), but can survive a few days with hand feeding. Similarly, mice expressing a truncation of the C-terminal domain of the GluN2B subunit die perinatally (Sprengel et al., 1998). Alternatively, GluN2A or GluN2C KOs are viable (Lu et al., 2001; Fagiolini et al., 2003; Logan et al., 2007; Zhao and Constantine-Paton, 2007; Zhang et al., 2012), suggesting an indispensable role for GluN2B in perinatal development, feeding and other physiological functions. Utilizing autaptic hippocampal cultures, voltage clamp recordings show that GluN2BKO animals express a NMDAR-mediated EPSC, but have faster deactivation kinetics and are less sensitive to glycine. These neurons are more sensitive to blockade by zinc and less sensitive to the GluN2B-slective antagonist

ifenprodil (Tovar et al., 2000). These data suggest that GluN2A can partially compensate for the loss of GluN2B subunits at the synapse. In the first perinatal days, GluN2BKO mice show impaired whisker-related barrelette structure and primary afferent terminal clustering in the brain stem. In the hippocampus of these mice NMDAR-meditated responses are absent as well as NMDAR-dependent LTD (Kutsuwada et al., 1996).

Differing studies have implicated GluN2B subunits in striatal plasticity and pathology. In animal models of Parkinson's disease dopamine depletion of the striatum leads to a reconfiguration of GluN subunits with a reduction particularly in GluN2B levels (Oh et al., 1999; Dunah et al., 2000; Hallett et al., 2005; Gardoni et al., 2006). Antagonists of the GluN2B-containing receptor have shown antiparkinsonian actions in both rodents and monkeys (Nash et al., 2000; Steece-Collier et al., 2000; Nash et al., 2004). Other studies suggest a therapeutic benefit for GluN2B antagonism reducing dyskinesias in animal models of Parkinson's disease, while GluN2A antagonists may exacerbate dyskinesias (Hallett and Standaert, 2004; Morissette et al., 2006; Ouattara et al., 2009; Rylander et al., 2009). In Huntington's disease models (HD) transgenic mice show potentiated GluN2B currents, suggesting the disease may involve abnormal GluN2B activity (Li et al., 2003). HD mice show increased GluN2B surface expression, current and toxicity (Shehadeh et al., 2006; Fan et al., 2007) and GluN2B overexpression enhances MSN cell loss (Heng et al., 2009). More recent studies suggest elevated extrasynaptic GluN2B subunit number in the striatum of transgenic mice expressing the full length version of human

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huntington protein and that these alterations are seen prior to motor dysfunction and neuronal loss (Milnerwood et al., 2010). Interestingly, the dorsal lateral striatum has a higher GluN2A to GluN2B ratio than the ventral medial striatum (Chapman et al., 2003), possibly playing a role in the shift in normal goal-directed to habit-based learning seen with repeated training (Yin et al., 2009). Overall, GluN2B is important in synaptic plasticity and learning and memory, and some striatal-associated diseases show disrupted GluN2B signaling.

Lines of evidence support a role for GluN2B favoring the induction of LTP. Many experiments have used ifenprodil, a relatively GluN2B selective antagonist, to determine if GluN2B subunits play a role in LTP. Ifenprodil blocks the induction of LTP using a pairing protocol in immature hippocampal slice cultures. Overexpression of GluN2A, leading to a replacement of GluN2B subunit containing NMDARs, also attenuates the induction of LTP (Barria and Malinow, 2005). Although other reports suggest GluN2A subunits mediate LTP (Liu et al., 2004; Massey et al., 2004), these studies utilized the drug NVP-AAM077, which was thought to be selective for the GluN2A receptor over the GluN2B receptor. However, NVP-AAM077 can block approximately 20% of the NMDAR-mediated current in GluN2AKO mice indicating lower selectivity (Berberich et al., 2005). Ifenprodil also has pharmacological caveats due to its complex pharmacology. Ifenprodil selectively blocks GluN2B-containing receptors over GluN2A-containing receptors at high levels of glutamate, while it actually potentiates NMDAR currents at low glutamate concentrations (Kew et al., 1996). The role of GluN2A and GluN2B subunits in LTP will need further study with region and

developmental age drawing important consideration when interpreting results.

With the advent of conditional GluN2BKO mice we should gain a better

understanding of the role of these subunits in plasticity. Indeed, conditional

GluN2BKO in the whole forebrain or hippocampus leads to deficits in LTP and

memory tasks (von Engelhardt et al., 2008).

Hippocampal LTD has been shown by three independent labs to be

insensitive to GluN2B blockade with ifenprodil (Morishita et al., 2007). This

suggests that hippocampal LTD induction does not require GluN2B receptors.

Other studies suggest that the GluN2B antagonist blockade of LTD is state

dependent, in that LTD is blocked by ifenprodil at a basal state, but not following

depotentiation (Massey et al., 2004). Recently, ifenprodil has been shown to

block deficits in spatial working memory and the induction/expression of an *in

vivo* LTD following acute exposure to endocannabinoids at CA3-CA1 synapses

(Han et al., 2012).

Long-term Synaptic Depression in the Striatum

Long-lasting neuroadaptations at glutamatergic synapses are not limited

to the hippocampus, but include brain regions like the striatum as well (Figure 3,

Table 1). Indeed, plasticity at excitatory synapses on MSNs in the striatum has

been clearly demonstrated. While plasticity in the striatum shares similarities

with plasticity seen in the hippocampus there are some notable exceptions,

including a much more prominent neuromodulatory role for dopamine. Long-

lasting changes in the strength of synaptic connections in the dorsal lateral

striatum are likely to influence striatal control over motor activity. Induction of

LTP or LTD is dependent on the age of the animal and subregion within the

dorsal striatum examined (Partridge et al., 2000). Unlike the hippocampus and

cerebral cortex, *ex vivo* studies in adult rodent brain slices using high frequency

stimulation (HFS) with or without concurrent depolarization typically leads to the

induction of LTD rather than LTP at glutamatergic synapses in the dorsal lateral

striatum (Calabresi et al., 1992c; Calabresi et al., 1996; Kerr and Wickens, 2001;

Bonsi et al., 2003) and this form of LTD is independent of NMDAR activation

(Calabresi et al., 1992c). Instead, induction of striatal HFS-LTD is dependent on

membrane depolarization, activation of voltage-gated calcium channels,

increases in postsynaptic calcium, coactivation of D1R and D2R signaling and

metabotropic glutamate receptors (mGluRs)(Calabresi et al., 1992c; Calabresi et

al., 1996; Choi and Lovinger, 1997a; Sung et al., 2001). Indeed, either D1R or

D2R antagonists are able to block HFS-LTD and mGluR antagonist significantly

attenuate LTD magnitude (Calabresi et al., 1992c). Additionally, this form of

LTD is absent in mice that lack the D2 receptor (Calabresi et al., 1997; Choi and

Lovinger, 1997a) or the dopamine-regulated phosphatase regulator DARPP-32

(Calabresi et al., 2000). Alternatively, muscarinic acetylcholine receptor

antagonists enhance the magnitude of LTD (Bonsi et al., 2008), while nicotinic

acetylcholine receptor antagonists prevent LTD induction (Partridge et al., 2002).

Striatal LTP

HFS, No Mg²⁺ ACSF

Striatal LTD

HFS, repetitive stimulation
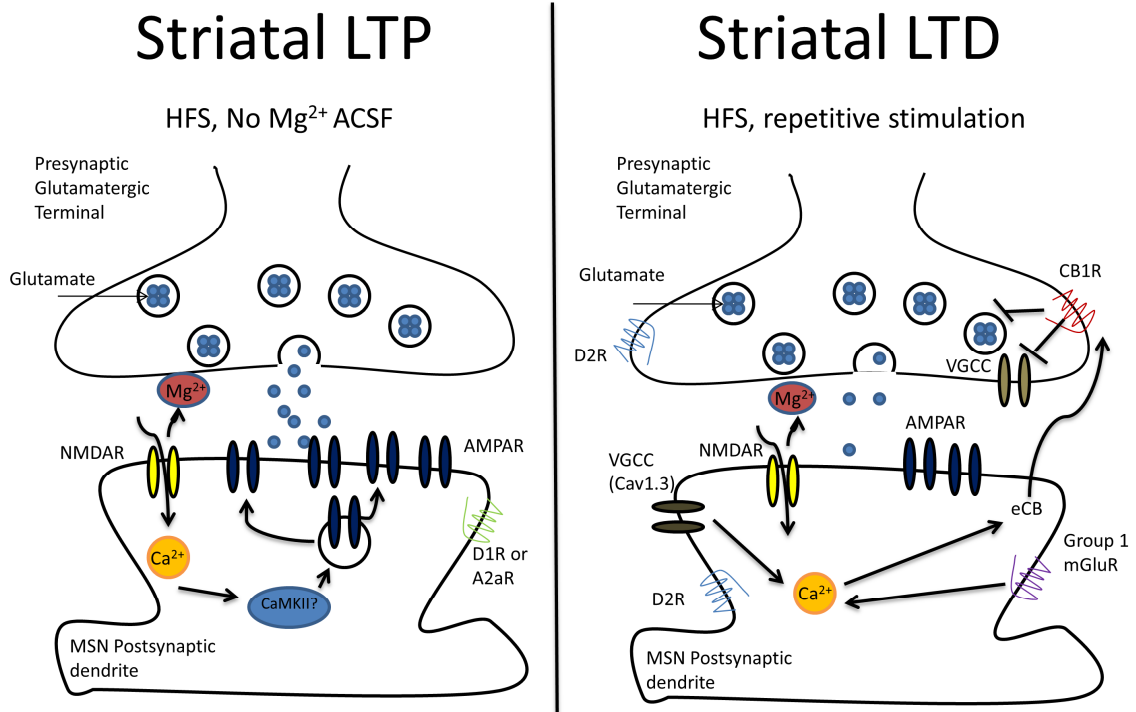
Figure 3. Striatal LTP/LTD. (left) Schematic diagram of mechanisms thought to be involved in striatal LTP. LTP is thought to involve the activation of NMDARs, along with either A2aR or D1R activation in indirect or direct pathway neurons, respectively. Increases in calcium may activate CaMKII and drive new AMPARs to the synapse. (right) Schematic diagram of the mechanisms thought to be involved in striatal LTD. Postsynaptic depolarization activates Cav1.3 voltage-gated calcium channels (VGCC) while metabotropic glutamate signaling converges to activate endocannabinoid (eCB) release. eCB act on CB1Rs to decrease the probability of release (via VGCC or vesicle fusion).

Striatal LTP	Striatal LTD
(Modulators or Mediators)	(Modulators or Mediators)
NMDAR activation (No Mg^{2+} ACSF)	Depolarization
D1R activation	Increases in intracellular calcium levels
A2aR activation	Group 1 mGluR1s activation
mACh (M1R signaling)	L-type VGCC activation (Cav1.3)
BDNF	Endocannabinoids (eCB)
PKA/PKC	D2R activation
DARRP-32	CB1R activation
Animal age	DARPP-32
Subregion of dorsal striatum	mACh (M1R signaling)
	nACh
	Animal age
	Subregion of dorsal striatum

Table 1. Known Mediators and Modulators of Dorsal Striatal LTP/LTD

At least some of these receptors/signaling molecules likely modulate, rather than mediate LTD in striatum (Table 1). For example, Lovinger's group found that L-type calcium channel activation with modest depolarization and synaptic activity is sufficient for LTD, bypassing both D2Rs and mGluRs (Adermark and Lovinger, 2007b).   Studies from this group further revealed that the mechanism underlying expression of striatal LTD is a reduction in the probability of release as indicated by increases in the paired-pulse ratio (PPR, a measure of short-term plasticity where a second EPSC is triggered shortly after the first leading to increases or decreases in the second response compared to the first) and coefficient of variation (CV, the SD of the EPSC amplitude normalized to the mean amplitude where the inverse square of CV is directly proportional to quantal content) of evoked excitatory responses following LTD induction (Choi and Lovinger, 1997a, b). Also supporting a presynaptic site of expression, LTD is associated with a decrease in mEPSC frequency, but not amplitude. This form of LTD is thought to be induced postsynaptically yet expressed presynaptically, evoking the need for a retrograde messenger.

Common retrograde messengers include endocannabinoids (eCB) such as anandamide and 2-arachidonyl glycerol. Retrograde signaling via eCBs has become a prominent theme in synaptic plasticity throughout the brain. In the striatum, unlike other brain regions, depolarization alone is not sufficient to release eCBs to modulate glutamatergic transmission. Rather, there is an additional requirement for mGluR activation to induce eCB release (Kreitzer and Malenka, 2005).   Additionally, striatal D2 receptor activation is known to mobilize

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endocannabinoid release as well (Giuffrida et al., 1999). The release of eCB postsynaptically can activate presynaptic CB1 receptors, which are Gi/o-coupled G-protein coupled receptors that suppress release at excitatory and inhibitory synapses (Szabo et al., 1998; Gerdeman and Lovinger, 2001; Huang et al., 2001). Studies using cannabinoid receptor (CB1R) agonists and antagonists and CB1R knockout mice support a role for postsynaptic endocannabinoids acting on presynaptic CB1Rs in a retrograde manner to induce LTD (Gerdeman et al., 2002; Kreitzer and Malenka, 2005). However, activation of CB1R alone is not enough to generate LTD, additional presynaptic stimulation is needed (Adermark and Lovinger, 2007a; Singla et al., 2007). This suggests that downstream signaling from CB1Rs synergize with depolarization-induced mechanisms, like calcium entry, to produce LTD. Indeed the striatum is enriched with CB1Rs. Within the striatum CB1R mRNA is expressed with a gradient in the striatum with expression levels higher in the lateral striatum with a gradual decrease moving to the medial striatum with little expression in the ventral striatum (Matyas et al., 2006; Martin et al., 2008). CB1R mRNA is expressed in cortical pyramidal neurons that project to the striatum (Tsou et al., 1998). While there is convincing functional data supporting the ability of CB1 receptors to suppress glutamatergic release (Gerdeman and Lovinger, 2001; Huang et al., 2001; Kofalvi et al., 2005), controversy exists over the ability of antibodies to recognize the expression of CB1 receptors on presynaptic glutamatergic receptor terminals (Matyas et al., 2006). Additional work and better antibodies are needed for a clearer understanding of eCB signaling in the striatum.

It is also unclear whether endocannabinoid-mediated LTD (eCB-LTD)

exists on both direct and indirect populations of neurons or if eCB-LTD can only

exist on indirect, D2R containing MSNs. Lovinger's and Surmeier's groups

provided data consistent with a model in which both direct and indirect pathway

MSNs support eCB-LTD via a cholinergic interneuron D2 receptor-dependent

reduction in M1 receptor tone (Wang et al., 2006). The resulting reduction in M1

receptor tone promotes the opening of Cav1.3 calcium channels, which then

enhances endocannabinoid production and CB1 receptor activation. More

recently the idea that a reduction in both ACh release and M1R signaling is

critical for the induction of eCB-LTD in both direct and indirect pathway MSNs

has been shown (Tozzi et al., 2011). However data from the Malenka lab

suggests that only indirect pathway MSNs can elicit eCB-LTD (Kreitzer and

Malenka, 2007). These discrepancies could possibly be explained by the use of

D1R-EGFP versus M4R-EGFP mice to label direct pathway MSNs. Another

possibility for the role of D2 signaling in eCB-LTD might be the need to inhibit

A2a adenosine receptor signaling impeding efficient EC synthesis and therefore

eCB-LTD (Fuxe et al., 2007). More recently, evidence agreeing with this idea

that antagonism of A2a receptors promotes eCB-LTD in indirect pathway MSNs

(Lerner et al., 2010). Additional conflicting data is reported by Calabresi's group

showing that stimulation of M1 receptors can facilitate the induction of striatal

LTP in ACSF lacking magnesium (Calabresi et al., 1999). A recent publication

showed that following repeated administration of Δ9-Tetrahydrocannabinol

(THC), eCB-LTD was abolished for 3-4 days following treatment. This loss of

LTD was associated with the development of THC tolerance with repeated administration, because a single injection of THC did not alter eCB-LTD. Functional tolerance in this model is reflected by a loss of available CB1R or an uncoupling of downstream G-protein signaling. Indeed, reduced field EPSCs following CB1R agonist application and reduced CB1R radioligand binding were observed in the THC tolerant group. Using a negative spike-timing dependent plasticity (STDP) protocol they were able to induce LTD of EPSPs with layer 5 cortical stimulation in D2R-expressing MSNs, but not in D1R-expressing MSNs. The STDP-LTD was lost in chronically treated THC mice. Additionally, these effects were specific to the dorsal lateral striatum, because LTD in the dorsal medial striatum, which was mediated by a different signaling mechanism, was not affected by repeated THC. It was found that the induction of eCB-LTD depended on the activation of L-type VGCC, but not NMDARs. Furthermore SK channel inhibition rescued eCB-LTD in tolerant mice (Nazzaro et al., 2012). Additional studies will be needed to better understand the reasons for the discrepancies in the literature for eCB-LTD in D1R-expressing and D2R-expressing MSNs.

An additional form of LTD induced via low frequency stimulation (LFS, 5-10min, 10-13Hz) can also be detected in the dorsolateral striatum (Kreitzer and Malenka, 2005; Ronesi and Lovinger, 2005). Like HFS-LTD, LFS-LTD is blocked by D2R and CB1R antagonists or blunted by blockers of L-type calcium channels. However, LFS-LTD differs from HFS-LTD in that it unaffected by postsynaptic depolarization, postsynaptic calcium chelation, mGluR antagonists,

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and an intracellular anandamide membrane transport inhibitor. The induction of

LFS-LTD does not occlude the induction of HFS-LTD suggesting both forms of

LTD can occur at the same synapses. This presents a scenario where HFS-LTD

is favored under strong bouts of cortical stimulation along with correlated

postsynaptic depolarization, while LFS-LTD would be favored under moderate

frequency cortical stimulation uncorrelated with postsynaptic depolarization.

LTD is also the major form of plasticity observed in the ventral striatum.

Typical stimulation protocols consist of 10-13Hz stimulation applied for several

minutes in the presence of $GABA_A$ receptor antagonists, leading to induction of a

robust LTD of evoked excitatory transmission (Robbe et al., 2002b; Hoffman et

al., 2003; Mato et al., 2004). This form of LTD, which is blocked by CB1R

antagonists and in CB1RKO mice, is mediated by the postsynaptic release of

eCB, which activate presynaptic CB1Rs on glutamatergic terminal afferents to

the NAc (Robbe et al., 2001). The activation of $G_{i/o}$-coupled CB1Rs leads to the

decrease in probability of glutamate release (Robbe et al., 2001). Consistent

with this finding, eCB-mediated LTD is associated with a decrease in the sEPSC

frequency, but not amplitude. Additionally, intracellular calcium-chelation and

antagonism of mGluR5 receptors blocks the induction of eCB-LTD, but NMDAR

antagonists do not affect this form of LTD. The group1 mGluR agonist, DHPG,

elicits LTD in the nucleus accumbens and 13Hz eCB-LTD is occluded following

DHPG-LTD suggesting a similar shared mechanism. Interestingly, this form of

LTD is blocked by prior chronic cocaine administration (Fourgeaud et al., 2004).

A second form of LTD is observed in the nucleus accumbens, mediated by presynaptic mGluR2/3 receptors and inhibition of P/Q-type calcium channels by the cAMP/PKA pathway (Robbe et al., 2002c). Both mGluR2/3 agonists and tetanic stimulation (three times for 1 sec at 100Hz, 20sec intervals) leads to mGluR2/3LTD in a majority of experiments. mGluR2/3 LTD, like eCB-LTD, leads to an increase in the paired pulse ratio indicative of a decrease in the probability of release of glutamate. mGluR2/3 LTD induced by tetanic stimulation is also independent of NMDARs, postsynaptic intercellular calcium concentrations and was occluded by prior mGluR2/3 agonist-induced chemical LTD.

Finally, a third form of LTD is observed in the nucleus accumbens. This form of LTD can be induced by three bursts of 5Hz stimulation for 3min paired with depolarization to -50mV or 1Hz for three minutes (Thomas et al., 2000; Thomas et al., 2001). This low frequency stimulation paired with depolarization induces LTD, which unlike other forms is dependent on NMDARs (Thomas et al., 2001). This form of LTD is dependent on internal calcium, but independent of mGluRs and D1 and D2 receptor activation (Thomas et al., 2000).

Long-term Potentiation in the Striatum

In addition to LTD, LTP can also occur at glutamate synapses on MSNs, but it is in general less well characterized. Interestingly, induction of striatal plasticity depends on the postnatal age of the animal and subregion of the dorsal striatum. In the dorsal lateral striatum HFS stimulation in young animals (P12-14) leads to the induction of LTP, while in older animals (P15-34) it leads to LTD.

Conversely, in dorsal medial striatum LTP is induced across both age ranges (Partridge et al., 2000). While the story is currently far from complete, striatal LTP may share some features with CA1 hippocampal LTP described above (Bliss and Collingridge, 1993), in particular that it is dependent on NMDARs for induction (Calabresi et al., 1997; Yamamoto et al., 1999; Partridge et al., 2000; Kerr and Wickens, 2001; Dang et al., 2006; Popescu et al., 2007), and has been observed both *in vitro* and *in vivo* (Charpier and Deniau, 1997; Charpier et al., 1999; Dos Santos Villar and Walsh, 1999). Interestingly, high frequency stimulation and depolarization concurrently with pulsatile application (Wickens et al., 1996), but not bath application (Arbuthnott et al., 2000), of dopamine in normal aCSF (perhaps mimicking the natural pulsatile release of dopamine) leads to the induction of LTP instead of LTD in the dorsal striatum.

As mentioned above, less is known about striatal LTP relative to LTD, largely because of difficulty in identifying consistent means by which to induce and record the LTP at a single cell level. For example in many striatal LTP studies, aCSF lacking magnesium is used to both unblock the NMDA receptor and likely increase release probability (Calabresi et al., 1992a; Centonze et al., 1999; Calabresi et al., 2000; Nazzaro et al., 2012). Potassium channel blockers have also been used as a means of facilitating striatal LTP (Wickens et al., 1998; Norman et al., 2005). Like LTD, striatal LTP appears to be heavily modulated by dopamine, apparently in a receptor-specific manner. D2 receptor knockout mice are reported to exhibit LTP with high frequency stimulation in normal aCSF (Calabresi et al., 1997). Moreover, application of a D2 receptor antagonist can

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enhance LTP induced in magnesium-free aCSF in wildtype mice, suggesting D2

receptors exert negative control on LTP induction (Calabresi et al., 1997).

Pharmacological inhibition of D1 receptors, genetic knockout of D1 receptors or

postsynaptic inhibition of PKA/PKC activity blocks the induction of LTP (Akopian

et al., 2000; Calabresi et al., 2000; Centonze et al., 2001; Kerr and Wickens,

2001; Centonze et al., 2003; Ding and Perkel, 2004; Fino et al., 2005). DARRP-

32 knockout mice, presumably via loss of PP1 inhibition, also prevent the

induction of LTP in magnesium-free aCSF (Calabresi et al., 2000). Striatal LTP

may also depend on the activation of M1 acetylcholine (Calabresi et al., 1999;

Lovinger et al., 2003) and mGluRs (Gubellini et al., 2003). In agreement with a

role for M1 acetylcholine receptors in striatal LTP, blockade of M2 acetylcholine

receptors enhances LTP (Calabresi et al., 1998). Another player in striatal LTP

has been identified recently, the activation of TrkB receptors by its ligand BDNF

(Jia et al., 2010). BDNF is released by corticostriatal terminals and TrkB

receptors are found on both direct and indirect pathway MSNs (Lobo et al.,

2010). Recently, repeated administration of THC was shown not to have an

effect on LTP induction, but blocks subsequent depotentiation (Nazzaro et al.,

2012), a process dependent on NMDAR and protein phosphatase-mediated

signaling (Picconi et al., 2003).

　　　　With D1 receptor stimulation necessary for LTP induction and D2

stimulation necessary for LTD induction, how can distinct populations of MSNs

expressing only D1 or D2 receptors elicit both LTP and LTD? This question was

addressed by Shen *et al.* using spike-timing dependent plasticity (STDP) in D1-

and D2-EGFP BAC transgenic mice (Shen et al., 2008). STDP involves the

precise timing of converging synaptic activity and back-propagating action

potentials and has been studied in many systems after its initial discovery

(Magee and Johnston, 1997; Markram et al., 1997). If an evoked excitatory

postsynaptic potential (EPSP, a depolarization of a neuron induced by

presynaptic stimulation increasing the likelihood of generating an action potential)

precedes postsynaptic spiking by a few milliseconds then this continued pairing

leads to LTP, whereas reversing the order induces LTD. Shen *et al.* report that

disruption of adenosine A2a receptors - not D1R - blocked the induction of LTP

on D2R MSNs. LTD in D2R MSNs induced with STDP, like conventional HFS-

LTD, can be blocked by the single addition of either D2R antagonists, CB1R

antagonists or mGluR5 antagonists. In D1R MSNs LTD was only induced in the

presence of D1R antagonists, while D2R antagonists had no effect on LTD. So it

seems that in D1 MSNs D1 receptors and mGluR5 receptors have antagonistic

actions while in D2 MSNs it seems that adenosine A2a receptors substitute for

D1 receptors to antagonize mGluR5. Thus, although this study showed that

dopamine plays a key role in determining the directionality of plasticity, dopamine

is not essential for the induction of striatal LTP/LTD under these conditions.

Another study using STDP concluded that both LTP and LTD induction in the

striatum is D1R-dependent and NMDAR-dependent with directionality

determined by timing and order of EPSPs and backpropagating action potentials

(Pawlak and Kerr, 2008; Pawlak et al., 2010). Like the hippocampus, this study

also suggested LTP involved a postsynaptic insertion of AMPA receptors via the

unsilencing of synapses, but additional work is needed to confirm if a similar mechanism functions in the striatum.

As in the dorsal striatum, LTP is observed in the ventral striatum, but with some notable differences. High frequency stimulation (100Hz), in physiologically normal magnesium concentrations, elicits LTP in field recordings in the NAc (Pennartz et al., 1993; Kombian and Malenka, 1994; Mazzucchelli et al., 2002; Schramm et al., 2002; Li and Kauer, 2004; Yao et al., 2004). This form of LTP is blocked by acute amphetamine, dopamine, or by dopamine receptor agonists (Li and Kauer, 2004). However after chronic administration of amphetamine the attenuation of LTP is lost. Another induction protocol uses pairings of depolarization via an intracellular patch pipette with low frequency stimulation to induce LTP (Kombian and Malenka, 1994). Induction of LTP using this pairing approach, while sensitive to intracellular calcium concentration, is not blocked by acute amphetamine treatment (Li and Kauer, 2004). These results suggest that dopamine receptor activation normally decreases responses to high frequency stimulation without disrupting excitatory synaptic transmission at low frequency. Indeed D1R stimulation decreases glutamatergic transmission in the NAc response to both single and multiple stimuli (Pennartz et al., 1993; Harvey and Lacey, 1996; Nicola et al., 1996; Beurrier and Malenka, 2002). Additionally, NMDARs are required for this form of LTP induction in the NAc (Kombian and Malenka, 1994). Interestingly, the activation of glutamatergic basolateral amygdala afferents that innervate ventral striatum enhances LTP at cortical glutamatergic synapses on MSNs (Popescu et al., 2007).

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In summary, it seems that repetitive stimulation of corticostriatal afferents can lead to the development of LTP or LTD depending on conditions. Therefore it will be crucial to better understand and identify the factors that control the directionality, induction and maintenance of plasticity in the striatum. Separating the direct and indirect projection neurons role in plasticity as well as determining effects of local interneurons and local networks as well determining how differing methodologies affect plasticity in the striatum will all be important in better understanding the mechanisms underlying motor function and disease.

Glutamatergic Plasticity and Motor Skill Learning

Long lasting changes in synaptic efficacy at synapses leads to long lasting changes on the cellular and network levels, which correlate with modifications in animal behavior and performance. Deletion of the obligatory GluN1 subunit of the NMDAR in the striatum leads to a disruption of motor learning on the accelerating rotarod and a disruption in dorsal striatum LTP and ventral striatum LTD (Dang et al., 2006). Yin *et al.* were able to correlate the acquisition and consolidation of a long-lasting motor skill to alterations in plasticity in the dorsal striatum. Differing regions of the dorsal striatum subserve different forms of motor and procedural learning. The dorsal medial region of the striatum, or associative striatum, is preferentially involved in the rapid acquisition of action-outcome contingencies while the dorsal lateral, or sensorimotor striatum, is involved in the gradual acquisition of habit based learning (Hilario and Costa, 2008). Experiments recording activity of MSNs in both in the dorsal medial and

dorsal lateral striatum *in vivo* in freely moving mice during a motor learning task found regional differences in neural activity at differing time points during learning (Yin et al., 2009). During early time points on the rotarod the dorsal medial striatum showed the largest increase in activity, while at later time points when learning had reached a plateau dorsal lateral striatum activity peaked. In agreement with these data, excitotoxic lesions in the dorsal medial striatum only affected early motor skill learning, while dorsal lateral striatum excitotoxic lesions only affected late learning. To determine if these changes corresponded to changes in synaptic strength, saturation experiments were performed by inducing LTD. Yin *et al.* found that it was more difficult to saturate LTD in the dorsal medial striatum at early time points and found it more difficult to saturate LTD in the dorsal lateral striatum at late time points in motor skill acquisition. These data suggest that early in motor skill acquisition the dorsal medial striatum undergoes synaptic potentiation while at later time points in acquisition the dorsal lateral striatum undergoes potentiation. Additionally, using whole cell voltage clamp they saw an increase in the sEPSC amplitude in the dorsal lateral striatum following extended training suggesting a postsynaptic increase in function/number of AMPARs which is reflective of synaptic potentiation. Furthermore, recordings from D1 and D2-EGFP BAC mice during motor skill acquisition suggested that late in training the potentiation of excitatory transmission in the dorsal lateral striatum occurs predominantly in D2 receptor expressing MSNs while becoming less dependent on the activation of D1 receptors (Yin et al., 2009). In all these data show that LTP of glutamatergic

transmission in the dorsal striatum is necessary for the acquisition of motor skill learning.

Exciting new research has dissected the learning of new action sequences and its disruption in nigrostriatal disorders. In operant tasks where mice learn to press a lever to obtain a food reward, researchers have found striatal neural activity corresponding to the initiation or termination of an action sequence. About one third of MSNs showed lever-press related activity as well as an increase or decrease on initiation or completion of the action sequence, which actually increased over repeated training. Using a striatal-specific NMDAR1 knockout mouse they were able to show that start/stop activity and sequence learning were both disrupted (Jin and Costa, 2010).

Role of the Striatum in Habit Learning

The neural circuits underlying the acquisition of goal-directed actions (action-outcome learning) or actions sensitive to value of the outcome have been shown to be different than the circuitry underlying the formation of habits (stimulus-response learning). The dorsal striatum has been shown to play a critical role in both (Yin and Knowlton, 2006). Extended training, different reinforcement schedules and drugs of abuse can shift goal-directed behaviors to habitual responding. While habits can be beneficial, losses in voluntary control and compulsive drug seeking highlight a maladaptive form of habit learning – addiction.

To study whether instrumental behavior is being performed because of its outcome or not, researchers developed experimental tools to parse out goal-directed actions from habits. Initially animals are trained on a fixed ratio (FR) schedule, where a certain number of lever presses equate to reward delivery, to acquire operant responding for food. Following initial training the animals can be switched to differing training schedules. Random ratio (RR) schedules have been shown to lead to goal-directed schedules, while random interval schedules promote habitual behavior (Balleine and Dickinson, 1998). Additionally, overtraining helps to transition goal-directed to habit-based learning. To determine whether an action is habitual, a devaluation procedure can be used (Hilario et al., 2007). If an action is habitual, then it should not be dependent on the outcome of the reward. First an animal is trained to press a lever for a specific food reward that is different than the food given under restriction conditions. Following acquisition the animals are given free access to the reward prior to a session leading to sensory-specific satiety. If the behavior is goal-directed then the animals responding under extinction conditions will fall off compared to control, but if responding is not sensitive to pre-feeding then it is thought that the behavior is habitual (Figure 4). While behaviors that are impervious to devaluation may be suggestive of habit learning additional tests are used to confirm this initial assessment. Contingency degradation or disrupting the contingency between the performance of the action and the outcome is another way of determining if behavior is habit based (Hammond, 1980; Dickinson et al., 1996; Corbit et al., 2002). Under contingency conditions

animals are given "free rewards" or rewards that are delivered without an operant

response. Like before if operant responding is maintained following contingency

degradation then the behavior is thought to be habitual. Another task used to

look at goal-directed versus habit learning is omission (Davis and Bitterman,

1971; Yin et al., 2006). Initially animals are trained that pressing a level equals

food reward. Following training during omission in order to obtain the reward

animals must omit from pressing the lever that was initially paired with reward.

Omission is one of the most rapid ways for reducing the performance of goal-

directed actions.

Devaluation   Contingency Degradation
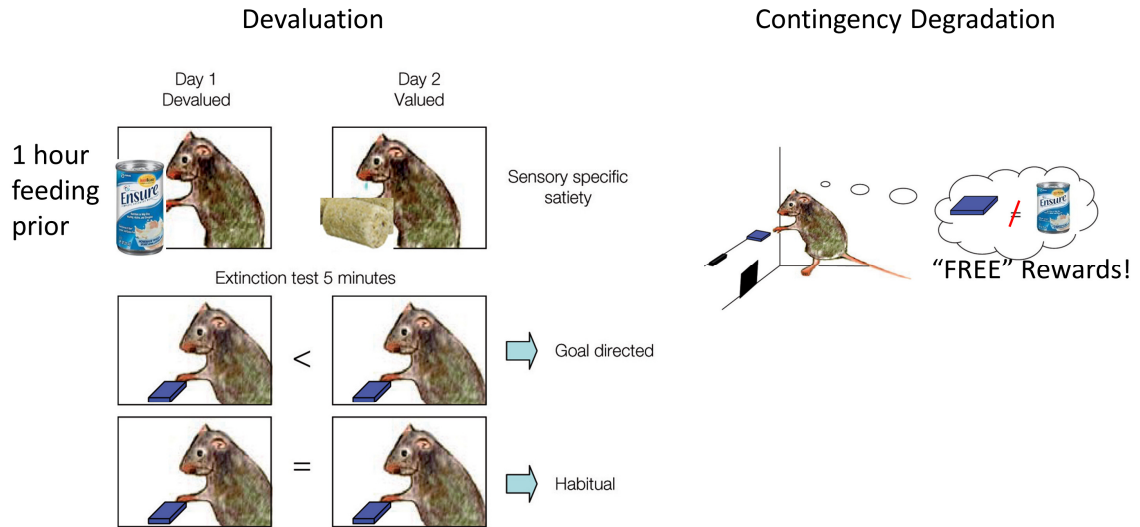
Figure 4. Parsing Goal-directed and Habit-based Learning in Rodents.
Devaluation and Contingency Degradation are two assays to determine if an animal's behavior is goal-directed or habit based. Following acquisition of operant training with a distinct reinforcer (Ensure) animals are given free 1 hour access to either Ensure or another generalized reinforcer chow in the home cage. In contingency degradation the animal is offered "free" rewards which are delivered non-contingently. In a five minute extinction test following devaluation or contingency degradation if the behavior is goal directed, then lever press responding should decrease, but if the behavior is habitual then responding should remain the same. Adapted from (Hilario and Costa, 2008)

Goal-directed actions have been shown to be controlled by dorsal medial striatum and its pre-limbic cortex and medial dorsal thalamic inputs (Corbit and Balleine, 2003; Corbit et al., 2003; Yin et al., 2005a; Yin et al., 2005b; Shiflett et al., 2010). In contrast, habit learning involves the dorsal lateral striatum and infralimbic cortex and associated inputs (Killcross and Coutureau, 2003; Yin et al., 2004). Interestingly, a lesion of the nigrostriatal input to the dorsal lateral

striatum seems to disrupt habit learning and instead support goal-directed

behaviors (Faure et al., 2005). Also repeated administration drugs of abuse like

cocaine and amphetamine can increase activity in the dorsal lateral striatum and

favor a shift to habitual behavior (Nelson and Killcross, 2006; Nordquist et al.,

2007; Takahashi et al., 2007). Evidence suggests that goal-directed learning and

habit learning compete with each other, with lesions of dorsal medial striatum

turning instrumental responding habitual (Yin et al., 2005b). Work in freely

moving rodents analyzing task-related oscillations in field potential activities

recently suggested that during early instrumental learning *in vivo* spiking in the

ventromedial striatum is associated with gamma-band (70-90Hz) bursts, however

after repeated training when behavior becomes habitual there is an increase in

global beta-band bursts (15-28Hz) with gamma-band bursts waning (Howe et al.,

2011).

Studies have tried to better understand the molecular players and

signaling cascades involved in habit learning. Data suggests that signaling

through CB1R is necessary for habit formation (Hilario et al., 2007). Additionally,

A2a adenosine receptor knock-out mice show disrupted habit learning (Yu et al.,

2009). Recently, a study examined the effects of prolonged THC exposure on

striatal plasticity and behavior. Behaviorally, the chronically treated THC mice

exhibited greater habitual responding compared to controls during devaluation

and omission tests. Infusions of apamin, a SK channel antagonist, rescued goal-

directed behavior in the THC tolerant mice (Nazzaro et al., 2012). Another

recent study using selective lesioning has suggested that the anterior portion of

the central amygdala is involved in the acquisition of habits (Lingawi and Balleine, 2012). Without a direct projection from the central amygdala (CeA) to the dorsal lateral striatum it seems plausible that an indirect route via the thalamus or substantia nigra pars compacta underlies the CeA role in habit learning. Overall, the concerted interplay of molecules and circuits underlying the formation of goal-directed and habits are just beginning to be understood making it an exciting area of research in coming years.

Alterations in Striatal Glutamatergic Synapses Following Dopamine Depletion

Lesions of the nigrostriatal dopamine neurons that innervate the striatum lead to a disruption in habit based learning. The hallmark of PD is the loss of the majority of dopamine neurons. It will be interesting to understand the potential mechanistic overlap in habit learning and the maladaptive alterations that occur in PD.   With the known interplay of dopamine and glutamate in the striatum it is important to understand how the loss of dopamine in the striatum alters glutamatergic synaptic transmission to better understand PD. I review some of the literature below.

Unilateral lesion of the nigrostriatal dopaminergic pathway with 6-OHDA is one of the most widely used animal models of PD leading to profound biochemical, morphological, electrophysiological and behavioral changes that in many cases mimic alterations described in patients with Parkinson's disease. Behaviorally, unilateral dopamine depletion leads to deficits in rotarod

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performance, locomotor performance and increased limb-use asymmetry

(Picconi et al., 2004a). Morphologically, the loss of dendritic spines from MSNs

also is seen in both dopamine depletion models (Ingham et al., 1989; Ingham et

al., 1993) and in patients with Parkinson's disease (Anglade et al., 1996). While

spine loss is occurring following dopamine depletion the remaining spines

undergo changes as well. For example, an increase in the number of perforated

synapses or synapses with bifurcated active zones - often seen after

manipulations that enhance excitatory transmission (Edwards, 1995)- is

observed following dopamine depletion (Ingham et al., 1998) and in PD patients

(Anglade et al., 1996). Additional studies also point to dopamine depletion

leading to an increase in glutamatergic transmission. Indeed, increased

glutamate release from corticostriatal synapses (Lindefors and Ungerstedt, 1990)

and increases in spontaneous glutamatergic transmission are seen following

dopamine depletion (Galarraga et al., 1987; Calabresi et al., 1993; Tang et al.,

2001; Gubellini et al., 2002; Picconi et al., 2004a). This increased sEPSC

frequency following dopamine depletion can be renormalized by the addition of a

D2 receptor agonist (Picconi et al., 2004a). This would suggest that dopamine

tone normally exerts negative modulation on glutamatergic transmission.

Conversely, work out of Surmeier's lab demonstrated that decreases, rather than

increases, in spontaneous glutamatergic transmission - coinciding with spine loss

- were only seen following dopamine depletion in indirect pathway MSNs (Day et

al., 2006). These differences could be due to differing experimental methodology

like the inclusion of cesium in the patch pipette which blocks potassium channels

and improves space clamp and voltage control at distal synapses. Another point for comparison was that this study only looked at sEPSC frequency following reserpine treatment, a drug known to reversibly deplete all monoamines without dopamine cell and terminal loss. Yet, another more recent study examining the effects of 6-OHDA dopamine depletion in the mouse found the opposite results where D1R-expressing MSNs showed reductions in sEPSC frequency about a month following lesion (Warre et al., 2011). Time after lesion, methodical considerations and compensatory mechanisms following dopamine loss may underlie these discrepancies.

Calabresi's group amongst others has suggested that dopamine depletion leads to a global inability to induce striatal LTP in magnesium-free aCSF (Centonze et al., 1999; Kerr and Wickens, 2001; Picconi et al., 2003; Picconi et al., 2004b) and prevents the induction of LTD ex vivo (Calabresi et al., 1992c). Alternatively, Malenka and colleges have shown that LTD is absent only in indirect pathway MSNs in reserpine and 6-OHDA treated animals. This eCB-LTD, in addition to locomotor activity and catalepsy, could be rescued by a D2 receptor agonist or inhibitors of endocannabinoid degradation (Kreitzer and Malenka, 2007). This finding is intriguing since D2R-dependent expression of LTD has been reported in both D1R and D2R-containing MSNs (Wang et al., 2006). Recently, Shen *et al.* showed that in animals lesioned with 6-OHDA, STDP leads to a flip in the polarity of plasticity so that LTP induction protocols induce LTD now in D1 MSNs (Shen et al., 2008). While in D2 MSNs LTP remained following dopamine depletion and could be rescued with a D2R

agonist. However, *ex vivo* slice data contrasts with *in vivo* LTD where alpha-

methyl-para-tyrosine-induced dopamine depletion (Reynolds and Wickens, 2000)

or blockade with D1R antagonists (Floresco et al., 2001) does not block LTD

induction.

Alterations in Glutamatergic Synapses Following Subsequent Levodopa

Administration

Administration of levodopa plus a peripheral dopa-decarboxylase inhibitor

like benserazide is currently one of the most effective therapeutic options in

Parkinson's disease.   The therapeutic efficacy of levodopa is presumed to be

due to its ability to counteract neuroadaptations that occur in the absence of

dopamine innervation (Obeso et al., 2000). Experimentally, levodopa

administration renormalizes many behavioral, biochemical, and

electrophysiological deficits seen following dopamine depletion. However after

chronic administration with dopamine replacement therapies the formation of

abnormal involuntary movements or dyskinesias appears. Independent of the

degree of dopamine denervation, dyskinesias are surprisingly observed only in a

subpopulation of rats and monkeys that undergo unilateral dopamine depletion

followed by levodopa administration. Data suggests that early administration of

levodopa (4 weeks post lesion) decreases the percentage of animals that

eventually develop dyskinesias (Marin et al., 2009). Normal animals and patients

that are given levodopa do not develop dyskinesias, suggesting that changes

that take place following dopamine depletion are important in revealing

dyskinesias. Behaviorally in nondyskinetic rats chronic levodopa administration

rescues rotarod performance and limb-use asymmetry (Picconi et al., 2003;

Picconi et al., 2004b). The loss of striatal LTD seen following dopamine

depletion can be rescued by application of dopamine or a combination of D1 and

D2 receptor agonists (Calabresi et al., 1992b; Calabresi et al., 1992c). Also the

ex vivo induction of striatal LTP is rescued following levodopa administration at

dopamine depleted synapses whether or not the rats exhibit dyskinesias (Picconi

et al., 2003). However, in animals that showed dyskinetic behaviors,

depotentiation – a reversal of LTP induced by low frequency stimulation and

mediated by phosphatases - could not be produced (Picconi et al., 2003). This

same study showed that levodopa increased the phosphorylation of DARPP-32

at Thr34, to presumably inhibit PP1, in dyskinetic rats versus non-dyskinetic rats.

Additionally, in 6-OHDA lesioned rats levodopa reverses hypersensitivity of D2

receptors and renormalizes glutamatergic spontaneous EPSC frequency (Picconi

et al., 2004a). Biochemical data implicate abnormalities in the subcellular

localization, levels and phosphorylation state of the NMDAR GluN2B subunit in

animals eliciting levodopa-induced dyskinesias (Oh et al., 1999; Dunah et al.,

2000; Hallett et al., 2005; Gardoni et al., 2006). Moreover, GluN2B antagonists

have shown efficacy in the reduction of dyskinesias (Hallett and Standaert,

2004). With these data it is conceivable that pharmacological modulation of

striatal glutamatergic synaptic plasticity might prove useful in the treatment of

motor symptoms observed in PD.

CaMKII Inhibition as a Therapeutic Target in Parkinson's Disease

As mentioned above, CaMKII is a key signaling molecule in synaptic plasticity. Both the Colbran and Calabresi labs found increases in the phosphorylation state of threonine 286 in CaMKIIα following dopamine depletion (Picconi et al., 2004b; Brown et al., 2005). In both studies, levodopa administration was able to restore levels of phosphorylated CaMKIIα to normal. The increased Thr286 phosphorylation of CaMKIIα persisted for up to 20 months post lesion, but increased phosphorylation of GluR1^{Ser831}, a CaMKII substrate, was only detected 9-20 months after dopamine depletion. Additionally Picconi *et al.* showed that intra-striatal injection of CaMKII inhibitors (KN93 or a membrane-permeant CaMKII inhibitor peptide) rescued LTP deficits following dopamine depletion, as well as limb-use asymmetry and rotarod performance. Together these data suggest an important role for downstream signaling molecules like CaMKII in the aberrant plasticity and disrupted motor output following dopamine depletion.

Effects of Drugs of Abuse on Plasticity in the Striatum

Psychostimulants like cocaine and amphetamine, as well as other drugs of abuse, increase dopamine levels by blocking dopamine re-uptake in the nucleus accumbens and in the dorsal striatum (Everitt et al., 2008). Animals chronically treated with drugs of abuse show long-lasting modifications in excitatory transmission in the striatum. In the nucleus accumbens shell chronic administration of cocaine leads to the depression of glutamatergic synaptic

strength reflected by decreases in the AMPAR/NMDAR current ratio (an index of

the relative AMPA-and NMDA-mediated currents contribution to EPSCs), the

amplitude of miniature EPSCs, and the magnitude of LTD (Thomas et al., 2001).

This suggests the cocaine administration leads to the induction of LTD *in vivo.*

Indeed, this form of NAc LTD was blocked by a peptide that disrupts clathrin-

mediated endocytosis or by a GluA2-derived peptide that blocks regulated AMPA

receptor endocytosis a major mechanism underlying LTD. This GluA2-derived

peptide also disrupted the development of behavioral sensitization, an

enhancement in the locomotor activating effects of cocaine with repeated

administration, which has been shown to correlate with LTD (Brebner et al.,

2005). eCB-LTD is abolished following single injection of cocaine and this effect

is blocked in D1 receptor KO mice and when D1 receptor antagonist are

administered with cocaine (Fourgeaud et al., 2004). Additionally this form of LTD

is blocked in morphine withdrawn animals (Robbe et al., 2002a), during cocaine

self-administration (Martin et al., 2006), and after the chronic treatment with

cannabis derivatives (Hoffman et al., 2003). These data suggest that LTD is

induced *in vivo* following chronic drug administration, therefore occluding

subsequent LTD *ex vivo.* In addition to electrophysiological changes following

psychostimulant exposure long lasting increases in spine density are observed

following cocaine or amphetamine administration in the nucleus accumbens

(Robinson and Kolb, 2004).

Recently, it has been demonstrated that extended withdrawal following

chronic cocaine administration leads to synaptic potentiation in the NAc shell

region and that subsequent re-exposure to cocaine reverses a synaptic potentiation to a synaptic depression (Kourrich et al., 2007). These data suggest that drug history determines the directionality of the plasticity in the NAc shell. Additionally, exposure to cocaine leads to the development of silent synapses in adulthood, accompanied by the insertion of new GluN2B subunits of the NMDA receptor (Huang et al., 2009). Along with changes in synaptic transmission, drugs of abuse like cocaine and amphetamine can impart long lasting changes in the intrinsic excitability of NAc MSNs. Chronic cocaine or amphetamine exposure leads to decreases in excitability in the nucleus accumbens shell region that starts within 1-3 days and persists for at least 2 weeks. The same drug regimen leads to an increase in intrinsic excitability in the NAc core region during early withdrawal (1-3 days), but returns to baseline after protracted withdrawal (2 weeks). These bidirectional changes in intrinsic excitability seem to be mediated by changes in the A-type potassium current (Kourrich and Thomas, 2009).

Interestingly, chronic cocaine administration leads to a downregulation of the postsynaptic density scaffolding protein PSD-95. Downregulation of PSD-95 correlates with synaptic potentiation and a PSD-95 targeted deletion enhances LTP and augments the locomotor activating effects of cocaine (Yao et al., 2004). Cyclin-dependent kinase 5 (CDK5) is a downstream target gene of the transcription factor deltaFosB, which accumulates in striatal neurons following cocaine administration. Enhanced cyclin-dependent kinase 5 (CDK5) expression in the NAc occurs following short access to self-administered cocaine (Seiwell et al., 2007), while inhibition of CDK5 augments both the development and

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expression of cocaine sensitization and enhances the incentive-motivational effects of cocaine (Taylor et al., 2007).

In all, drugs of abuse have been shown to modulate both the induction and directionality of plasticity in the nucleus accumbens. Like in the dorsal striatum the interactions between glutamatergic and dopaminergic systems are important for plasticity in this region. While many drugs have a common end point of elevating dopamine acutely in nucleus accumbens, chronic drug administration often leads to long lasting modification of synapses, emphasizing the importance of drug history, including withdrawal, when interpreting whether LTP or LTD is expressed.

Striatal MSN Intrinsic Excitability

MSNs are known for a hyperpolarized resting membrane potential (-80mV or lesser), low input resistance, and a prolonged delay for first action potential firing (Kita et al., 1984; Kawaguchi et al., 1989). Several types of potassium conductances shape the firing patterns of MSNs and underlie their hyperpolarized basal resting membrane potential (Nisenbaum and Wilson, 1995). One prominent player, the inwardly rectifying potassium channel (Kir), is a voltage sensitive potassium channel that is permeable to potassium at hyperpolarized potentials, but blocked by intracellular polyamines at depolarized potentials. Inwardly rectifying potassium conductances, predominately Kir2, are open at rest contributing to a hyperpolarized resting membrane potential, rapid membrane time constants, and low input resistance (Uchimura et al., 1989; Jiang

and North, 1991; Nisenbaum et al., 1996; Mermelstein et al., 1998). Upon

depolarization or upstate transition, inwardly rectifying potassium channels

inactivate and both A-type potassium currents, KV4.2 (fast inactivating) and

KV1.2 (slow inactivating), as well as a persistent Kv7 conductance activates and

generates a slow depolarization and long delay to first AP (Surmeier et al., 1989;

Surmeier et al., 1991; Nisenbaum et al., 1996; Tkatch et al., 2000; Shen et al.,

2004; Shen et al., 2005). The voltage ramp to first AP is shaped by Kv1 and Kv7

(KCNQ) potassium channels, as well as Nav1 sodium channels and Kv4

potassium channels (Tkatch et al., 2000; Shen et al., 2004; Shen et al., 2005;

Carrillo-Reid et al., 2009). Additionally, depolarization activates both small (SK)

and large (BK) calcium-activated potassium channels along with Kv1 and Kv7

channels which together act to counter depolarization and slow action potential

firing (Bargas et al., 1999; Galarraga et al., 2007). More recently it has been

found that spatially convergent synaptic inputs can recruit Cav3 calcium channels

and NMDARs to produce a regenerative event (Plotkin et al., 2011).

Alterations in CaMKII activity is linked to alterations in intrinsic excitability.

Autonomously active CaMKII suppresses neuronal excitability by increasing cell-

surface expression of an A-type K^+ channel, Kv4.2, via phosphorylation (Roeper

et al., 1997; Park et al., 2002; Varga et al., 2004). In addition, CaMKII inhibition

in medial vestibular nucleus neurons increases intrinsic excitability via a

reduction in BK-type calcium activated potassium currents (Nelson et al., 2005).

In dissociated cortical neurons, CaMKII inhibition induces hyperexcitability and

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neurotoxicity via dysregulated glutamate/calcium signaling (Ashpole et al., 2012b).

While the D1R-containing and D2R-containing MSN subclasses have many similar properties, notable differences have arisen. Initial hints of these differences were observed using post hoc reverse-transcriptase polymerase chain reaction (RT-PCR) to identify direct and indirect pathway MSNs (Mermelstein et al., 1998). More recently, a novel translational profiling approach using BAC transgenic mice allows for the affinity purification of polysomal mRNAs from direct or indirect pathway MSNs assisting in the identification of molecular changes (Heiman et al., 2008). Indirect pathway neurons had Kir channels that inactivated at more hyperpolarized potentials and had smaller amplitudes than direct pathway MSN. Further studies confirmed that D2R-containing MSNs fire at nearly twice the rate of D1R-containing MSNs in response to depolarizing current injection (Kreitzer and Malenka, 2007). Other differences include indirect pathway MSNs exhibit greater inhibition by muscarinic M1 receptors activation than directly pathway MSNs (Shen et al., 2007). Additionally, indirect pathway MSNs showed increased input resistance and a more depolarized resting membrane potential and these differences persist throughout development (Gertler et al., 2008). MSNs also differ in their activity level during cortically driven up state transitions (Wickens and Wilson, 1998). While indirect pathway MSN Kir channel expression contributes to enhanced excitability, differences still persist following inactivation with substantial depolarization suggesting other players are involved. More recent observations

using bacterial artificial chromosome (BAC) mice suggest that the underlying reason for enhanced excitability in indirect pathway MSNs is smaller dendritic trees than direct pathway MSNs (Gertler et al., 2008).

Neuromodulators like dopamine and acetylcholine shape the firing properties of MSNs through alterations in sodium, potassium and calcium channels. Activation of D1R on direct pathway MSNs reduces sodium currents (Schiffmann et al., 1995) and enhances Kir (Pacheco-Cano et al., 1996) together reducing MSN excitability. Yet in the upstate D1R activation can enhance L-type calcium currents and block slowly inactivating potassium current which should enhance spiking (Surmeier et al., 1995; Nisenbaum et al., 1998; Carter and Sabatini, 2004). These seemingly contradictory findings allow D1R signaling in the downstate to act as a filter opposing up state transitions, however once in the up state D1R can enhance MSN firing. D2R activation on indirect pathway MSNs leads to opposing effects. D2R stimulation reduces up state transitions and reduces spiking in the upstate by inhibiting L-type calcium channels (Hernandez-Lopez et al., 2000). M1R activation in MSNs inhibits Kir channels via phospholipase C activation, blocks persistent potassium currents mediated by Kv7 channels, and inhibits N- and P/Q- type calcium channels which couple to SK and BK channels (Shen et al., 2005; Shen et al., 2007). In all M1R activation increases MSN excitability and increases the likelihood of up state transitions.

Conclusions

The striatum represents a major site of plasticity in the basal ganglia. Interactions between fast excitatory glutamatergic synaptic transmission and slower dopaminergic and cholinergic modulation are critical for plasticity in this region. This is a region of complex anatomy that exhibits a wealth of long-lasting synaptic modifications. Dopamine's importance is reflected by altered plasticity seen following dopamine depletion, subsequent dopamine replacement therapies and chronic administration of drugs of abuse. We are just beginning to understand how plasticity in the striatum influences normal behaviors and its role in disease. The advent of BAC D1-EGFP and D2-EGFP transgenic mice to separate direct and indirect pathway MSNs along with targeted whole cell recordings of specific interneuron populations will continue to aid our understanding. New techniques utilizing channelrhodopsin or halorhodopsin, which can control firing rate of transfected cells with light *in vitro* or *in vivo*, will accelerate our knowledge of basal ganglia function and open the possibility of specific neural circuit control (Gradinaru et al., 2009). Gaining a more complete understanding of mechanisms underlying synaptic plasticity in the basal ganglia will hopefully allow for basic understanding of basal ganglia associated behaviors as well as open new avenues for therapeutic intervention in disease.

Hypothesis

Inhibition of striatal CaMKII alters glutamatergic synaptic transmission and

intrinsic excitability

Specific Aims

1. Test the hypothesis that genetic inhibition of CaMKII regulates synapse number in medium spiny neurons
2. Test the hypothesis that genetic inhibition of striatal CaMKII alters MSN intrinsic excitability
3. Test the hypothesis that inhibition of CaMKII alters striatal-related behaviors

Chapter II

MATERIALS AND METHODS

Generation of EAC3I-4 Transgenic Mice

For generation of double transgenic EAC3I-4 X tTA animals, heterozygous transgenic mice carrying the tTA gene driven by an alpha CaMKII promoter fragment were bred to heterozygous mice carrying the EAC3I transgene fused to EGFP driven by the tetO promoter. The CaMKIIα-tTA mice were obtained from Dr. Eric Kandel's lab and are maintained at Vanderbilt University. The autocamtide-3 derived inhibitory peptide (EAC3I) sequence (KKALHRQEAVDAL) mimics the autoinhibitory region of the CaMKII regulatory domain (residues 278-290) and acts by competitively binding to the catalytic site. In *in vitro* biochemical assays AC3-I blocks the phosphorylation of an autocamtide-2 substrate by purified rat CaM kinase with an IC_{50} of 3μM (Braun and Schulman, 1995; Wu et al., 2002), with a ≥100-fold reduced potency toward protein kinase C, CaM kinase I or CaM kinase IV (Braun and Schulman, 1995; Patel et al., 1999; Vest et al., 2007). EAC3-I is made up of the AC3-I peptide fused N-terminal to enhanced green fluorescent protein (EGFP) to stabilize and mark cellular and tissue distribution. In a previous study, EAC3I was transgenically expressed in the heart and total CaMKII activity in extracts was reduced by ≈40% (Zhang et al., 2005). This level of inhibition is likely to be a substantial underestimate of *in vivo*

inhibition, because proteins were diluted upon homogenization due to mosaic transgene expression.

I quantified the level of mosaicism in our EAC3-I mouse by staining with a NeuN antibody (1:1000, Millipore) to label all neurons (See supplemental for detailed immunohistochemical labeling). Manual counts of the number of EGFP positive neurons versus total number of NeuN stained neurons in z-stacks in the dorsal lateral striatum were made in Metamorph (Molecular Devices; Sunny Vale, CA), providing an estimate of the percent of cells expressing the transgene. TetO-linked transgene expression is controlled using mouse chow containing 200mg/kg Doxycycline (DOX) (Bio-Serv; Frenchtown, NJ). For DOX rescue experiments pregnant dams were fed DOX and weaned pups continued with the same food. At 6 weeks DOX was removed and the transgene was allowed to be expressed for 4-5 weeks. All DOX recordings were made between 10-11 weeks. All mice had been inbred onto a C57BL/6 background for more than seven generations. GluA1 knockout mice and wildtype littermates 8-16 weeks of age were utilized.

Brain Slice Preparation

All procedures were performed according to Vanderbilt University Institutional Animal Care and Use Committee approved procedures. Male and female EAC3I-4 transgenic mice or littermate controls (9-13 weeks or 3-4 weeks

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animals when indicated) were decapitated under anesthesia (Isoflurane). The brains were quickly removed and placed in ice-cold sucrose-artificial cerebrospinal fluid (ACSF): (in mM) 194 sucrose, 20 NaCl, 4.4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 1.2 NaH2PO4, 10.0 glucose, and 26.0 $NaHCO_3$ saturated with 95% O_2/5% CO_2. Hemisected coronal slices 300 µm in thickness were prepared using a Tissue Slicer (Leica). Slices containing dorsal lateral striatum were collected rostral to the crossing of the anterior commissure (Bregma 1.10–0.2 mm) (Franklin and Paxinos 1997). Slices were then stored in a heated (approximately 28°C), oxygenated (95% O_2-5% CO_2) holding chamber containing 'normal' ACSF [ACSF: (in mM) 124 NaCl, 4.4 KCl, 2 $CaCl_2$, 1.2 $MgSO_4$, 1 NaH_2PO_4, 10.0 glucose, and 26.0 $NaHCO_3$] for 1 hour and then transferred to a submersion-type recording chamber (Warner Instruments) where they were superfused with heated (28°C) oxygenated ACSF at a rate of about 2-3 ml/min. Preparation of GluA1KO animals and controls brain slices used very similar methodology except the high sucrose ACSF contained (in mM): 194 sucrose, 30 NaCl, 4.5 KCl, 1 $MgCl_2$, 26 $NaHCO_3$, 1.2 NaH_2PO_4, and 10 glucose and 250 µm thick brain slices were placed in 30°C oxygenated ACSF containing (in mM): 124 NaCl, 4.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 26 $NaHCO_3$, 1.2 NaH_2PO_4, and 10 glucose for 30 minutes followed by 30 minutes at room temperature before moving hemisections to the recording chamber.

Whole-Cell Voltage Clamp Recordings

MSNs of the dorsal lateral striatum were directly visualized with infrared video microscopy (Olympus BX51WI with QImaging Rolera-XP Camera). Only highly expressing EGFP-containing MSNs were selected for study and compared to neighboring MSNs visually devoid of EGFP expression. Recording electrodes (3-6 MΩ) were pulled on Flaming-Brown Micropipette Puller (Sutter Instruments) using thin-walled borosilicate glass capillaries (WPI). EPSCs were evoked by local fiber stimulation with bipolar nichrome electrodes. Stimulating electrodes were placed on the border of the corpus callosum and dorsal lateral striatum 100-300 μm dorsal to the recorded neuron, and electrical stimulation (5-20 V with a 100-150 μs duration, Grass Instruments) were applied at 0.05Hz unless otherwise noted. This location most likely stimulates both cortical and thalamic glutamatergic axons onto MSNs. Recording electrodes (3-6 MΩ) were filled with (in mM) Cs+ gluconate (117), HEPES (20), EGTA (0.4), TEA (5), $MgCl_2$ (2), ATP (4), GTP (0.3) pH 7.35, 285-290 mOsm. Series resistance averaging 16 MΩ (ranging 8-30 MΩ) was monitored and experiments with changes greater than 20% were omitted. AMPAR EPSCs and sEPSCs were isolated by adding 25μM picrotoxin and recording at a holding potential of -70mV in normal ACSF. To isolate mEPSCs 1uM TTX was added in addition to sEPSCs recording conditions. In all experiments a time period of at least 5 minutes post break in was allowed for internal solution exchange and stabilization of membrane properties. GluA1KO sEPSC recordings were conducted similarly except

recording ACSF contained 50 µM picrotoxin and internal solution contained (in mM) 120 $CsMeSO_3$, 5 NaCl, 10 TEA-Cl, 10 HEPES, 5 QX-314, 1.1 EGTA, 0.3 Na-GTP, and 4 Mg-ATP, 295-300 mOsm. GluA1 WT and KO littermate mice were 8-16 weeks of age at time of recording. In PPR experiments evoked 100-200 pA responses were elicited with the interstimulus interval set at 40 ms, 50 ms and 60 ms. For MK-801 experiments NMDAR currents were pharmacologically isolated (25 µM picrotoxin, 10 µM NBQX) and held at +40 mV while a stable ten minute baseline (0.05 Hz) was acquired. After a stable baseline was acquired the stimulator was switched off and 10 µM MK-801 was washed on for seven minutes. Following the wash-in period the stimulator was turned on (0.1 Hz) and the time constant for decay of the integral of the NMDAR current was calculated using nonlinear regression one-phase decay. For baclofen modulation of PPR, 20 sweeps of approximately 200pA EPSCs (0.05 Hz) were collected to establish a baseline. Following a wash-in of 10uM baclofen for 8 minutes, an additional 20 sweeps were collected. Additional 20 sweeps were taken at 10 minutes and 20 minutes post washout. CV was calculated by dividing the SD of the amplitude of the evoked EPSCs by the mean. For rectification experiments evoked AMPAR-mediated EPSCs were isolated in 25 µm picrotoxin and 100 µm DL-APV containing aCSF while the voltage was stepped from -70mV to +40mV in 10mV steps. 0.1mM spermine was included in the standard cesium internal solution to avoid dialysis of endogenous polyamines. The rectification index (RI) was calculated as the ratio of the amplitude of AMPAR-mediated currents evoked at -70mV over +40mV. All signals were acquired via a Multiclamp 700B amplifier

(Axon Instruments), digitized at 10 kHz, filtered at 2 kHz and analyzed via pClamp 10.2 software (Axon Instruments). Holding current and series resistance were all monitored continuously throughout the duration of experiments. Experiments in which changes in series resistance were greater than 20% were not included in the data analysis.

Statistical analyses were performed using Graphpad Prism 5.04. Two-tailed unpaired Student's t-test (t) were used unless variance differed significantly (Bartlett's test for equal variances) then non-parametric Mann-Whitney (U) tests were used. One or Two-way analysis of variance (ANOVA) (F) were used when indicated with Neuman-Keuls Multiple Comparison post hoc test. Non-parametric Kruskal-Wallis tests (H) were used with Dunn's Multiple Comparison post hoc test when variances differed significantly (Bartlett's test for equal variances). All values given are presented as average ± SEM. Cumulative probability plots were analyzed with Kolmogorov-Smirnov (KS) test.

Whole-Cell Current Clamp Recordings

Slices were prepared as before, but perfused with ACSF containing (in mM): NaCl (124), NaH_2PO_4 (1.25), KCl (2.5), $CaCl_2$ (2.5), $MgSO_4$ (2), $NaHCO_3$ (26), Glucose (11) pH=7.35, 300-305mOsm. Recording electrodes were filled with in (mM): K+ gluconate (120), NaCl (4), HEPES (10), Mg-ATP (4), Na-GTP (0.3), KCl (20), Na+ Phosphocreatine (10) pH= 7.3, 285-290 mOsm. MSNs

were identified by their intrinsic membrane properties (i.e. resting membrane typically more negative than -80 mV, inward and outward rectification in response to somatic positive and negative current injections, and a long depolarizing ramp to delayed first spike discharge (Kawaguchi et al., 1989). Recordings were rejected if the initial Vm was more positive than -75 mV. Resting membrane potential or zero current potential was determined right upon break-in. Spontaneous excitatory postsynaptic potentials after 5 minutes post break in were recorded for 4 minutes with the cell dynamically current clamped at -85mV. Resting membrane potential was monitored and current was injected to maintain the resting potential at -85 mV. For current-voltage (IV) relationships positive or negative current injections were given in 20 pA steps until the cell reached threshold and fired a single AP. Five additional current injections steps (20 pA each) were given above threshold. Input resistance was monitored throughout the experiment and the cell was rejected if the input resistance changed by more than 20%. Healthy cells showed APs that crossed +30 mV and stable resting membrane potentials.

Lucifer Yellow Intracellular Fills, Confocal Imaging and Measurement of Dendritic Structure

Transgenic EAC3I mice (3-4 months) were perfused with 10 ml room temperature phosphate buffered saline (PBS) followed by 100 ml of 4% paraformaldehyde solution delivered over 10-20 minutes. Brains were post-fixed

in ice cold paraformaldehyde solution for an additional hour before 200 μm thick coronal sections of the precommissural striatum were prepared on a vibrating microtome (Leica; Buffalo Grove, IL). MSN cell bodies in dorsolateral striatum were visualized at 40X. Randomly selected EAC3I-expressing (EGFP-positive) MSNs and non-fluorescent (EGFP-negative) MSNs in the dorsal lateral striatum were iontophoretically filled with an 8% solution of Lucifer yellow (LY; Sigma-Aldrich, in 50 mM Tris-HCl, pH 7.4) using hyperpolarizing current (3-5 nA for 8-10 minutes).   Slices were fixed in 4% paraformaldehyde/PBS overnight at 4°C and then coverslipped using Prolong Gold mounting solution (Invitrogen; Grand Island, NY).

Digital images of MSN dendritic segments located 80-100 μm distal to the cell soma were captured using a Zeiss LSM 710 confocal microscope with x63 oil immersion objective and x2.5 digital zoom. Spine density of three to four dendritic segments emanating from different primary dendrites was averaged to yield a mean spine density value per MSN. A total of 17 EAC3I-negative MSNs and 16 EAC3I-positive MSNs were analyzed; these were obtained from four different mice of each genotype. After the confocal images were coded by someone not involved in the study, another person unaware of the animal or genotype of the MSN being examined used Imaris (Version 5.5; Bitplane) to quantify dendritic spine density. A three-dimensional perspective in "surpass" mode of the software package was generated and images were processed with background subtraction thresholding and smoothed with a Gaussian filter.

Dendritic segments were modeled with the largest and smallest diameters set at 2 μm and 1 μm, respectively. Florescence in each dendritic segment was thresholded manually to capture all dendritic spines. The minimum terminal point spine diameter was set at 0.143 μm and the fluorescence contrast threshold was set at 1. Identified spines were counted and marked in 3D on a rotating version of the image. Finally, each structure identified as a spine by the Imaris software was visually verified. In order to determine total dendritic length and generate a Sholl analysis (number of dendritic branches intersecting circular rings drawn around the soma every 20 μm distal to the soma), we used Neurolucida Explorer (MicroBrightField; Willington, VT).

Immunohistochemistry

Mice were perfused with 10mL of ice cold 0.1M PBS followed by 20 mL of ice cold 4% paraformaldehyde /0.1M PBS. Brains were postfixed in the same fixative overnight at 4°C and then cyroprotected with 30% sucrose/0.1M PBS until the brain equilibrated. Parasagittal sections (100μm thick) or coronal slices (40μm) were taken on a Leica cryostat. The slices were counterstained with nissl stain (Neurotrace 530/615, Life Technologies). Free-floating, coronal slices were washed in 4X PBS and then permeabilized in 0.5% TritonX/PBS for 30 minutes. The slices were then blocked in 10% normal donkey serum/0.1% TritonX/PBS for 1 hour at room temperature. Some slices were stained with a primary antibody to NeuN (mouse clone A60; 1:2000, Chemicon/Millipore), a neuronal marker in

10%/NDS/0.1% TritonX/PBS and placed on a rocker at 4°C for 48 hours. Slices were then washed 4X10mins in PBS. A secondary antibody conjugated to CY5 (Jackson ImmunoResearch) and raised against the primary antibody host was added to the slices and incubated for two hours at room temperature. Slices were then washed 4X10 minutes and mounted on Fisher Superfrost Plus slides (Fisher Scientific). Slices were allowed to dry for 5-20 minutes and coverslipped with Aqua Poly/mount (Polysciences, Inc). EGFP and fluorescent probes were imaged with Zeiss 510 or 710 confocal microscopes.

Sample Collection and Quantification of Biogenic Amine Levels

Mice were anesthetized with isofurane and quickly decapitated. Brains were quickly dissected (less than a minute) and slices 1mm thick were made of the striatum and quickly frozen on dry ice. 1mm tissue punches were collected and placed at -80C. Detection and quantification of monoamine levels and metabolites was performed by the Vanderbilt Neurochemistry Core. Determinations are achieved using two dedicated waters high performance liquid chromatography systems equipped with autosamplers and either a Decade II electrochemical (monoamines) or 474 scanning fluorescence (amino acids) detector(Hnasko et al., 2006).

Pharmacology

Picrotoxin, (+)Mk-801 maleate ((5S,10R)-(+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,dicyclohepten-5,10-imine maleate), NBQX (2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline -7-sulfonamide), (R)-Baclofen ((R)-4-Amino-3-(4-chlorophenyl)butanoic acid), and DL-APV (DL-2-Amino-5-phosphonopentanoic acid) were purchased from Tocris (Ellisville, Missouri). DMSO (0.05%) (Sigma) was used as a vehicle for picrotoxin.

Rotarod

All animals were handled for three days prior to starting behavioral testing. Animals were placed on an accelerating rotarod (4-40rpm) for up to 5 minutes. Latency to fall was measured. Three trials with a 30 minute ITI were given on day 1 followed by five trials on day 7.

Locomotor Sensitization

Mice were habituated to handling and injection for three days prior to the beginning of the experiment. Sessions were performed using automated experimental chambers (27.9×27.9 cm; MED-OFA-510; MED Associates, Georgia, VT) under constant illumination within a sound-attenuated room. Analysis of locomotor activity was performed using Activity Monitor v5.10 (MED

Associates). For each of 5 days, mice were first injected with saline (10 ml/kg), and then placed in open field chambers for 30 min and activity was recorded. Immediately after the 30 min session, each mouse was injected with cocaine (10 mg/kg; 10 ml/kg) and placed back into the same chamber and activity was recorded for 30 min. Two weeks following the 5th day, mice underwent a challenge session that was identical to the other sessions.

Operant Testing

Operant training chambers were as described (Olsen and Winder *Neuropsychopharmacology* (2009)), with levers mounted 2.2 cm above the grid floor and cue lamps (yellow LEDs) mounted 2 cm above them. Mice were food restricted where chow was available 2 hours per day. Subjects first underwent a single day of magazine training (50 non-contingent reinforcers of 25% Ensure given on a VI-30 interval) where neither lever was available. In subsequent daily sessions, mice underwent operant conditioning in a food self-administration task. Operant sessions were conducted between 0900-1400 h. During operant training, mice were trained to lever press for access to a liquid reinforcer (25% Ensure) delivered by a liquid dipper (ENV-302W). The dipper was available for 10 s after head entry into the dispenser, and the cue lights remained illuminated until the end of the 10-s access.

At the beginning of each session, the house light was illuminated, the exhaust fan was turned on, and an initial noncontingent reinforcer was available concurrent with illumination of the cue lights. After retrieving the initial reinforcer, both levers were extended. Each mouse was assigned either the left or right lever to be the active lever, and the side of the active lever was counterbalanced between mice within each genotype.

Completion of the required ratio on the active lever resulted in illumination of the cue lights and elevation of a dipper cup containing ~40 ul of liquid food reinforcer (25% Vanilla Ensure®). The dipper was available for 10 seconds after head entry into the dispenser, and the cue lights remained illuminated until the end of the 10-second access. The experiment began with 6 30-min sessions where each active lever press was reinforced (Fixed Ratio-1, FR-1). Next, mice were advanced to a random-ratio 5 (RR-5) schedule of reinforcement, where each lever press had a 20% chance of being reinforced (30-min sessions, 50 reinforcers maximum). Mice underwent 4 days of RR-5 testing, then 4 days each of RR-10 (10% chance of reinforcement per lever press; 50 reinforcers maximum) and RR-20 (5% chance of reinforcement per lever press; 25 reinforcers maximum) testing. Following RR-20 testing, mice were tested using a devaluation procedure, where mice were prefed either the reinforcer (25% Ensure) or standard lab chow on one day, with pre-feeding of the alternate food type on the subsequent day (order counterbalanced within genotype). Prefeeding lasted one hour and occurred immediately prior to testing in the

operant chamber under extinction conditions. Next, mice had 4 days of RR-20

under standard food restriction schedule (2 hour/day access), and then mice

were tested under conditions of contingency degradation for 3 sessions. Under

these conditions, 25 reinforcers were given in a non-contingent manner at a rate

that the group (genotype) had previously earned under RR-20 conditions and

lever pressing was recorded (although there was no consequence of lever

pressing).

CHAPTER III

GENETIC INHIBITION OF CAMKII IN DORSAL STRIATAL MEDIUM SPINY NEURONS REDUCES FUNCTIONAL EXCITATORY SYNAPSES AND ENHANCES INTRINSIC EXCITABILITY

Introduction

The striatum is the major input nucleus of the basal ganglia (Yin and Knowlton, 2006). Dysfunction in this region is associated with drug addiction, Parkinson's disease and other disorders (Jenner, 2008; Kreitzer and Malenka, 2008; Milnerwood and Raymond, 2010; Redgrave et al., 2010; Luscher and Malenka, 2011; Wan et al., 2011; Yang and Lu, 2011). The striatum is primarily composed of projection GABAergic medium spiny neurons (MSNs) that integrate glutamatergic excitatory transmission with modulatory dopaminergic transmission. Since MSN firing is thought to be driven primarily by excitatory drive, understanding the basic mechanisms of glutamatergic transmission onto MSNs is necessary to understand how the striatum functions in health and disease.

Calcium-calmodulin-dependent kinase II (CaMKII) is a Ser/Thr kinase that is highly expressed in the striatum, constituting ~0.7% of total striatal protein (Erondu and Kennedy, 1985). CaMKII assembles into dodecameric complexes that in the striatum predominantly contain CaMKIIα and CaMKIIβ isoforms (Lisman et al., 2002). As a major constituent of the postsynaptic density (PSD) in the dorsal striatum (Fukunaga et al., 1988) as well as other forebrain regions

(Cheng et al., 2006; Baucum et al., 2012), CaMKII is activated by N-methyl-D-aspartate-receptor (NMDAR)-mediated calcium influx (Silva et al., 1992; Giese et al., 1998; Hinds et al., 1998). CaMKII is a key modulator of hippocampal and cortical pyramidal cell glutamate synapse function (Wayman et al., 2008; Lee et al., 2009; Lucchesi et al., 2011). CaMKII can phosphorylate many downstream substrates including the ionotropic glutamate receptors NMDARs and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) (Barria et al., 1997a; Mammen et al., 1997; Benke et al., 1998; Derkach et al., 1999; Lee et al., 2000; Strack et al., 2000). Indeed, in hippocampal pyramidal cells, CaMKII activation enhances synaptic trafficking of AMPARs and channel function (Lledo et al., 1998; Shi et al., 1999; Hayashi et al., 2000; Shi et al., 2001). In addition, a constitutively active form of CaMKII can decrease intrinsic excitability of hippocampal neurons as well as MSNs in the nucleus accumbens shell (Varga et al., 2004; Kourrich et al., 2012). While much is known about the role of CaMKII at glutamate synapses on glutamatergic projection neurons such as hippocampal and cortical pyramidal neurons, relatively little is known for GABAergic cells. Indeed, little CaMKII is expressed in GABAergic interneurons (Jones et al., 1994; Liu and Jones, 1996; Sik et al., 1998), making GABAergic projection cells such as MSNs, which are highly enriched in CaMKII, unique targets for studying the role of CaMKII in synaptic transmission and intrinsic excitability.

Previous studies have implicated striatal CaMKII in Parkinson's disease (PD) and addiction. CaMKII is hyperactivated after striatal dopamine depletion,

and CaMKII inhibition rescued striatal synaptic plasticity and motor deficits found in animal models of Parkinson's disease (Picconi et al., 2004b). Striatal CaMKII is also essential for the motivational effects of reward cues on goal-directed behaviors (Wiltgen et al., 2007) as well as curbing D1R-mediated cocaine hyperlocomotion (Stein and Hell, 2010) and modulating excitability following chronic cocaine administration(Kourrich et al., 2012). Thus, a better understanding of CaMKII's role in striatal glutamatergic synaptic transmission may suggest new approaches to treat PD and addiction.

In addition to its postsynaptic roles, CaMKII modulates a variety of presynaptic functions, including trafficking of synaptic vesicles (Llinas et al., 1985; Lin et al., 1990; Waxham et al., 1993; Stefani et al., 1997; Chi et al., 2001), P/Q type calcium channels (Elgersma et al., 2002; Hojjati et al., 2007; Jiang et al., 2008), voltage-gated sodium channels (Carlier et al., 2000; Wagner et al., 2006), catecholamine synthesis (Yamauchi et al., 1981; Atkinson et al., 1987) and dopamine transporter function (Fog et al., 2006; Binda et al., 2008). Thus, an investigation of the role of CaMKII within striatal MSNs requires a cell-specific approach. To accomplish this, we generated a transgenic mouse line that expresses a CaMKII inhibitory peptide selectively within dorsal striatal MSNs. Using this line, we found that CaMKII inhibition in dorsal striatal MSNs leads to a loss of functional glutamatergic synapses and an increase in intrinsic excitability. These findings shed light on the neural mechanisms underlying the development of striatal neural circuits, learning and memory, and motor behavior.

Results

Characterization and *in vivo* localization/expression of CaMKII inhibitory (EAC3I) peptide

To determine the role CaMKII plays in modulating glutamatergic transmission onto MSNs in the dorsal lateral striatum, we generated a transgenic mouse model with striatally enriched expression of a CaMKII inhibitory peptide fused to enhanced green fluorescent protein (EGFP) referred to hereafter as EAC3I (Braun and Schulman, 1995). The EAC3I peptide inhibits all isoforms of CaMKII, as well as both calcium-dependent and independent forms of the kinase, and the fusion with EGFP allows for visualization of the regional and cellular distribution of the transgenically expressed protein. EAC3I was previously utilized in another transgenic line to examine the role of CaMKII in the heart (Zhang et al., 2005). To spatially and temporally regulate EAC3I expression, the tetracycline transactivator (tTA) is driven by a CaMKIIα promoter fragment with the tTA gene product driving expression of EAC3I (Figure 5A). Constitutive expression of the EAC3I transgene has no overt effect on viability or on gross brain morphology, and is silenced by including doxycycline (DOX, 200 mg/kg) in the animals' chow for two weeks (Figure 5B). The alpha CaMKII promoter normally restricts transgene expression to the forebrain, but this founder line exhibits enrichment of EAC3I expression in the MSNs of the dorsal striatum (Figure 5C, 5D), presumably due to integration-site dependent effects. Little to no expression of the inhibitor was seen in cortex and thalamus (Figure 5C, 5D).

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The EAC3I peptide is expressed in a mosaic pattern throughout the dorsal striatum, with 34±4% of neurons containing the inhibitory peptide (Figure 5D). The mosaicism observed is common in transgenic animals and different founder lines utilizing the alpha CaMKII promoter fragment to drive transgene expression show differing expression patterns throughout the forebrain (Mayford et al., 1996; Kaufman et al., 2008). However, we observed no overlap of signal between EGFP positive neurons and striatal cholinergic and GABAergic interneuron markers such as ChaT, parvalbumin, NPY, and calretinin (Figure 6). At higher magnification the EAC3I inhibitory peptide was observed in the MSN cell soma, dendrites and dendritic spines (Figure 5E). Dense expression of EAC3I was detected in both the globus pallidus and in the substantia nigra pars reticulata (Figure 5C, 5F, 5H), but higher magnification images revealed the staining was localized to axons (Figure 5F), demonstrating that both indirect and direct pathway MSNs contain the inhibitory peptide (Figure 5C, 5F, 5H).
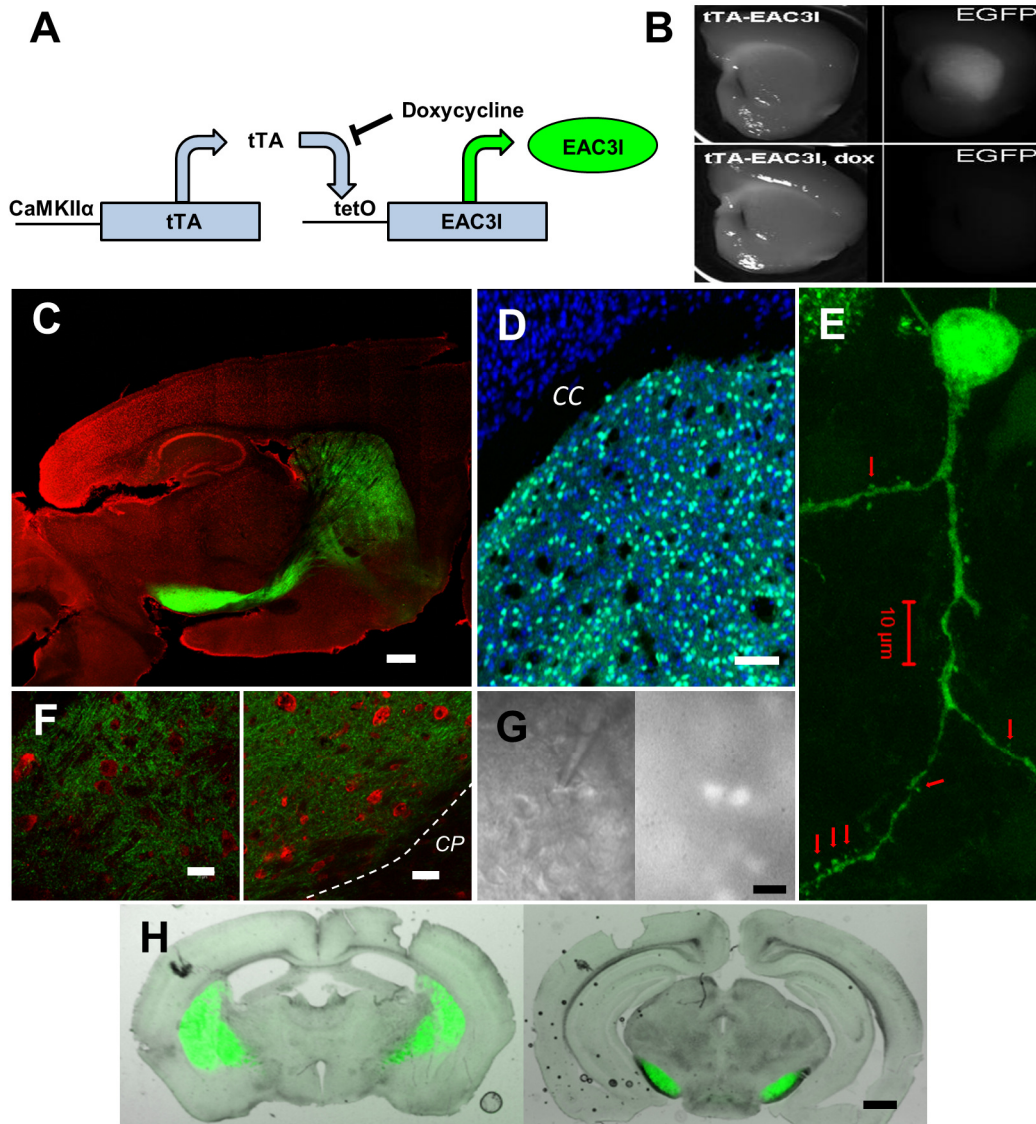
Figure 5. Characterization and *in vivo* localization/expression of CaMKII inhibitory (EAC3I) peptide in EAC3I mice. (A) Schematic of breeding strategy for production of EAC3I mice. CaMKIIalpha promoter drives expression of tTA which binds to the tetO promoter and drives expression of EAC3I peptide fused to EGFP. (B) (Top) Brain slice of EAC3I under brightfield (left) and EGFP epifluorescence (right). (Bottom) Brain slice of EAC3I as above following 3 weeks of doxycycline feeding (200 mg/kg). Note lack of EAC3I-EGFP expression. (C) Sagittal section of EAC3I mouse brain showing restricted endogenous EAC3I-EGFP expression (green) and nissl stain (red, neuronal marker). Scale bar 500µm. (D) Coronal image of mosaic expression of endogenous EAC3I-EGFP expression (green) in dorsal lateral striatum with a NeuN stain (blue, neuronal marker). Note little to no expression of EAC3I peptide in cortex. CC=corpus callosum, Scale bar 100 µm. (E) 63X image of unstained EAC3I-EGFP expressing MSN (green=endogenous EGFP signal). Note expression in soma, dendrites and dendritic spines (arrows). Scale bar 10 µm. (F) Images of globus pallidus (GP) (left) and substantia nigra pars reticulata (SNR) (right) showing MSN axon terminals (green) and nissl stain (red). GP and SNR cell somas (red, nissl stain) are devoid of EGFP signal. CP=cerebral peduncle. Scale bars 20 µm. (G) (left) DIC image of patch pipette on a MSN in whole cell mode, (right) epifluorescence image of left panel confirming EAC3I-EGFP expression. Scale bar 20 µm. (H) Overlaid coronal images showing EAC3I-EGFP MSN axon terminal field expression in globus pallidus (left) and substania nigra pars reticulata (right) confirming indirect and direct pathway expression, respectively. Scale bar 0.5 mm.

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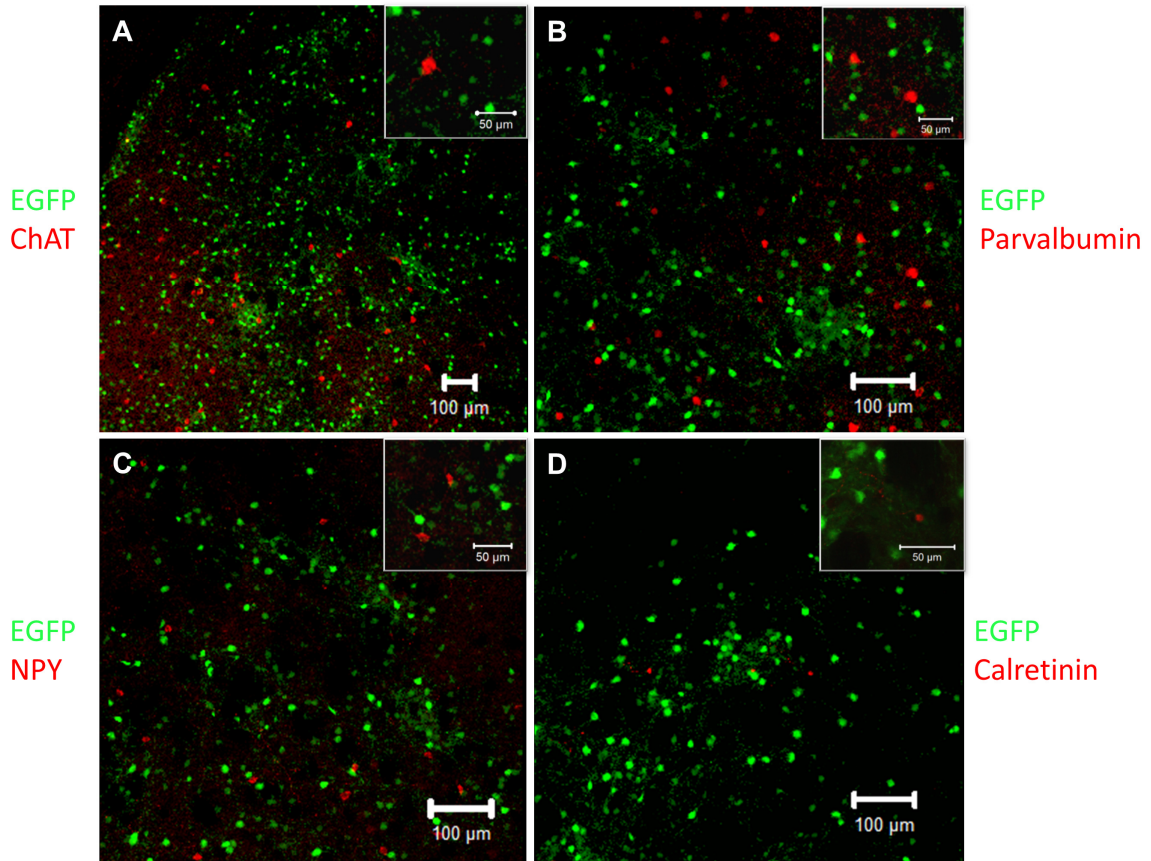
Figure 6. Striatal Interneuron Markers Do Not Colocalize with EAC3I Peptide. (A) 20X confocal image of dorsal lateral striatum from an EAC3I mouse of endogenous EGFP expression (green) and striatal ChAT immunopositive interneurons (red, 0/70 ChAT positive neurons contained EGFP). Scale bar 100µm, inset 50µm. (B) Like A, but striatal parvalbumin immunopositive interneurons labeled (red, 0/37 parvalbumin positive neurons contained EGFP). Scale bar 100µm, inset 50µm. (C) Like A, but striatal NPY immunopositive interneurons labeled (red, 0/22 NPY positive neurons contained EGFP). Scale bar 100µm, inset 50µm. (D) Like A, but striatal calretinin immunopositive interneurons labeled (red, 0/15 calretinin positive neurons contained EGFP). Scale bar 100µm, inset 50µm.

<u>Effects of CaMKII inhibition on MSN glutamatergic inputs in dorsal lateral</u>

<u>striatum</u>

We initially recorded spontaneous excitatory postsynaptic currents

(sEPSCs) from MSNs using whole-cell voltage clamp in the presence of

picrotoxin to isolate excitatory transmission. In this and subsequent

electrophysiological analyses, we compared three control groups of MSNs with

one experimental group: Control 1) MSNs from tTA- /tetO-EAC3I- mice (Wt),

Control 2) MSNs from tTA+ /tetO-EAC3I- mice (tTA), Control 3) EGFP negative

MSNs from tTA+/tetO-EAC3I+ mice (NON EGFP) and Experimental 4) EGFP

positive MSNs from tTA+/tetO-EAC3I+ mice (referred to as EAC3I MSNs

(EGFP)).

In adult mice no differences in sEPSC amplitudes were observed between

MSNs from the four groups described above (in pA, Wt 13.39±0.81; tTA

11.98±1.27; NON EGFP 14.97±1.38; EGFP 14.78±0.67) *[F (3, 33) =1.46; p=NS;*

Figure 8A, 8G]. However, sEPSC frequency was markedly reduced in EAC3I

MSNs relative to all control MSN groups (in Hz, Wt 3.20±0.24; tTA 2.85±0.17;

NON EGFP 2.99±0.63; EGFP 1.07±0.15) *[H (3, 33) =19.85; p=0.0002]; Figure*

8A, 8D]. Similar results were observed in sEPSC frequency in 3-4 week old mice

(in Hz, NON EGFP 3.94±1.05; EGFP 1.08±0.23) *[U (9) =3.000; p=0.0341; data*

not shown]. Additionally, we did not observe any differences in AMPAR-

mediated current voltage relationship between EAC3-I containing and lacking

MSNs (Rectification Index (RI) = -70mV/+40mV, NON EGFP 1.97±0.18; EGFP

1.75±0.24) *[t (8) =0.575; p=0.58; Figure 7A, 7B]*. To determine if the decrease in

sEPSC frequency was due to a change in the intrinsic electrical activity in the

slice, we also examined activity-independent miniature EPSCs (mEPSCs) in the

presence of TTX. Similar to the results with sEPSCs, mEPSC frequency was

also reduced in EAC3I MSNs (in Hz, Wt 3.62±0.32; tTA 4.88±0.67; NON EGFP

3.89±0.41; EGFP 0.97±0.40) *[F (3, 22) =9.688; p=0.0008]; Figure 8B, 8E]*,

suggesting that this effect is independent of presynaptic excitability.
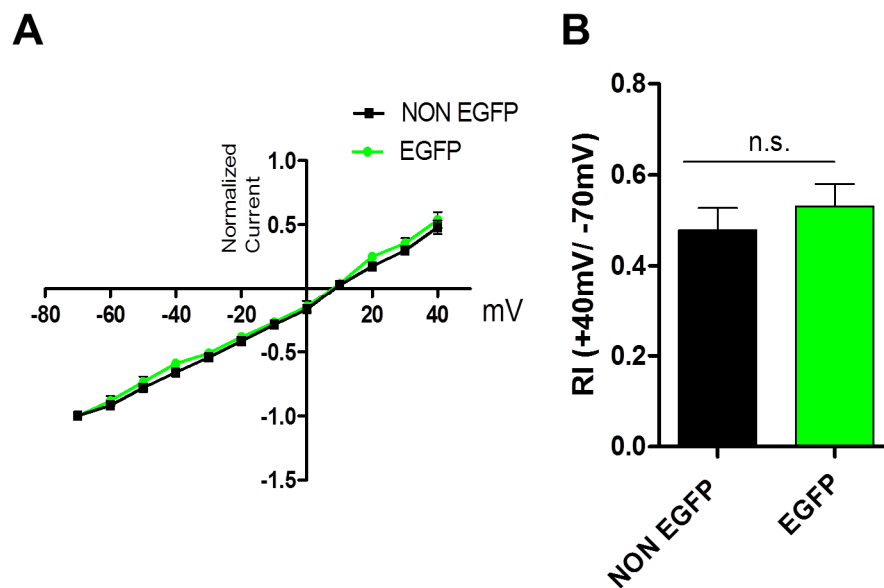
Figure 7. AMPA Current Voltage (IV) Relationships are Not Altered Between EAC3I-Containing and EAC3I-Lacking MSNs. (A) Averaged normalized whole-cell IV relationships (-70mV to +40mV, 10mV steps) from EAC3I-containing (EGFP) and neighboring EAC3I-lacking (NONEGFP) MSNs (NON EGFP: n=5; EGFP: n=6; p=0.1751). (B) Pooled data showing no change in rectification index (RI) (+40mV/-70mV) (NON EGFP: n=5; EGFP: n=6; p=0.4775). Error bars denote SEM, N.S. = not significant.
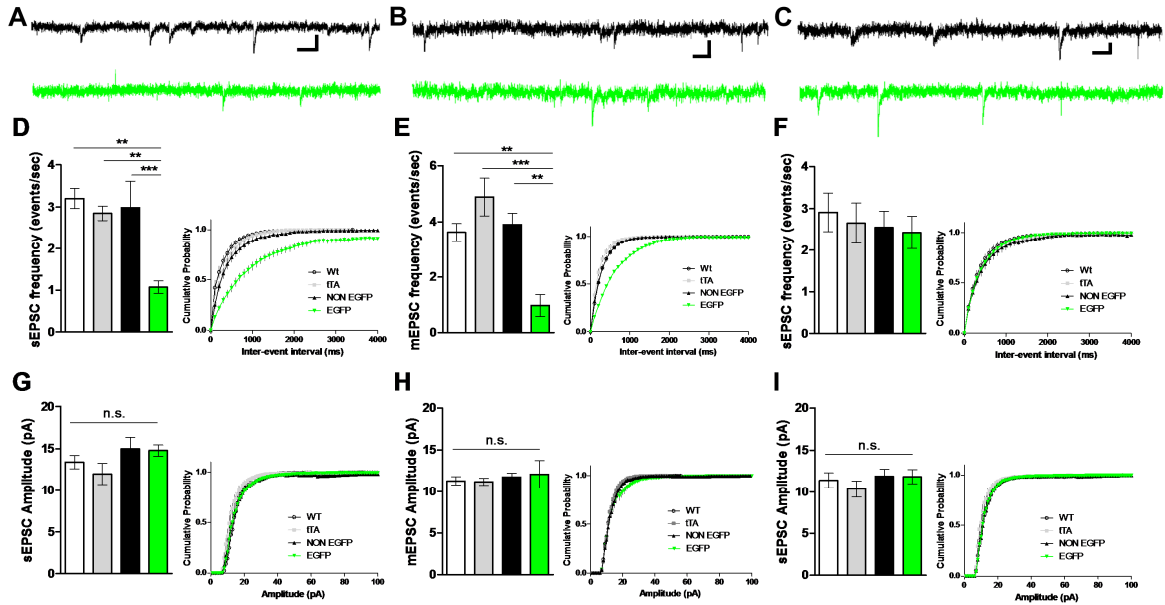
Figure 8. *In vivo* expression of EAC3I decreases s/mEPSC frequency in dorsal lateral striatum MSNs. (A) Representative sEPSC traces for NON EGFP (black) and EGFP (CaMKII-inhibited, green). Scale bars 50 ms and 10 pA. (B) Representative mEPSC traces for NON EGFP (black) and EGFP (CaMKII-inhibited, green). Scale bars 50 ms and 10 pA. (C) Representative sEPSC traces for NON EGFP (black) and EGFP (CaMKII-inhibited, green) MSNs from animals that were fed DOX from birth to six weeks and then removed to allow EAC3I transgene expression. Recordings were made 4-5 weeks following DOX removal at a similar age to previous. The scale bars are 50 ms and 10 pA. (D) (Left) Average sEPSC frequencies from EAC3I-containing MSNs compared to controls, (right) Cumulative probability distributions of sEPSC inter-event intervals (Wt: n=7, p=0.0001; tTA: n=5, p=0.0001; Non EGFP: n=9, p=0.0009; versus EGFP: n=13). (E) (left) Average mEPSC frequency from EAC3I MSNs compared to controls, (right) cumulative probability distributions of mEPSC frequency (Wt: n=5, p=0.0022; tTA: n=6, p<0.0001; Non EGFP: n=6, p=0.0019; versus EGFP: n=6). (F) (Left) Average sEPSC frequencies from EAC3I-containing MSNs versus controls, (right) cumulative probability distributions of sEPSC inter-event intervals (Wt: n=8, p=0.99; tTA: n=7, p=0.99; Non EGFP: n=8, p=0.99; versus EGFP: n=10). (G) (left) Average sEPSC amplitudes, (right) cumulative probability distributions of sEPSC amplitude (Wt: n=7, p=0.91; tTA: n=5, p=0.26; Non EGFP: n=9, p=0.99; versus EGFP: n=13). (H) (left) Average mEPSC amplitude, (right) cumulative probability distributions of mEPSC amplitude (Wt: n=5, p=0.90; tTA: n=6, p=0.71; Non EGFP: n=6, p=0.96; versus EGFP: n=6). (I) (left) Average sEPSC amplitudes, (right) cumulative probability distributions of sEPSC amplitude from animals that were fed DOX from birth to six weeks and then removed to allow EAC3I transgene expression (Wt: n=8, p=0.99; tTA: n=7, p=0.34; Non EGFP: n=8, p=0.99; versus EGFP: n=10). * *P <0.05; ** P <0.01; *** P < 0.001;* error bars represent SEM, N.S. = not significant. Note: All neurons in panels A-I are held at -70mV during recordings.

The use of the tTA expression system provides DOX-dependent control of EAC3I expression (Figure 5B). We suppressed expression of the transgene by supplementation of food with DOX (200 mg/kg) for both the dam and her litter until 6 weeks of age, and then recorded from EAC3I-expressing MSNs 4 weeks after removal of DOX. Under these conditions, the global expression of EAC3I was markedly lower than in non-DOX exposed mice, as has been previously noted with the tTA expression system (Bejar et al., 2002). Notably, sEPSC frequency in the EAC3I MSNs was not significantly different from controls (in Hz, Wt 2.91±0.46; tTA 2.65±0.48; NON EGFP 2.55±0.39; EGFP 2.42±0.39) *[F (3, 32) =0.2434; p=NS]; Figure 8C, 8F]*. These data indicate that the sEPSC frequency phenotype observed in no-DOX EAC3I MSNs is not likely due to an insertion site artifact.

MSN CaMKII inhibition reduces excitatory transmission independently of changes in release probability

The canonical interpretation of a reduction in s/mEPSC frequency is via a reduction in the probability of glutamate release or the number of release sites/ number of synapses. In order to better understand the mechanism(s) underlying the reduction in s/mEPSC frequency in EAC3I MSNs we first examined paired-pulse ratios (PPR) of evoked EPSCs, a measurement that inversely correlates with neurotransmitter release probability (Zucker and Regehr, 2002). The PPR

of evoked EPSCs on control MSNs did not differ from that observed on EAC3I MSNs [*PPR 40ms ISI:* (Wt 1.10±0.03; tTA 1.16±0.15; NON EGFP 1.20±0.10; EGFP 1.01±0.07) *H (3, 33) =2.436; p=NS; PPR 60ms ISI:* (Wt 1.12±0.07; tTA 1.17±0.16; NON EGFP 1.01±0.07; EGFP 0.94±0.06) *F (3, 33) =1.377; p=NS; Figure 9A, 9B, 9C*]. To show that we could predictably manipulate PPR we used the GABA$_B$R agonist baclofen (10 µM). Baclofen acts presynaptically to reduce the probability of release and therefore increase PPR at a number of CNS synapses (Zucker and Regehr, 2002; Lei and McBain, 2003). Baclofen increased the PPR in EAC3I MSNs to a similar extent as in control MSNs suggesting that release probability was modifiable in the CaMKII-inhibited cells and not at a floor (NON EGFP baseline 1.13±0.11, 10 µM baclofen 2.29±0.28, 20 min washout 1.38±0.14; EGFP baseline 1.04±0.11, 10 µM baclofen 1.94±0.43, 20 min washout 1.30±0.14) *[F (1, 9) =0.5403; p=NS; Figure 9E]*. Similar results were observed with coefficient of variation (CV) measures of evoked EPSCs, where baclofen application enhanced CV to the same degree in EAC3I MSNs and controls (NON EGFP baseline 0.21±0.05, 10µM baclofen 0.56±0.07, 20 min washout 0.24±0.07; EGFP baseline 0.23±0.05, 10µM baclofen 0.52±0.11, 20 min washout 0.31±0.06) *[F (1, 9) =0.0047; p=NS; Figure 9D]*. Additionally, we found that baclofen significantly decreased the sEPSC frequency to a similar extent in both EAC3I MSNs and controls (NON EGFP baseline 100±22.4%, 10 µM baclofen 37.8±11.1%; EGFP baseline 100±33.2%, 10µM baclofen 40.4±10.2%) *[F (1, 9) =0.0025; p=NS; Figure 9F]*.
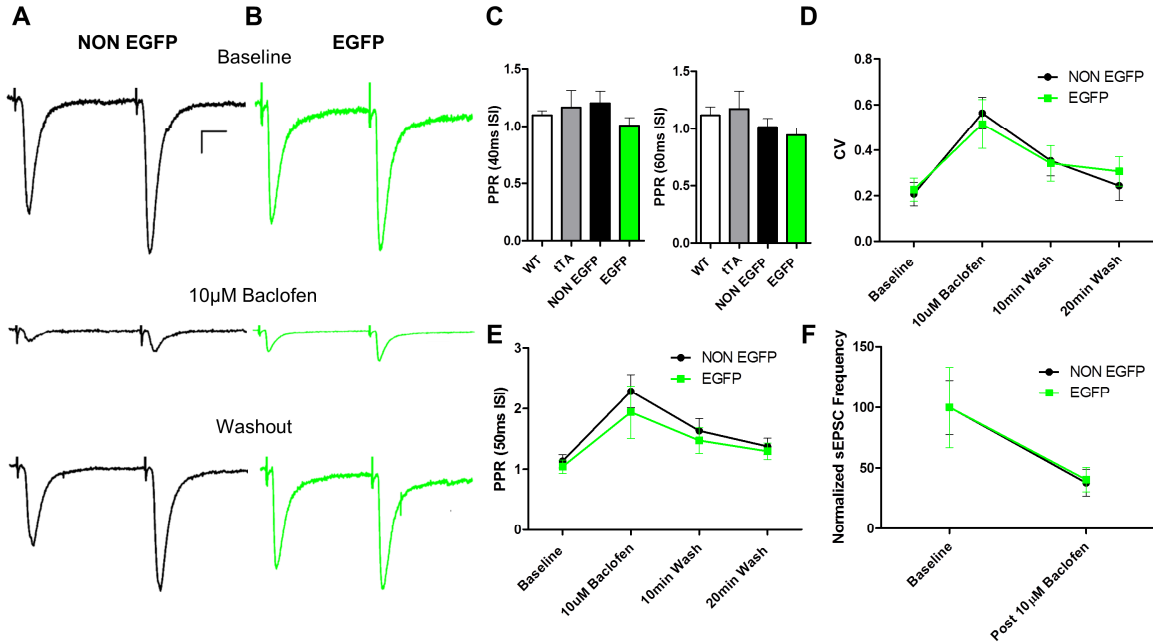
Figure 9. Dorsal lateral striatum MSN CaMKII inhibition reduces excitatory transmission independently of changes in release probability. (A) (Upper trace, black) NON EGFP (EAC3I-lacking) MSN PPR (50 ms ISI) example trace baseline (average of 20 sweeps, 0.05 Hz). (Middle trace, black) EAC3I-lacking MSN PPR (50 ms ISI) example trace post 10 µM Baclofen wash in (average of 20 sweeps, 0.05 Hz). (Bottom trace, black) EAC3I-lacking MSN PPR (50 ms ISI) example trace 20 minutes post wash out of drug (average of 50 sweeps, 0.05 Hz). (B) Same as in (A), but for an EGFP (EAC3I-containing) MSN. (C) Average PPR recorded by paired-pulse stimulation eliciting EPSCs with two different interstimulus intervals (40 and 60 ms) for EAC3I-containing MSNs versus all controls. (Wt: n=9; tTA: n=6; NON EGFP: n=9; EGFP: n=10; 40 ISI p=0.38, 60 ISI p=0.27). (D) Baclofen increases the CV of EPSCs. Coefficient of variation (CV=SD/Mean) change of EPSCs from before, during and after application of baclofen for EAC3I-containing and EAC3I-lacking MSNs (NON EGFP: n=6; EGFP: n=5; p=0.95). (E) Baclofen increases the PPR. PPR (50ms ISI) for EAC3I-containing and EAC3I-lacking MSNs before, during and after application of baclofen (NON EGFP: n=6; EGFP: n=5; p=0.48). (F) Baclofen decreases sEPSC frequency. Plot of normalized sEPSC frequency post 10 µM Baclofen compared to baseline for EAC3I-containing and EAC3I-lacking MSNs (NON EGFP: n=6; EGFP: n=5; p=0.96). Note: All MSNs held at -70mV during recordings.

Changes in s/mEPSC frequency could also theoretically be produced by changes in synaptic glutamate concentration. To address this possibility, we utilized MK-801, an uncompetitive, activity-dependent and irreversible antagonist of NMDARs (Huettner and Bean, 1988). After obtaining a stable baseline of evoked NMDAR-mediated EPSCs at +40mV in the presence of picrotoxin and NBQX, the stimulator was switched off and 10µM MK-801 was applied to the slice. After eight to ten minutes to allow the drug to equilibrate in the bath the stimulator was turned back on and the subsequent rate of inhibition of the NMDAR-mediated EPSC was calculated. The rate of inhibition was not significantly different between non-EGFP and EAC3I MSNs (tau in seconds, NON EGFP 80.82±9.65; EGFP 87.07±8.59) *[t (9) =0.6469; p=NS; Figure 10A, 10B]*, suggesting that the levels of glutamate at both synapses is not significantly different. Together, the lack of effect of CaMKII inhibition on PPR, baclofen modulation or MK-801 rate of blockade point to a postsynaptic mechanism for the decreased sEPSC frequency in the CaMKII-inhibited cells, which we interpret as a decrease in the number of functional synapses.
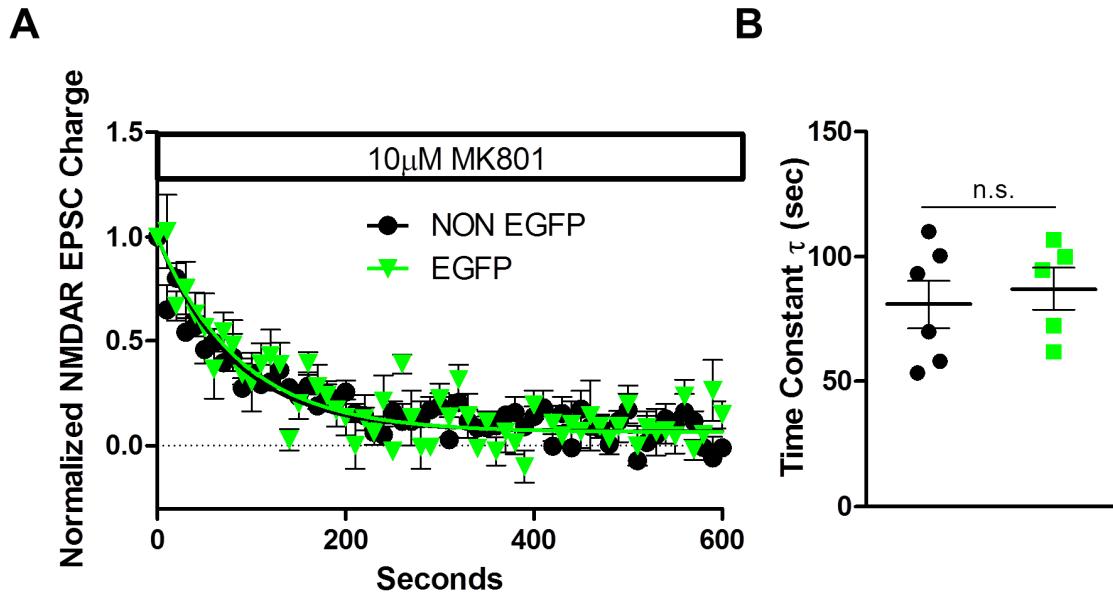
Figure 10. Dorsal lateral striatum MSN CaMKII inhibition does not alter the level of glutamate at the cleft. (A) Normalized average NMDAR-mediated EPSC Charge (AUC) measured with 0.1 Hz stimulation in the presence of 10 µM Mk-801 over time for EAC3I-containing and EAC3I-lacking MSNs. (B) The rate of NMDA-mediated EPSC decay is best fit with a single exponential decay function. The time constant (tau) in seconds was not significantly different between groups (NON EGFP: n=6; EGFP: n=5; p=0.65). All neurons held at +40mV to relieve NMDAR-dependent voltage blockade by magnesium.

<u>CaMKII inhibition does not alter dendritic spine density, but reduces dendritic</u>

<u>length and complexity</u>

We next examined dendritic spine density, dendritic length and branching complexity in CaMKII-inhibited versus neighboring non-inhibited cells. Previous work has suggested a correlation between changes in mEPSC frequency and dendritic spine density (Day et al., 2006; Fu et al., 2007; Lu et al., 2011b; Verpelli et al., 2011), although this is not always the case (Ding et al., 2012). Additionally, CaMKII has been shown to modulate dendritic length in the hippocampus (Fink et al., 2003). There was no difference in spine density related to expression of the transgene (NON EGFP 18.03 ± 0.52 spines per 10 µm vs.; EGFP 17.95 ± 0.85) *[t (31) =0.087; p=NS; Figure 11A, 11B]*. However, we observed a significant reduction in total dendritic length (NON EGFP 1538 ± 99 µm vs.; EGFP 1134 ± 78) *[t (31) =3.158; p=0.0035; Figure 11C, 11D]*. Sholl analysis revealed a significant overall decrease in dendritic branching (*F (1,31) =28.55; p<0.0001)*, with Bonferroni post-hoc analyses revealing specific significant decreases at 40 and 60 µm distal to the cell soma *(Figure 11E)*.
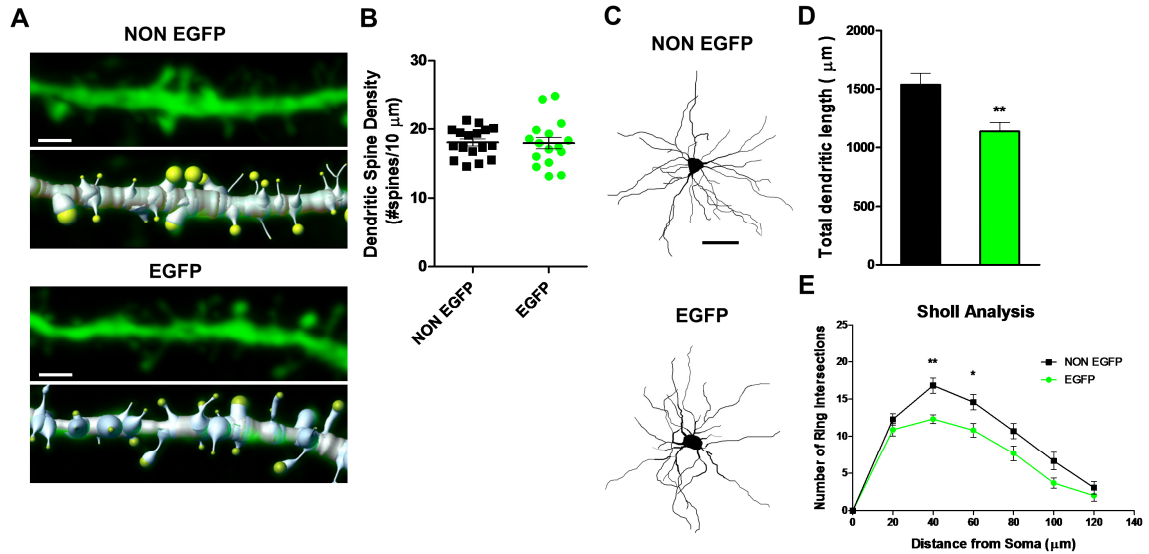
Figure 11. CaMKII Inhibition Does Not Alter Dendritic Spine Density, but Decreases Dendritic Length and Complexity. Rendering and quantification of a confocal image of a Lucifer yellow filled dendritic segment (80-100 µm from the cell soma) from a MSN from the dorsal lateral striatum. (A) (top) Confocal image of EAC3I-lacking MSN (NON EGFP) and the Imaris dendrite and spine model overlaid from segment above. (below) Same as above, but for an EAC3I-containg MSN segment. Scale bars 1.5 µm. Fluorescent signal (green) pertains to Lucifer yellow fill. (B) Average dendritic spine density (number of spines/10 µm) scatter plot for each neuron. NON EGFP n=17, EGFP n=16; p=0.93. (C) Neuronal reconstructions of representative EAC3I-lacking (NONEGFP) and EAC3I-containing (EGFP) dorsal striatal MSNs. Scale bar 50µm. (D) Average total dendritic length in EAC3I-lacking (black) and EAC3I-containing (green) MSNs. (E) Sholl analysis of dendritic complexity in EAC3I-lacking (black) and EAC3I-containing MSNs (green). ***p <0.01, *p <0.05;* error bars represent SEM. Thank you to Hui-dong Wang for data collection.

GluA1 KO mimics EAC3I decrease in sEPSC frequency

The AMPAR GluA1 subunit is critical for activity-dependent postsynaptic strengthening of excitatory synapses, and is inserted into the synaptic membrane in a CaMKII-dependent process in hippocampal neurons (Hayashi et al., 2000). Mice lacking the GluA1 subunit of the AMPAR have deficits in CA1 LTP (Zamanillo et al., 1999) (but see (Mack et al., 2001)) and deficits in learning and memory (Reisel et al., 2002; Bannerman et al., 2004; Schmitt et al., 2005; Wiedholz et al., 2008). Additionally, reductions in CaMKIIα mRNA and protein levels are seen in the hippocampus of GluA1 KO animals (Zhou et al., 2009). If the CaMKII inhibition-dependent reduction in functional glutamatergic synapses on striatal MSNs is due to defects in synaptic GluA1 insertion, then we predicted that GluA1 knockout mice should mimic EAC3I mice in terms of sEPSC frequency and amplitude. Thus, we measured sEPSC frequency and amplitude in adult GluA1 KO versus control mice in the dorsal lateral striatum. We found that the loss of the GluA1 receptor also led to a significant reduction in sEPSC frequency (in Hz, control 3.2±0.8; GluA1KO 1.4±0.3) *[U (31) =66; p=0.0133; Figure 12A, 12B],* but not sEPSC amplitude (in pA, control 18.3±0.5; GluA1KO 18.1±0.4) [*t (31) =0.3576; p=N.S.; Figure 12A, 12C].* The similarity in synaptic outcomes of the GluA1 KO and EAC3I expression reinforces the idea that a common pathway has been affected.
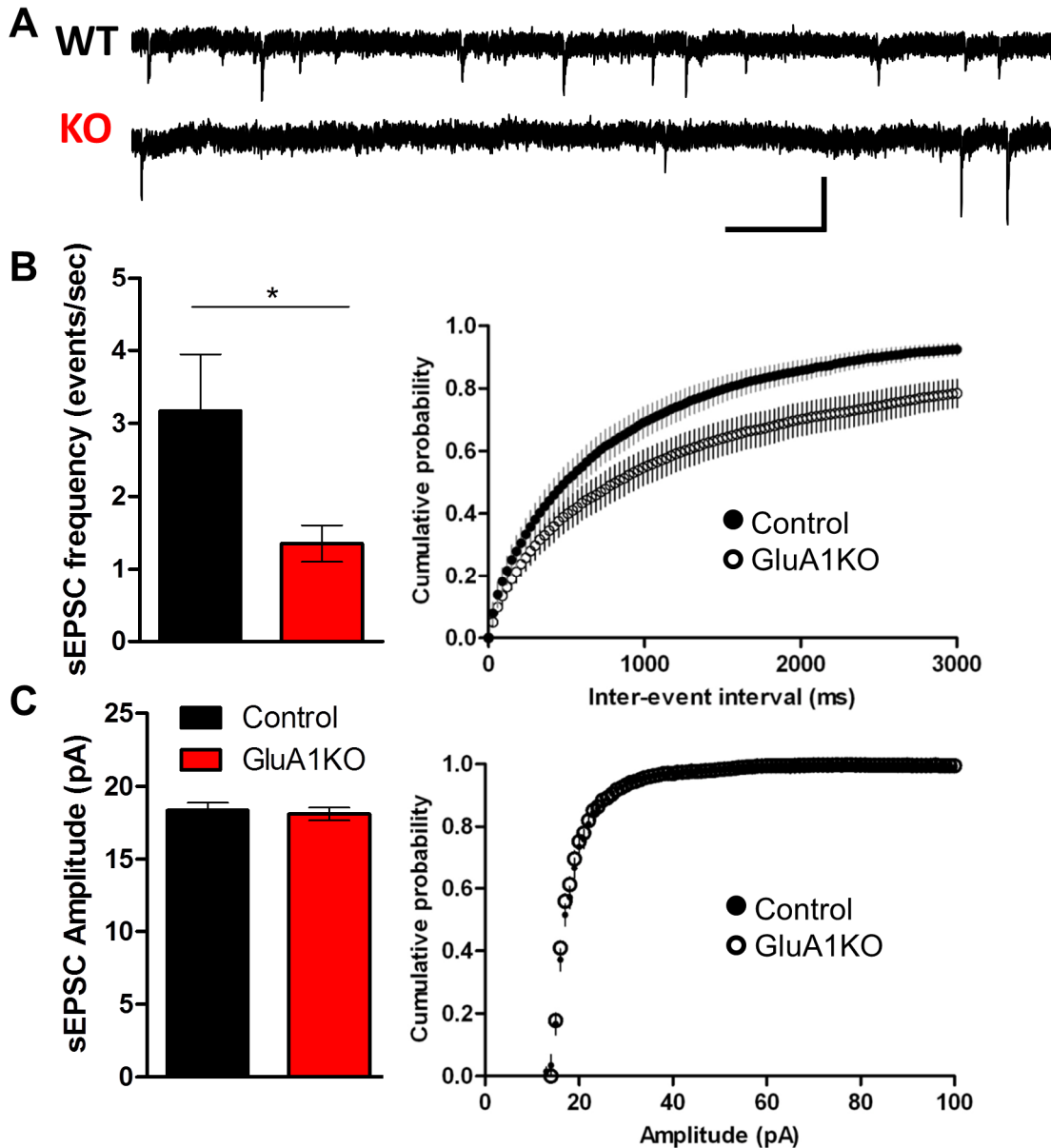
Figure 12. GluA1KO Mice Mimic the EAC3I Mice Decrease in sEPSC Frequency. (A) Example traces of sEPSCs collected from dorsal lateral striatum MSNs in Wt (top) and GluA1KO (bottom). Scale bars 0.5 sec, 30 pA (Control: n=15; GluA1KO: n=18; p=0.013). (B) (left) Average sEPSC frequency in GluA1KO versus controls. (right) Cumulative probability graph of inter-event interval. (C) (left) Average sEPSC amplitude in GluA1KO versus controls. (right) Cumulative probability graph of amplitude (Control: n=15; GluA1KO: n=18; p=0.72). *$P < 0.05$; error bars represent SEM. All MSNs were held at -70mV. Thank you to Brian Mather for data collection.

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MSN CaMKII inhibition leads to enhanced intrinsic excitability

      To further examine the impact of CaMKII inhibition on physiological responses of dorsal striatal MSNs, we next examined excitatory drive and excitability of these cells under current clamp conditions. As expected from our voltage clamp experiments with sEPSCs, we also observed a robust decrease in the frequency of sEPSPs in the CaMKII inhibited cells versus control neurons in current clamp (in Hz, Wt 3.18±0.29; tTA 3.09±0.06; NON EGFP 3.04±0.33; EGFP 1.78±0.18) *[F (3, 19) =4.704; p=0.0154; Figure 13A, 13C].* Surprisingly, however, we also observed a significant increase in sEPSP amplitude in the CaMKII-inhibited cells versus controls (in mV, Wt 0.48±0.05; tTA 0.47±0.10; NON EGFP 0.55±0.05; EGFP 0.77±0.09) [*F (3, 19) =3.605; p=0.0367; Figure 13A, 12D].* As similar effects were not observed with sEPSC amplitudes, this suggested a change in excitability of EAC3I neurons. In order to further test this idea we measured basal intrinsic excitability. EGFP cells possessed a significantly more depolarized resting membrane potential (in mV, Wt -86.30±0.50; tTA -85.61±0.50; NON EGFP -85.52±0.38; EGFP -83.61±0.68) *[H (3, 78) =8.588; p=0.0353; Figure 13E]* and had significantly increased input resistance compared to control cells (in MΩ, Wt 75.85±9.90; tTA 91.98±6.22; NON EGFP 70.48±5.54; EGFP 122.65±13.81) *[H (3, 79) =19.89; p=0.0002; Figure 13F].* This suggests that CaMKII inhibition moves the resting membrane potential closer to firing threshold and increases membrane resistance to enhance the propagation of depolarizing current from distal MSN dendrites. Next

we examined the voltage responses to differing current injections. While injecting minimal current to maintain resting membrane potential at -85 mV, a series of hyperpolarizing and depolarizing current injections were given in 20 pA steps. The threshold for 1st AP or rheobase current injection was significantly lower in the CaMKII inhibited MSNs versus control (in pA, Wt 357±32; tTA 285±28; NON EGFP 357±30; EGFP 194±17) *[F (3, 78) =9.393; p<0.0001; Figure 13G].* The firing threshold was not significantly different amongst groups (in mV, Wt -36.4±1.3; tTA -35.3±1.5; NON EGFP -33.4±0.8; EGFP -37.0±0.9) *[F (3, 78) =2.516; p=0.0649],* suggesting that input resistance changes are a major contributor to changes in rheobase. Also CaMKII inhibited cells exhibited increased spiking over a range of suprathreshold current injections *[F (3,60) =5.425; p=0.0023; Figure 13H]* versus control, reflecting a decrease in the interspike interval. Taken together, these data show that CaMKII inhibition enhances the intrinsic excitability of MSNs.
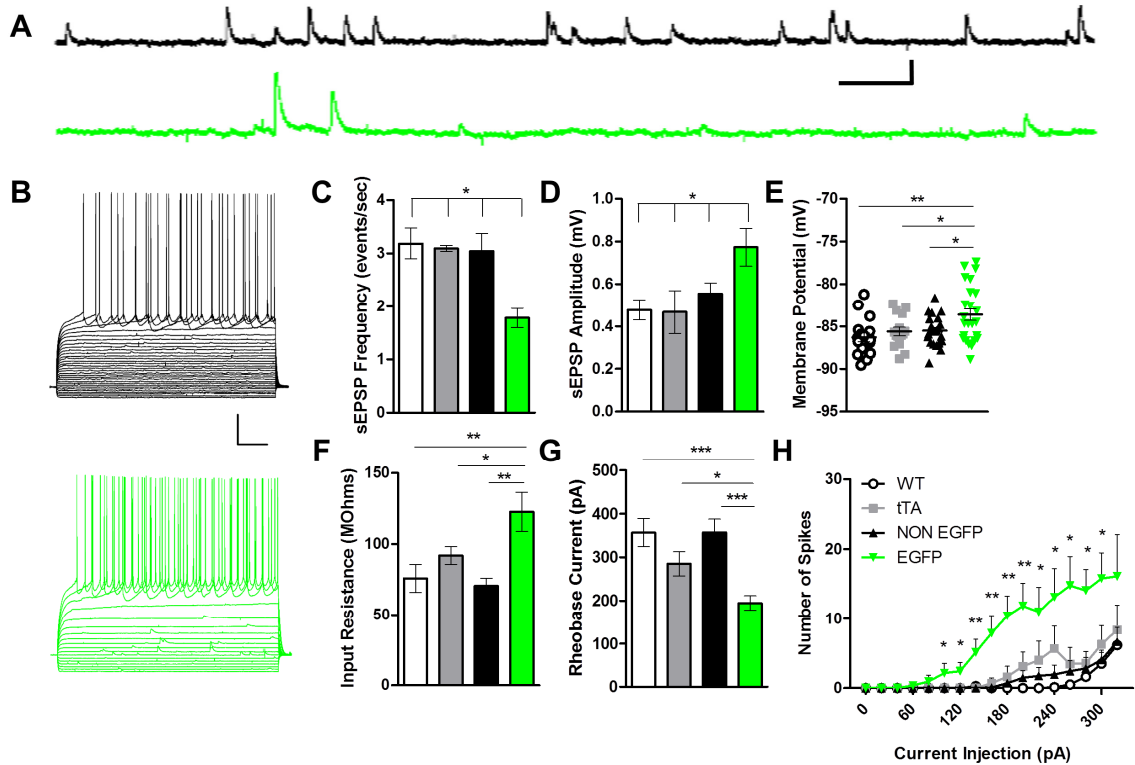
Figure 13. CaMKII inhibition enhances MSN intrinsic excitability. (A) Traces of EAC3I-lacking (NON EGFP, black) and EAC3I-containing MSNs (EGFP, green) sEPSPs recorded at -85 mV. Scale bars 0.6 mV, 200 ms. (B) Traces of EAC3I-lacking (black) and EAC3I-containing MSNs (green) with 20pA hyperpolarizing and depolarizing current injections (-120 pA to +100 pA above AP threshold, 20 pA steps). Scale bars 200 ms, 20 mV. (C) Average sEPSP frequency in EAC3I-containing MSNs versus controls (Wt: n=4; tTA: n=4; NON EGFP: n=9; versus EGFP: n=6; p=0.015). (D) Average sEPSP amplitude (current clamped at -85 mV) in EAC3I-containing MSNs versus controls (Wt: n=4; tTA: n=4; NON EGFP: n=9; versus EGFP: n=6; p=0.037). (E) Resting membrane potential (RMP) (mV) of EAC3I-containing and control MSNs (Wt: n=18; tTA: n=15; NON EGFP: n=22; versus EGFP: n=24; p=0.0043). (F) Input resistance of EAC3I-containing and control MSNs (Wt: n=18; tTA: n=15; NON EGFP: n=22; versus EGFP: n=24; p=0.0002). (G) Rheobase current injection or current injection to reach 1st AP in EAC3I-containing and control MSNs (Wt: n=18; tTA: n=15; NON EGFP: n=22; versus EGFP: n=24; p<0.0001). (H) Firing frequency (Hz) after 4 sweeps (20 pA steps) following threshold firing in EAC3I-containing and control MSNs (Wt: n=18; tTA: n=15; NON EGFP: n=22; versus EGFP: n=24; p=0.0023). *P <0.05; **P <0.01; ***P < 0.001; error bars represent SEM.

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Discussion

We present converging lines of evidence that dorsal striatal MSN CaMKII inhibition decreases functional synapse number and increases intrinsic excitability. Inhibition of CaMKII in MSNs leads to a decrease in sEPSC frequency, without a change in release probability, glutamate levels at the synaptic cleft or dendritic spine density. These observations are consistent with a decrease in the number of functional synapses. In addition to changes in excitatory transmission, inhibition of CaMKII leads to an enhancement of MSN intrinsic excitability. These data suggest that CaMKII coordinates opposing regulation of excitatory transmission and intrinsic excitability in MSNs, serving as a cellular rheostat.

CaMKII inhibitors such as KN62 and KN93 have been useful tools in probing CaMKII functions, but these drugs also inhibit voltage-gated K^+ and Ca^{2+} channels (Li et al., 1992; Ledoux et al., 1999), and do not inhibit the autonomous activity of Thr286-autophosphorylated CaMKII (Tokumitsu et al., 1990; Sumi et al., 1991). In addition, in dendritic spines where the concentration of calmodulin and CaMKII are extremely high (~100μM) (Faas et al., 2011; Feng et al., 2011), KN-62 (10 μm) only partially decreases CaMKII activity (Lee et al., 2009). The EAC3I peptide we used inhibits all isoforms of CaMKII, including CaM-stimulated and autonomous activity, with low micromolar potency (Braun and Schulman, 1995; Chen et al., 2001; Zhang et al., 2005). EAC3-I is ≥100-fold selective for CaMKII over protein kinase C, CaM Kinase I or CaM kinase IV (Braun and

Schulman, 1995; Patel et al., 1999). The AC3-I peptide sequence differs from another highly selective CaMKII-inhibitor, AIP, by only one amino acid residue (Vest et al., 2007). It is also important to consider the localization of CaMKII inhibition when interpreting these results. CaMKII is highly expressed in dopamine terminals, which densely innervate the striatum, where it stimulates dopamine efflux via the dopamine transporter in the presence of amphetamine (Fog et al., 2006). In addition, CaMKII is present in glutamatergic projections, which form the presynaptic terminal onto MSN spines and dendrites (Liu and Jones, 1996; Fog et al., 2006), where it may modulate release events (Chi et al., 2001; Elgersma et al., 2002; Hojjati et al., 2007; Jiang et al., 2008). Our transgenic strategy resulted in the selective expression of the CaMKII inhibitor in the postsynaptic MSN, where it cannot directly affect the function of the glutamatergic and dopaminergic terminals, consistent with the lack of change in glutamate release parameters in EAC3I MSNs (Figures 9, 10).

We demonstrated that CaMKII inhibition decreases s/mEPSC frequency with no changes in presynaptic function. The lack of a bimodal distribution in the s/mEPSC frequency data suggests that CaMKII inhibition similarly affect both direct and indirect pathway MSNs. Changes in s/mEPSC frequency are traditionally interpreted as alterations in presynaptic quantal content; the product of changes in release probability or synapse number. However, multiple lines of evidence suggest that presynaptic function is unaltered. Together these data suggest that CaMKII inhibition decreases functional synapse number. One

possibility is that CaMKII inhibition produces a loss of functional synaptic connections. Alternatively, there could be an increase in the number of silent synapses which contain NMDARs but no AMPARs and are typically abundant early in development (Liao et al., 1992; Isaac et al., 1995; Liao et al., 1995; Durand et al., 1996; Wu et al., 1996; Kerchner and Nicoll, 2008). A further possibility is that CaMKII inhibition could increase the numbers of silent modules of synapses or increase the number of AMPAR-lacking subregions of the synapse due to local nature of basal synaptic transmission (Raghavachari and Lisman, 2004; Lisman and Raghavachari, 2006). In the hippocampus, silent synapses can be unsilenced following NMDAR activation by the introduction of new AMPAR to the synapse, underlying a common mechanism of LTP of synaptic glutamatergic transmission (Shi et al., 1999; Hayashi et al., 2000; Park et al., 2004; Adesnik et al., 2005). Previous research has suggested a direct role for CaMKII in the unsilencing of synapses in the hippocampus (Pettit et al., 1994; Lledo et al., 1995; Shirke and Malinow, 1997; Pi et al., 2010a). Additionally, CaMKII is required for the formation of new synapses and/or morphological growth following hippocampal LTP induction (Toni et al., 1999; Jourdain et al., 2003; Lee et al., 2009; Ciani et al., 2011a).

Striatal CaMKII inhibition did not alter dendritic spine density, suggesting that the decrease in s/mEPSC frequency could best be explained by some spines lacking active presynaptic terminals or increased numbers of silent synapses. However, the significant increase in the input resistance of EAC3I-

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positive MSNs should enhance sampling of mEPSCs from more distal dendritic sites, potentially increasing s/mEPSC frequency. These data together suggest that decreases in s/mEPSC frequency in EAC3-I MSNs are potentially underestimated. Alternatively, some of these effects may be due to decreased dendritic length and complexity seen in EAC3I-positive MSNs. However, it is not clear whether distal MSN synapses are sampled in our s/mEPSC analyses due to cable filtering effects previously reported (Williams and Mitchell, 2008) . Hippocampal βCaMKII has been shown to modulate dendritic length and branching as well as synapse number (Fink et al., 2003). These results suggest that CaMKII plays important roles in modeling MSN dendritic morphology. The alpha CaMKII promoter fragment that drives EAC3I expression reportedly turns on around P5 (Kelly et al., 1987; Sugiura and Yamauchi, 1992, 1994a, b). This would lead to inhibition of CaMKII in early postnatal development and continuing into adulthood. The effects of this longer term genetic CaMKII inhibition contrasts with the typically minimal effects of acute, short term application of a related CaMKII inhibitor peptide, AIP, on basal glutamatergic transmission in the CA1 and CA3 region of the hippocampus (Sharma et al., 2008; Buard et al., 2010; Shen et al., 2010; Ciani et al., 2011b). Conversely, expression of CaMKII inhibitor peptides CaMKIIN or AIP over 2-6 days reduced hippocampal CA1 AMPAR-mediated, but not NMDAR-mediated EPSCs (Goold and Nicoll, 2010). Another report suggests a CaMKII inhibitor peptide, CN19, persistently decreased hippocampal CA1 field EPSPs or EPSCs amplitudes at higher concentrations disrupting the CaMKII/NMDAR complex (Sanhueza et al., 2011).

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These data suggest that disruption of the CaMKII/NMDAR complex, a complex that increases following strong synaptic stimulation (Leonard et al., 1999) and is necessary for LTP (Barria and Malinow, 2005; Zhou et al., 2007), may offer an alternative mechanism underlying our observed effects.

Endogenous CaMKIIα levels peak around 3 weeks postnatally, which is a crucial time for synaptogenesis and synapse maturation (Hanley et al., 1987; Kelly et al., 1987; Sugiura and Yamauchi, 1992). The apparent decrease in functional synaptic connections in CaMKII inhibited MSNs in adulthood may have four possible explanations: 1) CaMKII activity is necessary for the normal unsilencing of synapses in the adult; 2) Ongoing CaMKII activity is required to maintain functional synapses; or 3) CaMKII is needed early in synaptogenesis to turn initially silent synapses into functional ones; or 4) the CaMKII-mediated decrease in dendritic length may underlie the reduction in total synapse number. The decrease in sEPSC frequency in EAC3I MSNs was detected as early as three weeks postnatally, suggesting a large proportion of synapses were never unsilenced or perhaps never formed. In the hippocampus both electrophysiological and anatomical studies at light and electron microscopic levels suggest that in the first few weeks of life many synapses start as NMDAR-only synapses or silent synapses (Rao and Craig, 1997; Gomperts et al., 1998; Nusser et al., 1998; Petralia et al., 1999; Takumi et al., 1999). Synapse unsilencing involving the trafficking of new AMPARs (GluA4-containing) to the synapse in early postnatal development is dependent on activity, but is

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independent of CaMKII (Zhu et al., 2000; Esteban et al., 2003). Instead, PKA plays an important role early in postnatal development (<P9) in plasticity in the hippocampus being necessary and sufficient for GluA4 incorporation, but requiring additional CaMKII activity for GluA1 receptor incorporation (Esteban et al., 2003; Yasuda et al., 2003; Man et al., 2007). These data suggest that the similar synaptic phenotypes of GluA1KO and EAC3I mice arise from disruption of a common mechanism. However, it is interesting that the phenotype of the EAC3I cells is also virtually identical to that recently reported for MSNs in SAPAP3 knockout mice (Chen et al., 2011b; Wan et al., 2011). Intriguingly, SAPAP3 may be phosphorylated by CaMKII, possibly assisting in synaptic targeting of GluA1R (Dosemeci and Jaffe, 2010). Thus, it will be important for future studies to directly identify downstream MSN proteins regulated by CaMKII.

It is also possible that differences in the relative innervation of MSNs by cortical and thalamic inputs impacts the synaptic phenotypes. Indeed, differences in release probability have been observed between the two inputs (Smeal et al., 2007; Ding et al., 2008; Ding et al., 2010). Our measures of sEPSC and mEPSC frequency are likely comprised of both cortical and thalamic-mediated glutamate release, yet our data do not rule out the possibility that inhibition of MSN CaMKII may have a greater influence on one of these excitatory synapses over the other.

CaMKII inhibition also leads to alterations in intrinsic excitability that may serve to broadly counteract the reduced s/mEPSC frequency. Although we expected a decrease in sEPSP frequency in current clamp based on the sEPSC results, we observed significantly larger sEPSP amplitudes. This is likely due to enhanced intrinsic excitability. In CaMKII-inhibited neurons we observed more depolarized resting membrane potentials, increased membrane resistance, decreased rheobase current injection, and increased firing frequency. Autonomously active CaMKII has been shown to suppress neuronal excitability by increasing cell-surface expression of an A-type K^+ channel, Kv4.2, via phosphorylation (Roeper et al., 1997; Park et al., 2002; Varga et al., 2004). In addition, CaMKII inhibition in medial vestibular nucleus neurons increased intrinsic excitability via a reduction in BK-type calcium activated potassium currents (Nelson et al., 2005). Changes in Kv4.2 or BK activity following CaMKII inhibition could account for the differences in firing that we observed in EAC3I cells. However, modulation of Kir 2 channels may be responsible for the changes in input resistance and resting membrane potential (Cazorla et al., 2012). A recent study showed that acute CaMKII inhibition in cortical cultures leads to increased excitability, but also increased cell death (Ashpole et al., 2012a). We did not note increased cell death, nor did membrane properties hint at unhealthy EAC3I-expressing cells. The differences in these studies may be attributed to the fact that MSNs are GABAergic cells bypassing potential excitotoxicity vulnerabilities seen with recurrent excitatory connections in the cortex. Alternatively, cortical cultures – which are often more excitable - may

have a more difficult time regulating extracellular glutamate levels, something that is not as problematic in *ex vivo* slice preparations.

The opposing regulation of excitatory transmission and excitability observed in these studies suggests that CaMKII may serve as a molecular fulcrum to counterbalance changes in enhanced excitatory input with decreases in excitatory output. It is important to note in the present dataset we cannot rule out that possibility that one of these adaptations is compensatory to the other, rather than both being directly initiated by CaMKII inhibition. Regardless, this likely has important implications for the modulation of basal ganglia circuitry underlying habit learning, addiction and neurodegenerative disease. CaMKII plays a role in setting the number of functional synapses and therefore may provide a substrate for experience dependent plasticity in the striatum. Dorsal striatal CaMKII may be crucial early in postnatal development as well as in adulthood entraining new motor repertoires and refining synaptic connections as those motor skills are refined into habits later in life. With the inhibition of CaMKII leading to a decrease in the number of functional contacts, CaMKII may function in the dendritic and synaptic maturation processes, from nascent filopodia to mature dendritic spine (Jourdain et al., 2003; Lee et al., 2009), or alternatively be important in maintaining existing synaptic connections. Further investigation will be needed to determine the precise role of CaMKII in striatal synaptic maturation.

CHAPTER IV

MOSAIC GENETIC INHIBITION OF CAMKII IN DORSAL STRIATAL MEDIUM SPINY NEURONS AFFECTS STRIATAL-BASED BEHAVIORS

Introduction

CaMKII is highly expressed in the striatum, the main input nucleus of the basal ganglia, and it is known to interact and phosphorylate numerous downstream substrates playing an important role in synaptic plasticity and learning (Erondu and Kennedy, 1985; Lisman et al., 2002). CaMKII abundance and localization places it in a position to control corticobasal ganglia circuits that play a role in voluntary movements and motivated behaviors. While an important role of CaMKII in hippocampal-mediated learning is well known (Lisman et al., 2012), little is known of its role in striatal learning. The dorsal striatum has been implicated in the formation of goal-directed and habit based learning as well as the formation of action sequences (Yin and Knowlton, 2006; Robbins et al., 2008). Additionally, this region is implicated in neurodegenerative diseases, like Huntington's and Parkinson's disease, as well as obsessive compulsive disorder, Tourette's, dystonia and addiction.

Striatal-dependent behaviors in mice can be studied using instrumental and non-instrumental learning paradigms. Instrumental learning paradigms require an animal to perform an operant behavior, like pressing a lever for a food reward. Differing operant schedules can promote differing types of behavioral learning. A random ratio schedule, where an animal is rewarded on a

percentage of total lever presses, is known to promote goal-directed learning (Hilario and Costa, 2008). Under certain conditions, like overtraining or using random interval schedules, habitual behavior develops. Goal-directed learning is sensitive to changes in the value of the reinforcer, while habitual learning is insensitive to devaluation of the reinforcer (Yin et al., 2004, 2006). This switch from flexible, goal-directed actions which involve the associative corticobasal ganglia network to a more rigid habitual action governed by the sensorimotor network is thought to involve synaptic plasticity at glutamatergic synapses in the dorsal striatum (Yin and Knowlton, 2006; Yin et al., 2009).

Hippocampal CaMKII is known to be both necessary and sufficient for the induction of long-term potentiation (LTP) of glutamatergic transmission, a neural correlate of learning and memory. Disruption or removal of CaMKII in the hippocampus disrupts spatial learning and working memory (Lisman et al., 2012). Differing forms of plasticity are thought to be involved in striatal-based learning; we hypothesized that CaMKII activity in medium spiny neurons of the striatum is necessary for motor learning, goal-directed and habitual behavior. To test this hypothesis, we ran transgenic mice expressing a CaMKII-inhibitory peptide (EAC3I) only in medium spiny neurons in the striatum through numerous behavioral tasks. We examined open field locomotor activity, motor learning on the rotarod, fixed ratio and random ratio responding for food reward in EAC3I transgenic mice and their transgene lacking littermates. Additionally, we used devaluation and contingency degradation to determine if CaMKII plays a role in goal-directed or habit based learning. If an action is habitual, then devaluation of

the reinforcer or breaking up the contingency between the action and the reward outcome by offering "free" rewards should have no effect on performance. Conversely, if the behavior is goal-directed then performance should be reduced following devaluation or contingency degradation. We observed that inhibition of CaMKII did not disrupt rotarod motor learning or behavioral sensitization to cocaine, yet disrupted goal-directed behaviors and prevented the goal-directed to habit learning transition.

Results

<u>Effects of dorsal striatal CaMKII inhibition on locomotion in the novel open field</u>

Rodents placed into a novel environment will initially exhibit elevated locomotor activity as they explore their new surroundings which then decreases over time as they habituate to the environment (Wiedholz et al., 2008). We examined both EAC3I mice and controls in a novel open field where locomotor activity was tracked over a sixty minute session. While both groups habituated over the sixty minute period, EAC3I double transgenic mice showed reduced total locomotor activity compared to wildtype and EAC3I single transgene containing mice. Similar results were observed when locomotor activity was separated into five minute bins [$F(3,23)=7.282$, $p=0.0017$; Figure 14A,14B]. However, single tTA mouse locomotor activity was equivalent to double transgenic EAC3I mice, suggesting that inclusion of the tTA transgene leads to a hypolocomotor phenotype as reported previously (McKinney et al., 2008). Additionally, there were no statistical differences in time spent in the center of the

open field, a crude measure of overall anxiety, between EAC3I mice and controls

[$F_{(3,23)}=2.121$, $p=0.13$; Figure 14C] (Pogorelov et al., 2005).
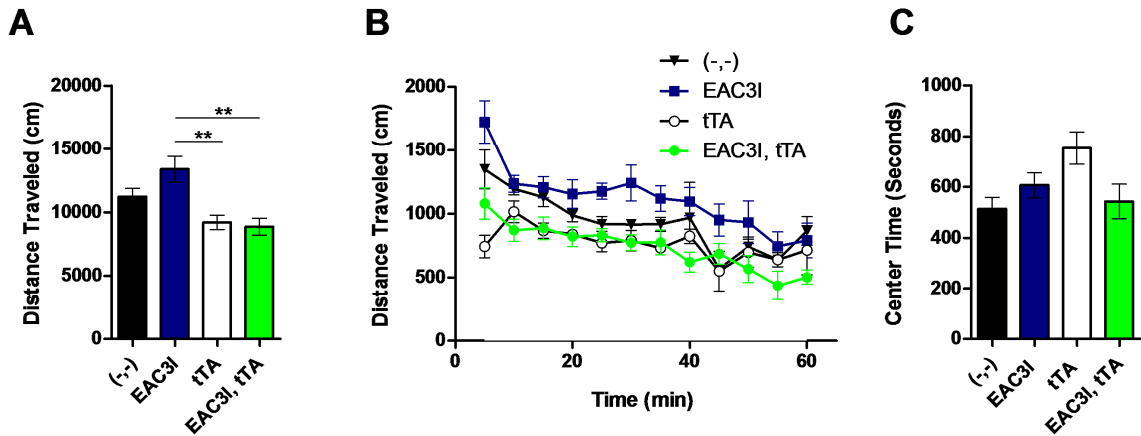
Figure 14. EAC3I Mouse Locomotor Activity and Center Time in the Novel Open Field. (A) Total distance traveled (cm) in the novel open field over 60 minutes. (B) Total distance traveled plotted in 5 minute bins over 60 minutes. (C) Amount of time spent in a 4X4 inch square in the center of the open field area. More time in the center is associated with reduced anxiety. ** = $p<0.01$, Error bars represent SEM.

Mosaic inhibition of dorsal striatal CaMKII does not affect simple motor learning

      To examine simple motor learning often a rotarod test is used consisting of an accelerating rotating bar that mice must maintain their balance. Mice performing in a rotarod task undergo rapid improvement in performance in early trials with more gradual improvements as performance stabilizes and the motor task is well learned. During the early learning phase of this task the dorsal medial striatum is thought to be engaged, but later once the skill is automatized the dorsal lateral striatum predominates (Yin et al., 2009). Previously, motor

learning on the accelerating rotarod was shown to be disrupted by striatal-

specific disruption of the GluN1 subunit of the NMDAR (Dang et al., 2006;

Beutler et al., 2011). CaMKII activation lies downstream of NMDAR activation,

so we tested the hypothesis that inactivation of dorsal striatal CaMKII would

disrupt motor learning on the accelerated rotarod. EAC3I and controls were run

on the accelerating rotarod for multiple trials to test for motor learning.

Surprisingly, both groups exhibited similar learning curves over the multiple trials,

suggesting normal motor learning in the EAC3I mice *[F(1,68) =0.7369; p=N.S.;*

Figure 15A]. This lack of an effect in behavior could be due to the mosaicism of

expression of the CaMKII inhibitory peptide throughout the dorsal striatum with

other non-expressing MSNs compensating for the loss of CaMKII. Alternatively,

striatal NMDARs may also have roles in rotarod motor learning outside of CaMKII

signaling.
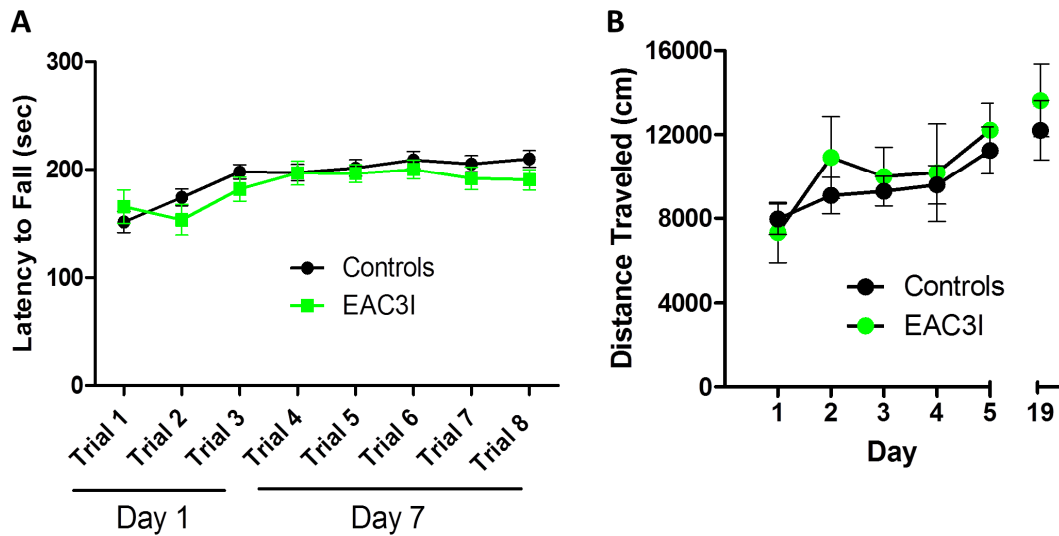
Figure 15. Mosaic Dorsal Striatal CaMKII Inhibition Does Not Alter Rotarod Motor Learning or Behavioral Sensitization to Cocaine. (A) Latency to fall on the accelerating rotarod measured over eight trials on two days separated by a week in EAC3I mice versus combined controls. (B) Cumulative distance traveled over 30 minutes following a 10mg/kg cocaine injection (i.p.) over five consecutive days followed by a challenge injection given two weeks after the fifth injection in EAC3I versus combined controls. Note mice are habituated to the locomotor chambers for thirty minutes prior to injection of cocaine. *P <0.05; ** P <0.01; *** P < 0.001; error bars represent s.e.m.

Behavioral sensitization by cocaine is normal in EAC3I mice

Behavioral sensitization produced by cocaine is dependent on dopaminergic projections from the ventral tegmental area (VTA) to the nucleus accumbens (NAc) (Thomas et al., 2008; Schmidt and Pierce, 2010; Wolf and Ferrario, 2010). Injection of a CaMKII inhibitor into the VTA enhances acute cocaine locomotor activity while reducing the expression of behavioral sensitization, indicating that inhibition of CaMKII affects both the acute and chronic locomotor effects of cocaine(Licata et al., 2004). Expression of the CaMKII inhibitory peptide is considerably reduced in the nucleus accumbens of

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EAC3I mice. As expected there was no difference in the degree of behavioral

sensitization to repeated cocaine injections (10mg/kg, i.p.) in EAC3I versus

control mice over five days, nor was there a difference in locomotor activity

following a challenge dose of cocaine 14 days later *[F(1,28) =1.161; p=N.S.;*

Figure 15B].

EAC3I mice have deficits in random ratio-supported operant behavior

We next tested the hypothesis that striatal CaMKII plays a role in goal-

directed learning. We used escalating random ratio (RR) operant schedules that

have been shown to be associated with goal-directed learning (Packard and

Knowlton, 2002; Yin and Knowlton, 2006; Wiltgen et al., 2007; Hilario and Costa,

2008; Dias-Ferreira et al., 2009). We trained EAC3I and control animals to lever

press under fixed ratio responding (FR-1) for highly palatable food (25% Ensure).

We initially trained them on FR-1 for six days where mice had access to both an

active lever and an inactive lever. There was no significant difference between

EAC3I and control mice in active or inactive lever pressing during FR-1 training

[active F(1,35) =2.506; p=N.S.; inactive F(1,35) =0.3947; p=N.S.; Figure 16A]

suggesting that operant learning and activity levels are similar.

Following six days of FR-1 training the mice were switched to escalating RR

schedules where a lever press equated to a probability of reinforcement of the

Ensure reward. RR schedules have been shown previously to promote the

acquisition of goal-directed behavior (Hilario et al., 2007). Animals were trained

for four days on a RR5 schedule of reinforcement (probability of reinforcement,

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P=0.2), followed by four days on a RR10 schedule (probability of reinforcement, P=0.1), and then four more days on a RR20 schedule (probability of reinforcement, P=0.05). With the increasing work load over the increasing random ratio schedules the EAC3I mice showed increasing deficits in random ratio responding *[RR-5, F(1,35) =19.27 p<0.0001; RR-10, F(1,35) =9.787 p=0.0026; RR-20, F(1,35) =10.85 p=0.0016; Figure 16B].* These data together suggest that inhibition of dorsal striatal CaMKII leads to deficits in a model of goal-directed learning. As an additional control for differences in reward sensitivity, we tested food conditioned place preference (CPP). Following four days of conditioning 25% ensure on a designated paired side of the chamber twice a day, both EAC3I and control mice showed similar levels of CPP on a probe test [*t(14) =1.108; p=N.S.; Figure 17*]. This suggests that the rewarding properties of ensure was similar across genotypes.

EAC3I mice show greater devaluation and contingency degradation

In order to investigate if lever pressing following a RR-20 schedule was goal-directed or habitual we performed a devaluation and contingency degradation tests. The data were normalized to the last day of RR-20 responding. During the devaluation test, EAC3I mice responded significantly less during the devalued condition, when the outcome they pressed for during training was devalued by sensory-specific satiety (Ensure), than during the non-devalued condition (chow) [F(1,33)=2.180; p<0.05; Figure 18A], suggesting greater goal-directed behavior. Although it seems unlikely that the previous

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results are were driven by differences in hedonics or value processing, we used a different task to determine if the EAC3I mice had impairments in performing actions based off the consequences of their behavior. We performed contingency degradation where the delivery of reward was given non-contingent to lever pressing. Under extinction conditions the EAC3I mice showed greater reduction in responding in the first session under contingency degradation suggesting greater goal-directed behavior [$F_{(1,33)}=3.636$; $p<0.05$; Figure 18B]. In all these data suggest that EAC3I mice are operating in a goal-directed manner after extended training, with CaMKII inhibition possibly disrupting the natural transition from goal-directed to habit based learning.
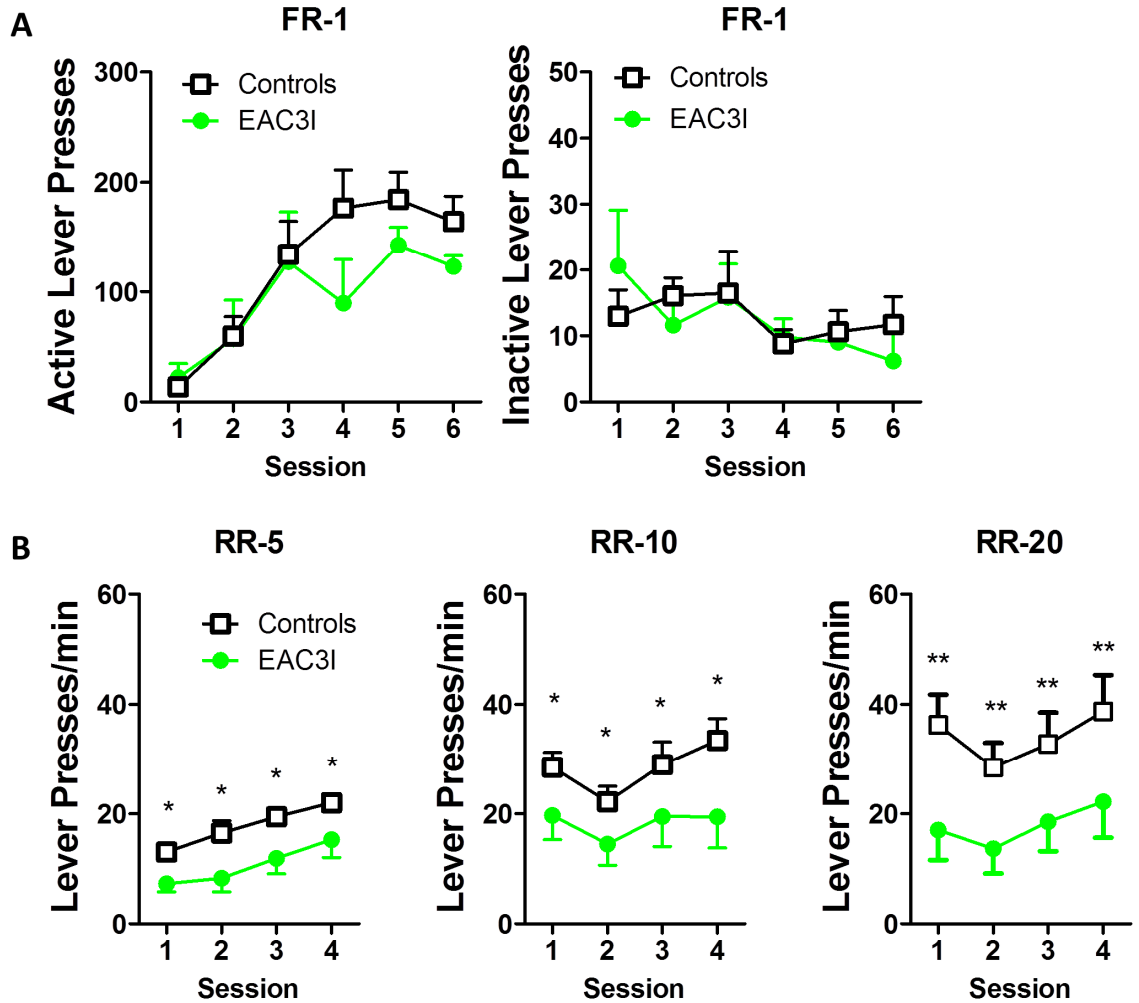
Figure 16. EAC3I Mice have Deficits in Responding on a Random Ratio Schedule for Food. (A) Number of active lever presses (left) and inactive lever presses (right) for EAC3I versus combined controls on a fixed ratio-1 schedule for 25% Ensure over six, 30 minute sessions. (B) Rate of lever presses per minute on a random ratio schedule (RR-5, RR-10, and RR-20; 20%, 10%, 5% chance of reinforcement, respectively) over four daily, 30 minute sessions. Note the enhanced deficit in EAC3I mice versus controls over increasing random ratio schedules. *P <0.05; ** P <0.01; *** P < 0.001; error bars represent s.e.m. Thank you to Chris Olsen and Erin Watt for data collection.

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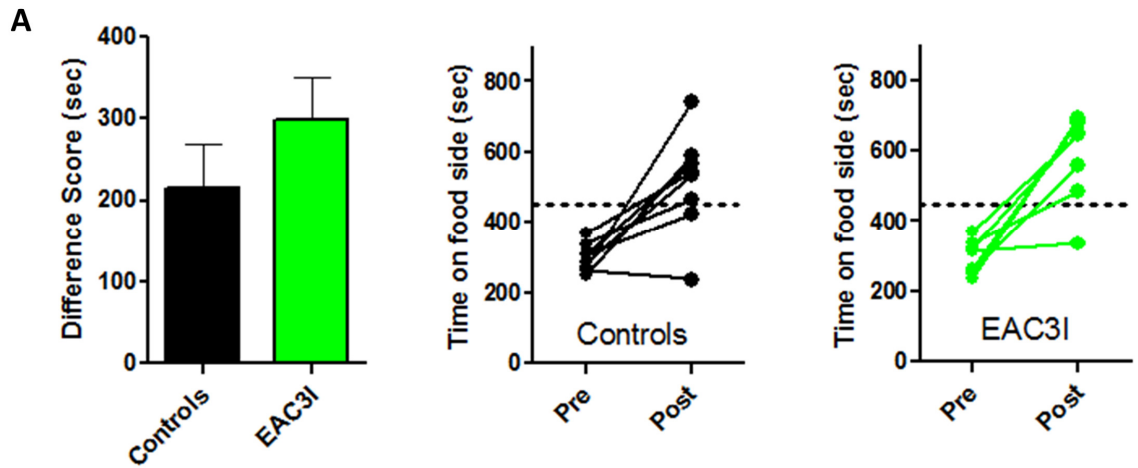
Figure 17. EAC3I mice show normal conditioned reinforcing responses to food in a CPP paradigm. (A) (left) Difference score between paired ensure side to non-paired side in EAC3I mice and combined controls. (middle) Time on food side pre and post CPP training in control mice. (right) Time on food side pre and post CPP training in EAC3I mice. Thank you to Chris Olsen for data collection.

A Devaluation

B Contingency Degradation
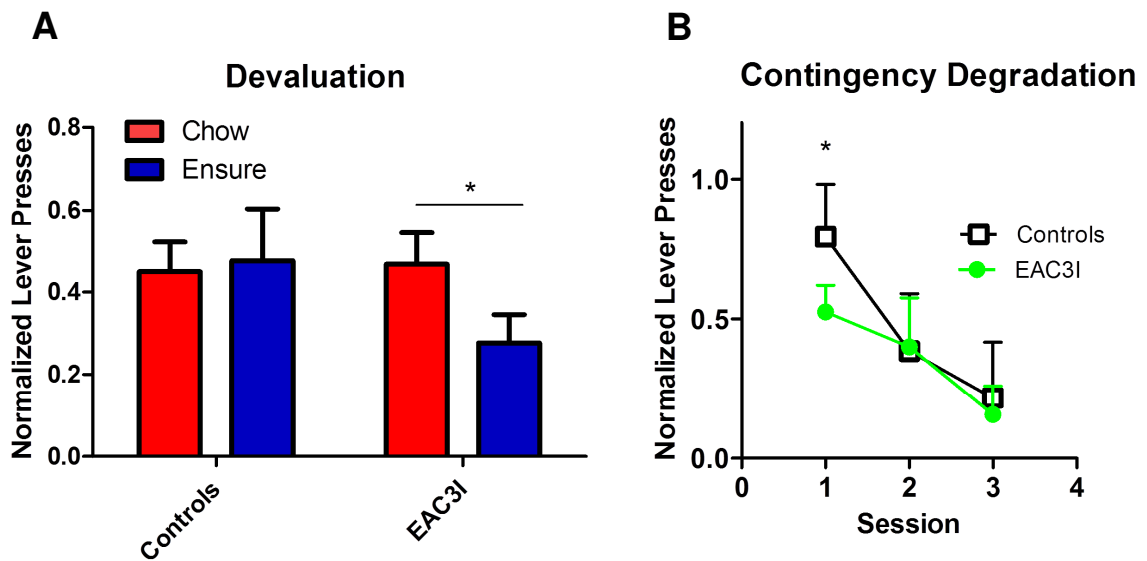
Figure 18. EAC3I mice show greater devaluation and contingency degradation. (A) Normalized lever presses to last RR-20 day in grouped controls (Wt and single transgenics) and EAC3I mice given 1 hour pre-feeding with Ensure (devalued) or chow (valued, control). Note greater devaluation in EAC3I group in the reinforcer specific Ensure group. (B) Normalized lever presses to last RR-20 day in grouped controls (Wt and single transgenics) and EAC3I mice following extinction conditions with non-contingent reward delivery. Note greater contingency degradation in EAC3I mice on the first session. *p<0.05, Error represents SEM.

Discussion

The expression of behavioral sensitization is thought to be dependent on the nucleus accumbens (Thomas et al., 2008). Since there was little to no expression of EAC3I in the nucleus accumbens, normal behavioral sensitization to repeated cocaine administration in the EAC3I mice is not unexpected. While previous reports suggest that rotarod motor learning is dependent on the striatal expression of GluN1 subunits of the NMDAR (Dang et al., 2006), we did not encounter any deficits in motor learning on the rotarod in the EAC3I mice versus controls. We hypothesize CaMKII inhibition in only a subpopulation of MSNs along with the simplicity of the motor task allows other non-inhibited MSNs to compensate and maintain normal motor learning. Alternatively, this motor learning task may require signaling downstream of the NMDAR that does not rely on CaMKII.

The everyday process of turning goal directed responses, requiring concentration and monitoring of the response, into an automatic habit is one way we balance the need for behavioral flexibility for efficiency of repeated motor scenarios. It is known that the cortico-basal ganglia circuits underlie normal goal-directed and habit based learning. The associative cortical inputs to the dorsal medial striatum are implicated in the formation and execution of goal directed actions, while the dorsal lateral aspect of the striatum is necessary for the formation of habits (Balleine and Dickinson, 1998; Yin et al., 2005b; Yin and Knowlton, 2006). Mosaic inhibition of CaMKII did not inhibit the acquisition of FR-1 responding for food and did not disrupt food CPP, suggesting that the

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EAC3I mice had similar reward sensitivity to controls in these tasks. However, following random ratio training, under a schedule that was previously shown to bias for goal-directed behavior (Hilario et al., 2007; Dias-Ferreira et al., 2009), EAC3I mice showed increasing deficits with increasing random ratio schedules. Additionally, the EAC3I mice showed greater devaluation and contingency degradation to the satiety-specific reinforcer suggesting the animals are responding in a goal-directed manner. In controls we did not observed these differences suggesting that habit learning has been engaged. Inhibition of MSN CaMKII may disrupt the natural goal-directed to habitual learning that takes place with extended skill training. Disrupted synaptic transmission combined with enhanced excitability of MSNs could underlie some of the deficits seen in random ratio responding, possibly disrupting normal goal-directed behavior and preventing or facilitating the normal transition to habit based learning. It begs the question why motor learning on the rotarod was intact, yet deficits in goal-directed behaviors were witnessed. Possibly goal-directed learning requires a larger local network of MSNs and inhibiting CaMKII in a minority of neurons prevents normal goal-directed actions from being established, while rotarod learning may be more diffusely stored requiring fewer neurons. Additional study will be needed to better understand the role of CaMKII in habit learning.

CHAPTER V

THE ROLE OF THE GLUN2B SUBUNIT OF THE NMDAR IN STRIATAL GLUTAMATERGIC SYNAPTIC TRANSMISSION AND ITS ROLE IN STRIATAL-BASED BEHAVIOR

Introduction

The dorsal striatum is an area of the brain that underlies the formation of goal-directed actions as well as the formation of habits (Yin and Knowlton, 2006). Its importance underlying normal, voluntary movements is underscored by neurodegenerative diseases such as Parkinson's and Huntington's disease, both of which disrupt synaptic transmission on MSNs and ultimately manifest behavioral deficits (Kreitzer and Malenka, 2008). More recently, additional attention has been given to the dorsal striatum's role in drug addiction, a maladaptive form of habit-based learning (Koob and Volkow, 2010). The GluN2B subunit of the NMDAR has received a lot of attention, being implicated in learning and memory, pain perception, stroke, feeding behaviors and numerous human neurological disorders including alcoholism, anxiety disorders, schizophrenia, Parkinson's disease, and Huntington's disease (Yashiro and Philpot, 2008; Wu and Zhuo, 2009; Gardoni et al., 2010; Chen et al., 2011a; Lai et al., 2011; Qiu et al., 2011; Raymond et al., 2011; Wang et al., 2011). The GluN2B subunit is highly expressed in the striatum (Chen and Reiner, 1996), yet its role in synaptic transmission, plasticity and behavior in this region is not well understood.

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The NMDA receptor is a heteromeric ligand-gated ion channel that passes

sodium, potassium and calcium as well as interacts with multiple intracellular

proteins. It is comprised of a combination of four subunits from seven known

subunits including obligatory GluN1 subunits (formerly NR1, ζ1), and some

combination of GluN2A-2D (formerly NR2A-2D or ε1-4), and/or GluN3A-3B

(formerly NR3A-3B) (Yashiro and Philpot, 2008). The predominant GluN2

subunits in the striatum are the GluN2A and GluN2B subunits, which in turn

dictate NMDAR-mediated EPSC kinetics as well as localization, binding partners,

and intracellular signaling (Traynelis et al., 2010). GluN2A and GluN2B subunits

are incorporated into a di-heteromeric (GluN1/GluN2A or GluN1/GluN2B) or in a

tri-heteromeric (GluN1/GluN2A/GluN2B), with around one-third of the subunits in

the tri-heteromer form in the adult hippocampus - yielding unique functional

properties (Al-Hallaq et al., 2007). Levels of GluN2B are high in most forebrain

regions in late embryonic development, but in the first weeks of postnatal

development GluN2B subunits are progressively replaced with GluN2A subunits

as the synapse matures (Sheng et al., 1994; Flint et al., 1997; Roberts and

Ramoa, 1999). This developmental switch from GluN2B to GluN2A is seen in

the striatum (Monyer et al., 1994; Wenzel et al., 1997). This GluN2B to GluN2A

switch has been shown to alter the threshold for synaptic plasticity induction and

has been shown to be bidirectionally regulated by activity and experience

(Quinlan et al., 1999; Bellone and Nicoll, 2007).

Studies using transgenic mice have lent some insight into the role GluN2B

plays in synaptic plasticity. Overexpression of GluN2B in the forebrain of

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transgenic mice leads to enhanced activation of NMDAR receptors enhanced

LTP to stimulation at both 10Hz and 100Hz. Additionally, GluN2B

overexpressing mice showed enhanced long term memory for novel objects ,

enhanced fear memory (contextual and cued), and enhanced spatial memory in

the Morris water maze (Tang et al., 1999). Alternatively, overexpression of

GluN2B c-terminal tails actually show reductions in LTP magnitude and exhibit

learning deficits, presumptively through disruption of the CaMKII-GluN2B

complex (Zhou et al., 2007).

While the role of GluN2B in synaptic plasticity in the hippocampus and

cortex has been studied, the contribution of GluN2B to NMDAR-mediated

synaptic plasticity and learning and memory remains a controversial field.

Conflicting reports propose different roles for hippocampal GluN2B in LTP and

LTD (Liu et al., 2004; Massey et al., 2004; Morishita et al., 2007). Understanding

the role of the GluN2B subunit has been clouded by nonselective inhibitors,

complex pharmacology (Neyton and Paoletti, 2006; Paoletti and Neyton, 2007)

and the fact that traditional GluN2B knock out animals die soon after birth

(Kutsuwada et al., 1996), To circumvent this issue and study the role of the

GluN2B subunit in the adult striatum, an area that has received much less

attention, we utilized a conditional GluN2BKO mouse line. The current work

explores excitatory glutamatergic transmission in the dorsal striatum in the

conditional GluN2BKO animal, dendritic spine density, as well as the effects of

GluN2B deletion on striatal-based behaviors. Additionally, we explore the effects

of deletion of GluN2B at early postnatal versus adult time points to better understand the effects of GluN2B on synaptogenesis and adult striatal circuits.

Results

Mouse Model and Validation of GluN2B deletion

We utilized a double transgenic mouse model where a fragment of the alpha CaMKII promoter drove expression of the tetracycline transactivator (tTA) and its expression product bound to the tetO promoter of CRE recombinase which would excise the floxed GluN2B gene (Wills et al., 2012) (Figure 19A). Overtly the mice did not exhibit gross morphological differences, yet the conditional GluN2B KO mice showed a strong trend for a decrease in weight [in grams, Controls 25.14 ± 0.76, GluN2BKO 22.65 ± 0.63; U(38)=111.5, p= 0.067; data not shown]. The alphaCaMKII promoter restricted the deletion of GluN2B to only the forebrain. Western blot analysis of 1mm tissue punches isolated from the dorsal lateral striatum revealed the virtual absence of GluN2B protein [percent of control, 1 ± 0.02; t(9)=6.298, p< 0.0001; Figure 19B], similar to other results utilizing a similar mouse model (von Engelhardt et al., 2008). Furthermore, consistent with previous studies utilizing GluN2BKO mice to examine hippocampal excitatory transmission (von Engelhardt et al., 2008; Brigman et al., 2010; Wills et al., 2012), we observed a significant decrease in the decay time of evoked NMDAR-mediated EPSCs [in ms, Control 242.1±31, GluN2BKO 56.34±7; t(14)=8.093, p< 0.0001; Figure 19C].

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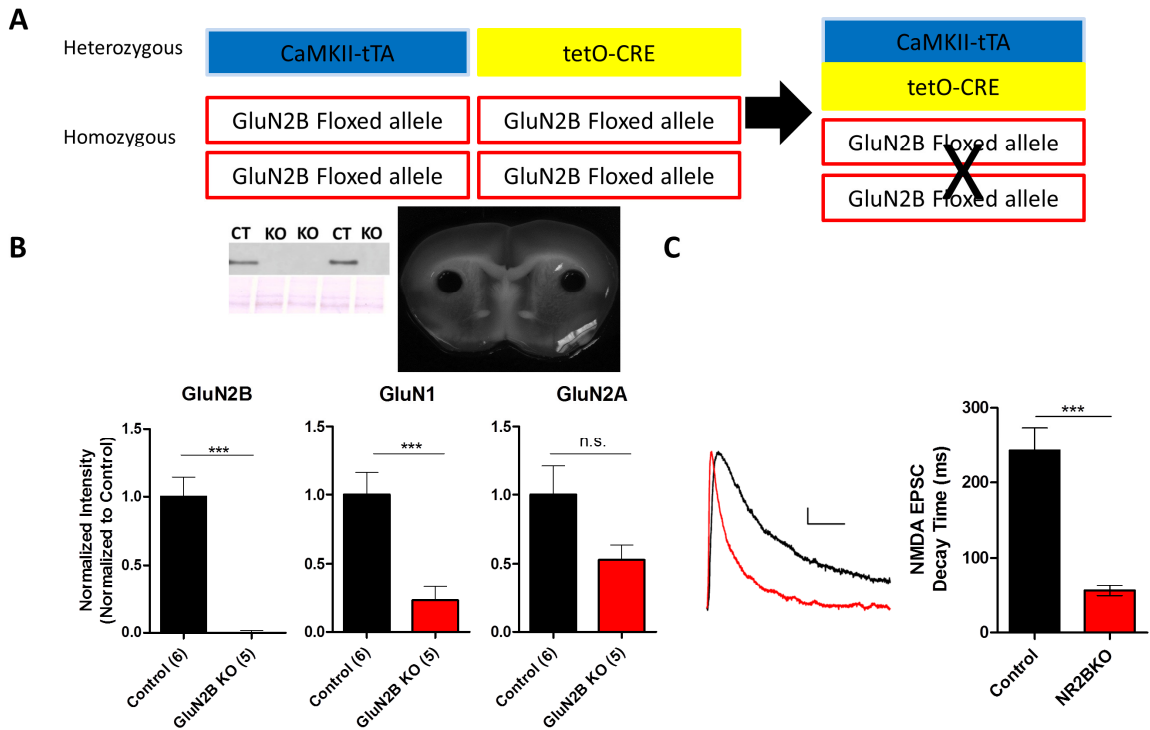
Figure 19. Breeding Strategy and Verification of GluN2BKO. (A) Schematic of breeding strategy for production of GluN2BKO mice. Mice are homozygous for the GluN2B (fl,fl) allele and heterozygous for either the CaMKIIα-tTA or tetO-CRE transgene. (B) Western blot analysis of GluN2B, GluN1, and GluN2A protein levels from 1mm punches of the dorsal lateral striatum (shown above) in control (single transgene and wildtype littermates) and GluR2BKO mice. Representative western blot of GluN2B protein levels shown in control (CT) and GluN2BKO (KO). (right) Representative traces and quantification of decay time for NMDAR EPSCs from control and GluN2B KO mice. Scale bars 30ms, 0.1 (scaled to 1) ***P<0.0001. Error bars represent SEM. Thank you to Julie Healy for western data.

Basal Glutamatergic Synaptic Transmission in GluN2B KO Mice

        To better understand the impact of GluN2B deletion on glutamatergic synaptic transmission we probed the levels of other NMDAR subunits and associated proteins in the dorsal lateral striatum. Similar to other GluN2BKO animals (Brigman et al., 2010; Badanich et al., 2011; Wills et al., 2012), GluN1 levels were significantly reduced [percent of control, 23 ± 0.05; $t(9)=9.307$, $p< 0.0001$; Figure 19B], while GluN2A showed only a trend for a decrease in protein levels [percent of control, 53 ± 0.11; $t(9)=1.883$, $p=0.093$; Figure 19B].

        Additionally, we took 1mm punches of M1 motor cortex, a region that sends excitatory glutamatergic afferents to the dorsal lateral striatum, in the same brain slice. Similarly, in the motor cortex we saw a highly significant reduction in GluN2B [percent of control, 20 ± 0.09; $t(10)=3.810$, $p< 0.0034$; data not shown] as well as GluN1 [percent of control, 51 ± 0.134; $t(10)=2.789$, $p< 0.0191$; data not shown]. GluN2A protein levels were not significantly reduced [percent of control, 84 ± 0.07; $t(10)=1.058$, $p< 0.3148$, data not shown].

        Next we assessed basal glutamatergic synaptic transmission onto dorsal striatal medium spiny neurons in adult animals using whole-cell patch-clamp recordings. AMPAR-mediated spontaneous excitatory postsynaptic current (sEPSCs) frequency was unchanged by GluN2B deletion [in Hz, Control 3.57 ± 0.45, GluN2BKO 4.04 ± 0.43; $t(28)=0.6824$, $p= 0.3401$; Figure 20A], yet there was a significant increase in sEPSC amplitude [in pA, Control 12.86 ± 0.72, GluN2BKO 15.69 ± 075; $t(28)=2.396$, $p=0.0235$; Figure 20B]. Additionally, we looked at the paired-pulse ratio (PPR), a measure of presynaptic release

probability. Over a range of interstimulus intervals there was no significant

difference in the PPR [$F_{(1,20)}=0.132$; $p=0.7205$; Figure 20E]. This suggests

that GluN2B deletion leads to an increase in the postsynaptic function and/or

number of AMPARs without altering the probability of release.
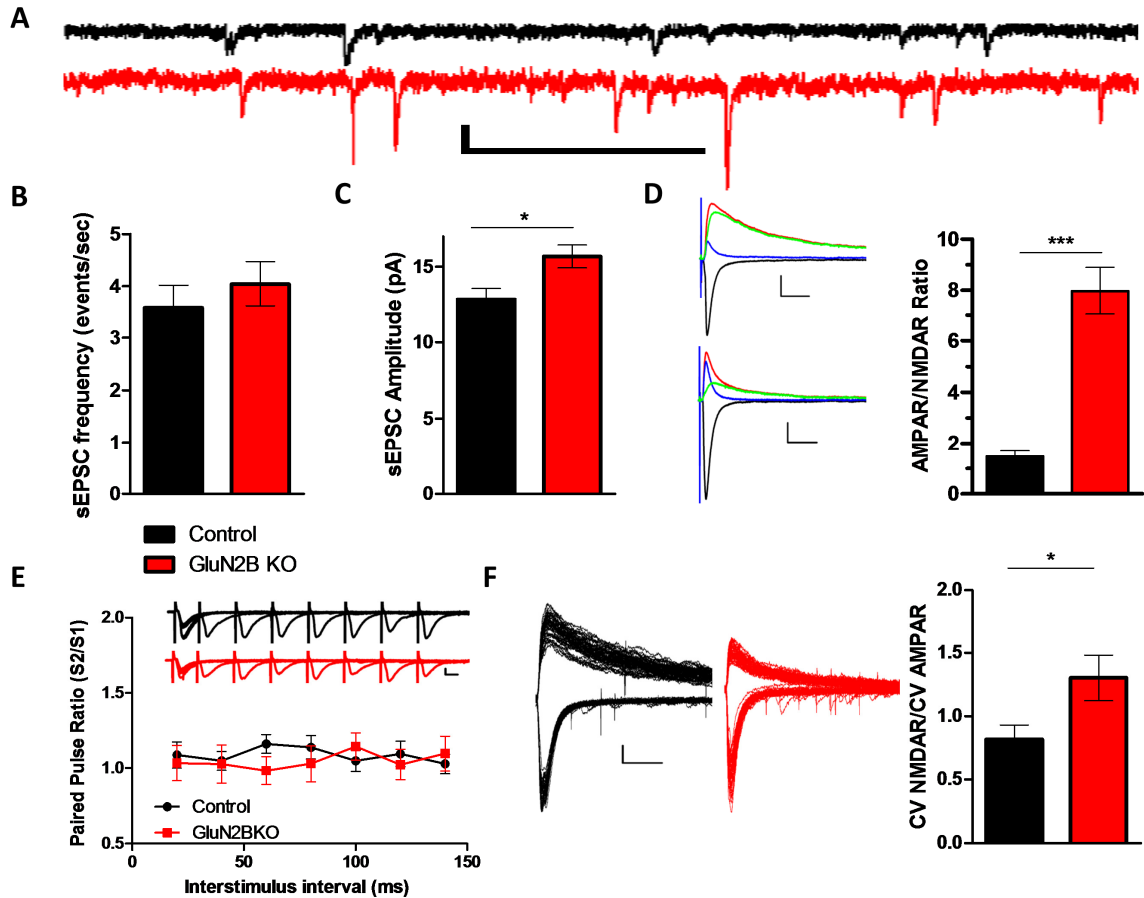
Figure 20. Basal Glutamatergic Transmission in Adult GluN2BKO Following Early Postnatal Deletion of GluN2B. (A) Representative traces of sEPSC currents at -70mV from controls (black) and GluN2BKO (red). Scale bars 300ms, 10pA (B) Quantification of sEPSC frequency and (C) sEPSC amplitude from controls and GluN2BKO. (D) (left) AMPAR/NMDAR ratio traces, AMPAR EPSC -70mV (black), dual AMPAR and NMDAR EPSC +40mV (red), AMPAR EPSC +40mV following 100µM DL-APV, NMDAR EPSC digital subtraction (green). Scale bars 20ms, 30pA. (right) Quantification of AMPAR/NMDAR ratio in controls and GluN2BKO mice. (E) Paired pulse ratio (S2/S1) over 20ms-140ms interstimulus intervals (20ms ISI steps). (Above) Evoked AMPAR EPSCs elicited at differing ISI for control (black) or GluN2BKO (red) mice. Scale bars 5ms, 50pA. (F) (left) 50 evoked EPSCs at -70mV (downward, AMPAR-mediated) and at +40mV (upward, NMDAR at +50ms) for control (black) and GluN2BKO (red) to compute coefficient of variation (CV) NMDAR/AMPAR ratio. Scale bars 20ms, 40pA. (right) Quantification of CV-NMDAR/CV-APMAR ratio in controls (black) and GluN2BKO (red)

To determine if deletion of GluN2B subunit of the NMDAR altered the composition of AMPARs, we examined AMPAR-mediated current voltage relationships in dorsal lateral striatum. In the presence of 0.1mM spermine in the patch pipette, to avoid dialysis of intracellular polyamines, the AMPAR-mediated current voltage relationships were not significantly altered [$F_{(1,14)}=0.1894$; $p=0.6706$; Figure 21A, 21B]. Both plots were linear, suggesting that the AMPARs were GluR2 containing and the AMPAR subunit composition was not significantly different between GluN2B KO MSNs compared to controls.
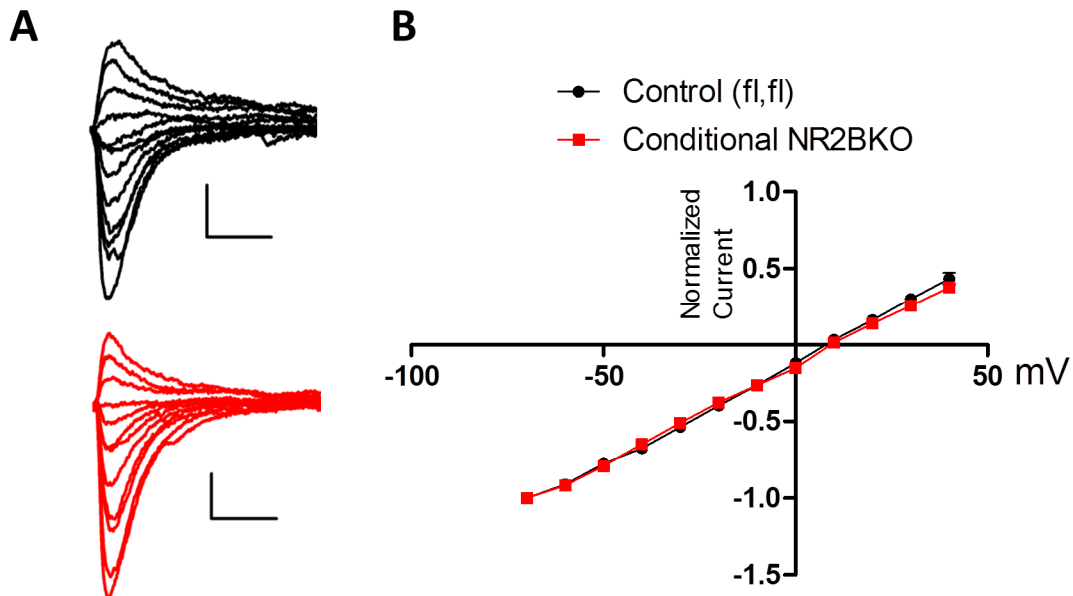
Figure 21. Deletion of the GluN2B Subunit of the NMDAR Does Not Alter Striatal AMPAR Current Voltage Relationships. (A) Evoked AMPA-mediated EPSCs for control (black) and GluN2BKO mice (red) isolated with 100μM DL-APV and 25μM picrotoxin at holding potentials ranging from -70mV to +40mV in 20mV steps. Scale bars 10ms, 100pA. (B) Average normalized AMPAR-mediated current at holding potentials ranging from -70mV to +40mV in 20mV steps in control (black) and GluN2BKO mice (red). Error bars represent SEM.

We also examined the ratio of NMDAR-mediated to AMPAR-mediated evoked EPSC amplitude to compute an AMPAR/NMDAR ratio as an index of excitatory synaptic transmission. We found that the AMPAR/NMDAR ratio was significantly enhanced using two differing methodologies [For +50ms method, Control 1.724 ± 0.17, GluN2BKO 5.169 ± 0.68; U(20)=8, p=0.0007; Figure 20D] [For digital subtraction method, Control 0.669 ± 0.11, GluN2BKO 1.747 ± 0.41; t(7)=3.463, p=0.0105]. These data are consistent with a previous study using a GluN2BKO mouse to study glutamatergic transmission in the hippocampus (von Engelhardt et al., 2008; Wills et al., 2012). This increase in AMPAR/NMDAR ratio could be due to an increase in AMPAR number and/or function, or be due to a decrease in NMDAR number and/or function, or potentially a combination of the two.

We also looked at the coefficient of variation (CV) of NMDAR- and AMPAR-mediated responses. CV is inversely proportional to quantal content, the probability of release and number of synapses. We observed a significant increase in the CVNMDAR/CVAMPAR ratio in the GluN2BKO group compared to controls [Control 0.8184 ± 0.11, GluN2BKO 1.3202 ± 0.18; t(14)=2.392, p=0.0313, Figure 20F]. This increase in CVNMDAR/CVAMPAR ratio was driven primarily by an increase in the NMDAR CV, suggesting a decrease in quantal content. Quantal content, which is inversely proportional to the CV, is made up of the product of the probability of release and number of release sites. We can assume that the decrease in quantal content is probably driven by a decrease in

the number of synapses given that PPR, a measure of release probability, was similar.

Genetic deletion of the GluN2B subunit of the NMDAR leads to a decrease in dendritic spine density

Given the increase in CVNMDA/CVAMPA ratio, we next assessed the role of GluN2B in the dorsal striatum on MSN dendritic spine density as a morphological correlate of synapse number. Following golgi staining, observers blinded to genotype counted MSN dendritic spine density within a 10μm segment, 80-100μm from the cell soma beyond the primary dendrite branch point. Dendritic spine density was significantly reduced in GluN2B KO animals compared to wildtype littermates [Segment means, Controls 10.96 ± 0.20, GluN2BKO 9.320 ± 0.23; t(54)=5.441, p<0.0001; Figure 22A] [Animal means, Controls 10.96 ± 0.39, GluN2BKO 9.489 ± 0.19; t(6)=3.368, p=0.0151]. Combined with the increased NMDA CV data, these data suggest that deletion of GluN2B in the dorsal striatum may lead to a reduction in NMDAR-only synapse number.

A
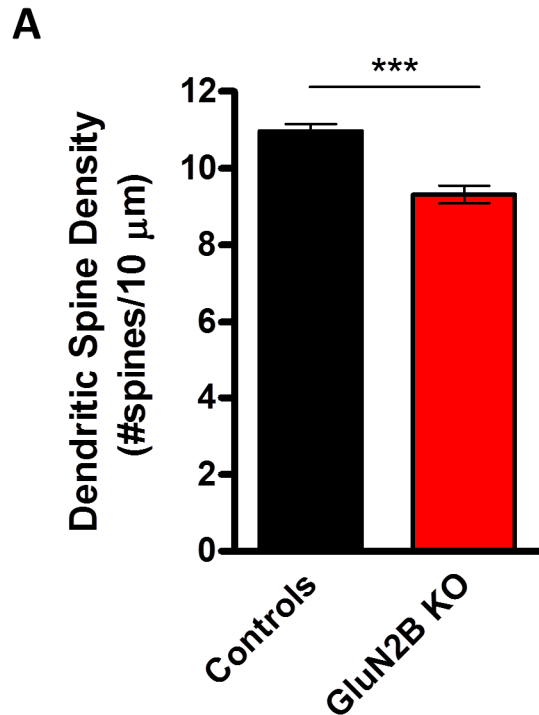
Figure 22. Deletion of the GluN2B Subunit of the NMDAR Leads to a Reduction in Dendritic Spine Density. (A) Average MSN Dendritic Spine Density (# of spines per 10µm), 80-100µ from the cell soma. ***P<0.0001. Error bars represent SEM. Thanks to Bonnie Garcia for collection of the data.

Deletion of GluN2B in adulthood reduces sEPSC frequency

To better understand the role of GluN2B in development, we delayed the deletion of the GluN2B by feeding pregnant dams and then GluN2BKO mice and their littermates doxycycline (200mg/kg) in their chow until P45. We first confirmed that indeed GluN2B was deleted in these mice at P80, similar to previous recordings, by measuring the speeding of NMDAR-mediated delay kinetics [in seconds, Control 256.8 ± 14.53, GluN2BKO 58.31 ± 2.75; $t(13)=10.93$, $p<0.0001$; Figure 23D] and confirmed the loss of GluN2B by

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western blot (Figure 23D, inset). Following the removal of doxycycline we again

assayed basal glutamatergic synaptic transmission in the mice at the same time

point in adulthood as previous experiments. We found that delayed deletion of

GluN2B again led to an increase in both sEPSC amplitude [Control 12.52 ± 0.37,

GluN2BKO 15.53 ± 0.81; t(12)=2.652, p=0.0211; Figure 23A,C] and the

AMPAR/NMDAR ratio [Control 1.472 ± 0.22, GluN2BKO 7.967 ± 0.93;

t(13)=8.175, p=0.0004; Figure 23E], as seen previously with GluN2B deletion

earlier in development. However, surprisingly we observed that the delayed

deletion of GluN2B lead to a decrease in sEPSC frequency [Control 4.594 ±

0.36, GluN2BKO 3.285 ± 0.18; t(12)=3.634, p=0.0034; Figure 23A,B]. This

suggests that GluN2B plays an important role in adulthood maintaining sEPSC

frequency, possibly via altering the number of synapses or the probability of

release.
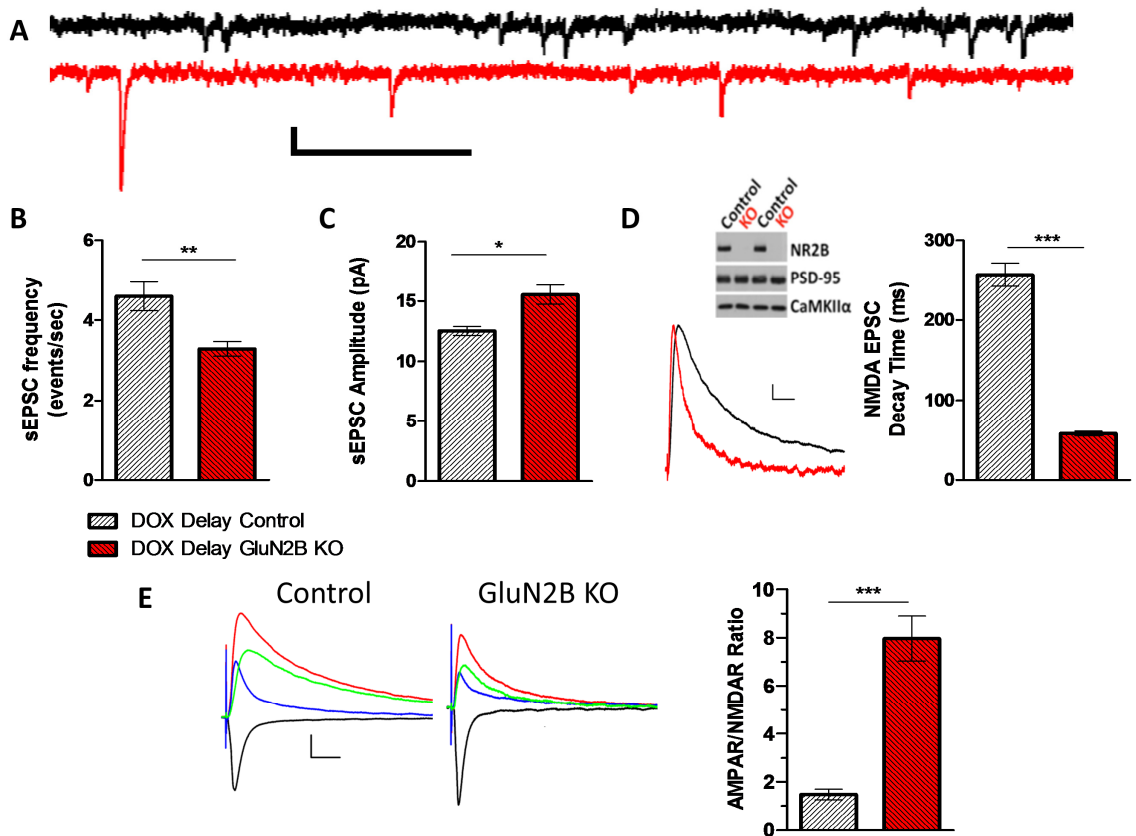
Figure 23. Delayed Deletion of the GluN2B Subunit Effects on Glutamatergic Synaptic Transmission in Dorsal Striatal MSNs. GluN2B deletion occurs at P45 with the removal of doxycycline (200mg/kg) chow. (A) Traces of AMPA-mediated sEPSCs at -70mV in control (black) and GluN2BKO mice (red). Scale bars 250ms, 10pA. (B) Average sEPSC frequency and (C) amplitude in controls (grey, hashed) and in GluN2BKO mice (red, hashed). (D) (left) Example averaged NMDAR EPSC traces for controls (black) and GluN2BKO (red). Scale bars 30ms, 0.1 (scaled to 1) (right) Average NMDAR EPSC decay time (ms) in controls (grey, hashed) and GluN2BKO (red, hashed). (inset) Western blot for GluN2B in controls and GluN2BKO mice (KO) showing the complete loss of GluN2B in the striatum with doxycycline delayed GluN2B deletion. PSD-95 and CaMKIIα are added as loading controls. (E) (left) AMPAR/NMDAR ratio averaged traces. Evoked AMPAR EPSC -70mV (black), Dual AMPAR and NMDAR EPSC +40mV (red), AMPA EPSC post 100μM DL-APV (blue), NMDAR EPSC digital subtraction (green). Scale bars 20ms, 40pA. (right) Average AMPAR/NMDAR ratio (+50ms) in controls (grey) and in GluN2BKO mice (red).

<u>Deletion of GluN2B subunit leads to a hyperlocomotive phenotype in the novel</u>

<u>open field that can be rescued by inhibiting GluN2B later in development</u>

We started to assess the effects of GluN2B deletion on behavior by observing locomotor activity in the novel open field in adult mice (16 weeks). Conditional GluN2B KO animals or wildtype littermates were placed in a novel open field for sixty minutes to track overall locomotor activity. GluN2B KO mice exhibited similar locomotor activity in the first five minutes in the novel open field. However, at later time points the GluN2BKO mice showed significantly escalating locomotor behavior [$F_{(1,48)}=36.53$; $p<0.0001$; Fig 24A], whereas controls locomotor behavior habituated to the environment as expected. Yet there was no significant difference in center time, a crude measure of overall anxiety levels in GluN2BKO mice versus control [in seconds, Controls 325.3 ± 49, GluN2BKO 270.6 ± 45; $t(25)=0.7793$, $p=0.4431$]. Surprisingly, this affect was rescued by feeding doxycycline to inhibit the deletion of GluN2B before P45 [$F_{(1,12)}=4.617$; $p=0.0572$; Fig 24B]. The doxycycline food was removed at P45 allowing for the deletion of GluN2B to occur (Fig 24B, inset) and the animals were then examined at the same age as Fig 24A. The rescue of locomotor activity by delaying the deletion of GluN2B suggests that GluN2B is crucial early in development for normal motor activity.

A

B

DOX Delay (P45)
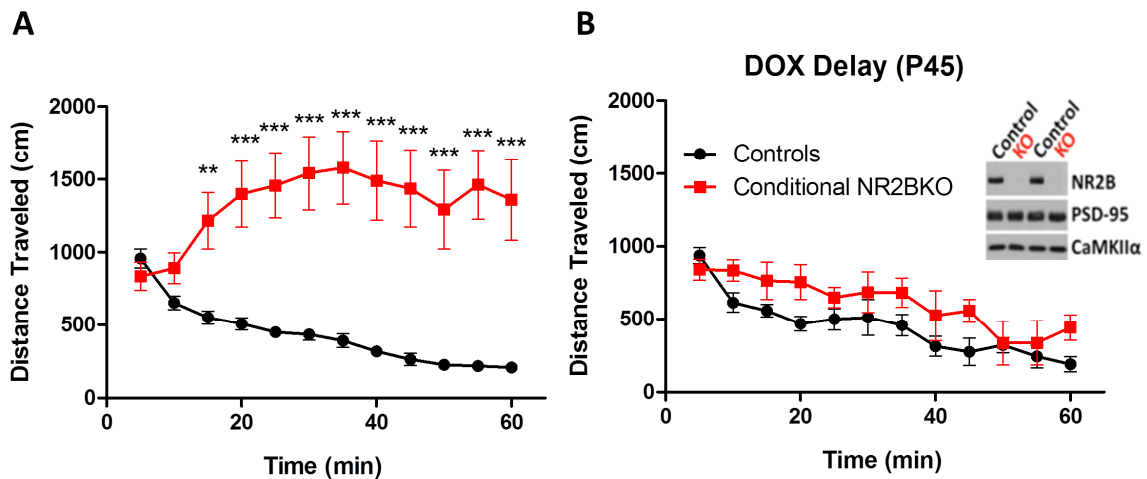
Figure 24. Effects of GluN2B deletion on Novel Open Field Locomotor Activity. (A) Distance traveled (cm) in the novel open field for controls (single transgene, and wildtype littermates) (black) and early perinatal GluN2BKO animals averaged in 5 minute bins for 1 hour. (B) Distance traveled in the novel open field for controls (single transgene, and wildtype littermates) (black) and GluN2BKO animals (deleted at P45) averaged in 5 minute bins for 1 hour. (inset) Western blot for GluN2B in controls and GluN2BKO mice (KO) showing the complete loss of GluN2B in the striatum with doxycycline delayed GluN2B deletion. PSD-95 and CaMKIIα are added as loading controls. **$P<0.01$, ***$P<0.0001$, Error bars represent SEM.

Genetic deletion of GluN2B partially disrupts motor performance on the rotarod

Previous work has shown that deletion of the obligatory GluN1 subunit of

the NMDAR just from the striatum disrupted normal motor learning on the rotarod

(Dang et al., 2006; Beutler et al., 2011). Additionally, transgenic mice where

MSNs are ablated do not show any improvement over trials in an accelerating

rotarod, showing the importance of the striatum in this task (Kishioka et al.,

2009). To understand the effects of GluN2B subunit deletion on rotarod motor

learning we ran mice on repeated trials (<5mins, 30 min ITI). While both the

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GluN2B KO mice and controls learned to perform better over repeated trials [Control 1 vs. 8, t(102)=8.862, p<0.0001; GluN2BKO 1 vs. 8, t(58)=4.051, p=0.0002], there was a trend for a decrease in performance in early trials and a significant reduction in performance in later trials in the GluN2B KO mice [F (1,80)=9.336; p=0.0031; Figure 25A]. These data suggest that GluN2B is not crucial for simple striatal motor learning, but may have some influence on overall motor performance. Additionally, we looked at the effect on motor learning when delaying the deletion of GluN2B until after P45 by feeding doxycycline. Surprisingly, delaying the deletion led to greater deficits in rotarod performance with significant reductions seen in early trials [F (1,41)=7.225; p<0.0001; Figure 25B]. Comparing the first trial to the last trial as a measure of motor learning controls showed a significant increase in the latency to fall measure indicative of motor learning over time [in seconds, Controls Trial 1, 113.7 ± 8.7; Controls Trial 8, 214.0 ± 10.7; t(64)=7.294; p<0.0001]. However, the GluN2BKO failed to show a significant increase in the latency to fall over measure over trials suggesting impaired motor learning [in seconds, GluN2BKO Trial 1, 78.50 ± 15.9; GluN2BKO Trial 8, 124.3 ± 25.9; t(64)=1.506; p=0.1495]. This suggests that early deletion of GluN2B is less detrimental than GluN2B deletion in adulthood in striatal motor learning, possibly allowing for early adaptation and compensation to the loss to the GluN2B subunit.
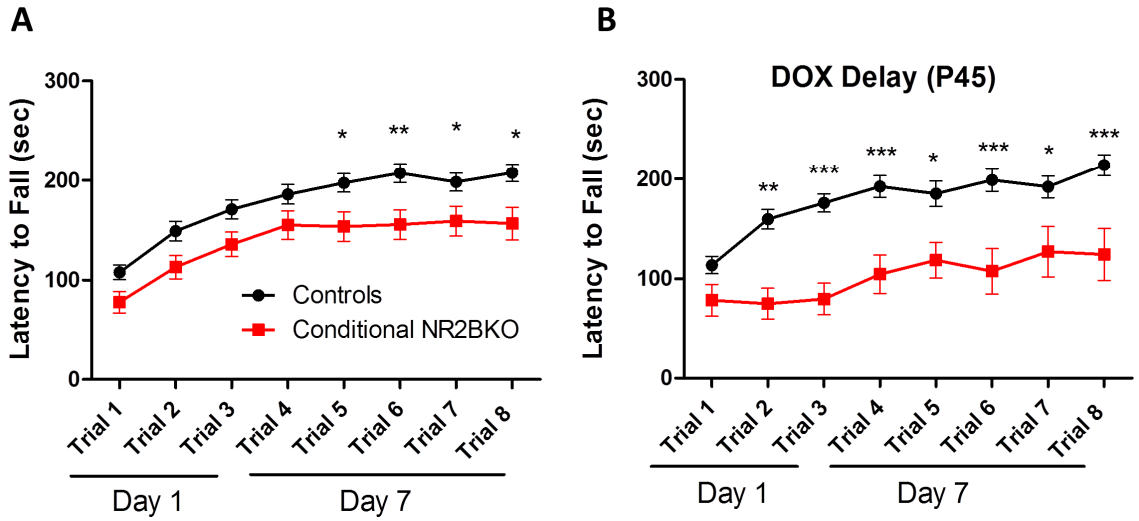
Figure 25. Effects of deletion of GluN2B subunit of the NMDAR on simple motor learning. (A) Averaged latency to fall (seconds) over eight trials on 2 days separated by a week in control (black) and early perinatal GluN2BKO mice (red). (B) Averaged latency to fall (seconds) over eight trials on 2 days separated by a week in control (black) and GluN2BKO mice (deleted at P45) (red). *P<0.05, **P<0.01, ***P<0.0001. Error bars represent SEM.

Discussion

These studies indicate a key role for the GluN2B subunit in striatal glutamatergic synaptic transmission as well as in striatal-based behaviors. We observed that deletion of GluN2B in the striatum early in postnatal development leads to an increases in sEPSC amplitude, AMPAR/NMDAR ratio, and CVNMDAR/CVAMPAR ratio coupled with decreases in dendritic spine density. The increase in sEPSC amplitude along with the increase in AMPAR/NMDAR ratio suggests an increase in number and/or function of AMPARs. The increased CVNMDAR/CVAMPAR ratio data combined with no change in the presynaptic release probability coupled and decreased dendritic spine density suggests a possible decrease in the number of NMDAR-only synapses or "silent" synapses. Behaviorally, GluN2B deletion early postnatally led to a hyperlocomotive phenotype and partial performance deficits on the rotarod, but did not disrupt motor learning.

Postponing the deletion of striatal GluN2B until P45 led to a similar synaptic phenotype with a notable difference. The delayed deletion decreased sEPSC frequency, suggesting a possible decrease in the number of synapses or the probability of release. Behaviorally, postponed deletion of GluN2B rescued the hyperlocomotive phenotype, yet worsened the behavioral deficit on the rotarod as well as blunting motor learning. These data suggest that the timing of the GluN2B subunit deletion is crucial in determining what electrophysiological and behavioral phenotype observed.

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Disrupted Glutamatergic Synaptic Transmission in the GluN2BKO mice

We observed an almost complete (99%) reduction in GluN2B levels in the striatum as well as a significant reduction in the obligatory GluN1 subunit (77%) necessary for heteromeric assembly suggesting that the loss of GluN2B reduced the number of NMDARs in total. Previous studies using GluN2B KOs suggest that total GluN1 mRNA levels were unaltered, but GluN1 protein levels were reduced suggesting a deficit in translation or degradation of the receptor (Brigman et al., 2010; Badanich et al., 2011). Indeed, the loss of GluN2B may impart greater NMDAR instability and greater turnover (Huh and Wenthold, 1999). Many other synaptic proteins such as GluA1, CaMKIIalpha and CaMKIIbeta were unaltered suggesting a selective alteration in glutamate receptors. This pattern was similar to previously reported with the loss of GluN2B in the CA1 region of the hippocampus (von Engelhardt et al., 2008; Brigman et al., 2010) as well as similar to what is seen in the striatum (Badanich et al., 2011). Additionally, confirming the loss of GluN2B the decay kinetics of evoked NMDAR-mediated EPSCs was significantly substantially accelerated.

We assume that all of the NMDAR remaining in the striatum are heteromers of GluN1/GluN2A. GluN2A containing NMDARs are known to have faster deactivation kinetics than GluN2B containing receptors fitting our data (Traynelis et al., 2010). Notably we did not observe any GluN2D-like long lasting decay kinetics, observed previously in striatal NMDAR subunit knockout animals (Logan et al., 2007). Increased sEPSCs amplitude with early postnatal GluN2B deletion suggests an increased number and/or function of synaptic AMPARs.

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While we did not see an increase in crude GluNA1 levels by western blot, this technique is a cruder measure of total AMPARs including extrasynaptic and internalized receptors and not an enriched synaptic fraction. Increased, AMPAR-mediated synaptic transmission fits with the idea that NMDAR activation in synaptogenesis suppresses AMPAR function (Hall et al., 2007). Additionally, AMPAR current voltage relationships were linear and unchanged between the GluN2BKO animals and controls, suggesting that the AMPARs are GluA2-containing and that GluN2B subunit deletion does not significantly alter AMPAR subunit composition. The increase in the AMPAR/NMDAR ratio could come from an increase in AMPAR-mediated transmission or a decrease in the NMDAR-mediated component or some combination of the two. In this case the increase in sEPSC amplitude along with a loss in GluN1 levels would suggest that both are in play. sEPSC frequency and the paired pulse ratio (PPR) were unchanged between controls and GluN2BKO animals suggesting that the number of synapses and the probability of release are unchanged, yet we observed a reduction in the dendritic spine density- a morphological correlate of synapse number. This reduction in dendritic spine density was similar to what was observed in the in CA1 or CA3 region of the hippocampus in a GluN2B KO animal (Akashi et al., 2009; Brigman et al., 2010) and in cultured hippocampal neurons after GluN2B RNA interference (Kim et al., 2005). However, this contrasts the finding in the hippocampus that GluN2B deletion increases functional synapse number (Gray et al., 2011). Utilizing a coefficient of variation AMPAR/NMDAR ratio (CV-NMDAR/CV-AMPAR ratio) a measure of quantal

content reflecting changes in the number of synapses and/or the probability of release we found a significant increase in this ratio primarily driven by an increase in the NMDAR CV. This suggests a decrease in the number of NMDAR-only containing synapses or silent synapses. Thus this decrease in "silent" synapses may be reflected by fewer dendritic spines observed and would not be picked up in AMPAR-mediated sEPSC frequency measures.

Delaying the deletion of GluN2B until P45 lead to a similar phenotype consisting of accelerated NMDAR-mediated decay time, loss of GluN2B from the striatum, increased sEPSC amplitude, and increased AMPAR/NMDAR ratio. However, the postponed deletion of GluN2B until adulthood resulted in a significantly reduced sEPSC frequency. This may suggest that deletion of GluN2B in adulthood may decrease the number of functional synapses or the probability of release at those excitatory synapses. This suggests that GluN2B subunits may play some synaptic maintenance role, protecting the synapse from deletion. The lack of a decrease in sEPSC frequency early in development may suggest a deficit in synaptogenesis of potential silent synapses.

Disrupted striatal-based behaviors in GluN2B KO mice

Alterations in synaptic transmission and dendritic spine number were associated with selective striatal-based behavior disruptions. GluN2BKO animals showed a hyperlocomotive phenotype with an escalating locomotor response in the open field compared to within session habituation observed in wildtype littermates. Previous reports using NMDA antagonists as well as

GluN2B-preferring antagonists such as Ro 25-6981 at higher doses produced

hyperlocomotion (Chaperon et al., 2003; Kiselycznyk et al., 2011). Additionally,

genetic replacement of the GluN2B subunit with the GluN2A subunit results in a

hyperlocomotive phenotype (Wang et al., 2011). Also GluN2BKO animals

showed elevated locomotor activity that did not habituate, but did not show

escalation in locomotor activity like we observed (Badanich et al., 2011).

However, in our case when we delayed the deletion of GluN2B until P45 the

hyperlocomotive phenotype was rescued. This suggests that early removal of

GluN2B in development leads to this phenotype. It is known that levels of

GluN2B are high in early postnatal development and then start to decline as

GluN2A levels rise (Monyer et al., 1994; Sheng et al., 1994). The delayed

removal of GluN2B may allow for proper synaptogenesis and synaptic

maturation.

When we examined striatal-based motor learning assayed on the

accelerating rotarod we observed that deletion of GluN2B early in postnatal

development lead to a modest decrease in performance especially in later trials,

but it did not disrupt overall motor learning. Surprisingly, postponing the deletion

of GluN2B until P45 actually led to a more severe deficit in rotarod performance

in GluN2BKO mice. GluN2BKO mice did not show any significant motor learning

over repeated trials compared to littermate controls, suggesting an important role

for GluN2B in motor learning in the striatum. The enhanced deficit seen when

the deletion of GluN2B is postponed until adulthood, suggests that compensation

may be able to prevent serious motor deficits early on, but disrupting a system

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which has utilized GluN2B signaling previously may lead to more serious deficits. In contrast to these data, a study using GluN2A or GluN2B antagonists injected into the dorsal striatum suggested that while GluN2A was crucial for motor learning on the accelerating rotarod, GluN2B was not (Lemay-Clermont et al., 2011). This discrepancy may be accounted for by issues with selectivity with the GluN2A antagonist, NVP-AAM077 or the greater alliance on the loss of GluN1 subunit also seen in our studies. Rotarod learning, especially the late component, seems to involve an increase in NMDAR-mediated synaptic transmission and is associated with an upregulation of GluN1 (Yin et al., 2009; D'Amours et al., 2011). Another study showed that disrupting the interaction between CaMKII and the GluN2B subunit, however did not disrupt rotarod or locomotor activity, suggesting that the actual loss of the GluN2B or GluN1 receptor plays the crucial role (Halt et al., 2012).

While the differing phenotype following the delayed deletion of GluN2B subunit may be linked to when the subunit is removed in development an alternate explanation may be that there are regional differences in the completeness of the forebrain wide KO. This differential regional deletion has been observed previously in similar GluN2BKO mice (Badanich et al., 2011).

In conclusion, the current study describes a genetic approach to understanding the role of the GluN2B subunit in striatal synaptic transmission, dendritic spine density and behavior. We found that the deletion of GluN2B early in postnatal development shortens NMDAR-mediated currents, leads to potentiated AMPA-mediated synaptic transmission and a decrease in the number

of dendritic spines on MSNs potentially via a loss in NMDA-only or "silent" synapses. A delayed deletion of GluN2B receptors in adulthood leads to a similar phenotype, but with a reduction in the number of functional receptors or probability of release. Behaviorally, early disruption of GluN2B leads to disruptions in locomotor activity, while adult deletion of GluN2B results in deficits in striatal motor learning. These data emphasize the importance of the GluN2B subunit of the NMDAR in striatal synaptic transmission and behavior.

CHAPTER VI

GENERAL DISCUSSION

The role of glutamatergic transmission in the dorsal striatum in health and its dysregulation in disease states is becoming increasingly recognized. Long-lasting alterations in glutamatergic transmission have been connected to the formation of motor repertoires and the organization of habitual behavior (Yin and Knowlton, 2006; Yin et al., 2009). Moreover, alterations in glutamatergic transmission in the striatum have been seen in models of Parkinson's disease, Huntington's disease and addiction (Picconi et al., 2004b; Kreitzer and Malenka, 2007; Milnerwood et al., 2010; Luscher and Malenka, 2011). Disruptive or maladaptive synaptic plasticity in the striatum could in part underlie the motor abnormalities seen in neurodegenerative disease and underlie the habitual nature of compulsive drug seeking in addiction. Importantly, molecules like CaMKII and the NMDAR are already tied to synaptic plasticity and learning and memory in the hippocampus. However, little is known about the regulation of glutamatergic transmission and its role in behavioral output in the striatum. Utilizing electrophysiological approaches along with pharmacological, genetic, immunohistochemical and behavioral manipulations this thesis has begun to elucidate some of the roles of striatal CaMKII and the GluN2B subunit of the NMDAR in synaptic transmission, synaptic plasticity and ultimately behavior.

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Role of Striatal CaMKII in Glutamatergic Transmission

The data presented in this document suggests that MSN CaMKII in the dorsal striatum plays a role in setting functional synapse number and intrinsic excitability, acting as rheostat to modulate the output of dorsal striatal MSNs (Figure 26).   We find that selective genetic inhibition of CaMKII in dorsal striatal MSNs leads to a robust reduction in s/mEPSC frequency without altering s/mEPSC amplitude. Alterations in s/mEPSC frequency are canonically interpreted as a presynaptic change in either release probability or the number of functional synapses/release sites. Experiments designed to test for alterations in release probability or the concentration of glutamate at the synapse, indicate that inhibition of postsynaptic MSN CaMKII did not alter these measures. Thus in total these data suggest that striatal CaMKII inhibition decreases the number of excitatory functional synapses. Reduced dendritic length and complexity in EAC3I mice may account for the reduction in the number of synapses without any significant changes in dendritic spine density. Alternatively, there could be an increase in the number of silent synapses or silent synapse modules - synapses which contain NMDARs, but lack AMPARs. This fits with the idea that CaMKII inhibition did not reduce dendritic spine density, suggesting that at least some of the observed postsynaptic dendritic spines in CaMKII inhibited cells are indeed silent. In the hippocampus silent synapses are abundant in early development and silent synapses can be unsilenced in an activity-dependent, but CaMKII independent manner. However, other studies using a constitutively active form of CaMKII suggests a direct role for CaMKII in the unsilencing of

synapses. Additionally, CaMKII has been shown to be required for the formation

of new synapses and/or morphological augmentation following hippocampal LTP

induction.
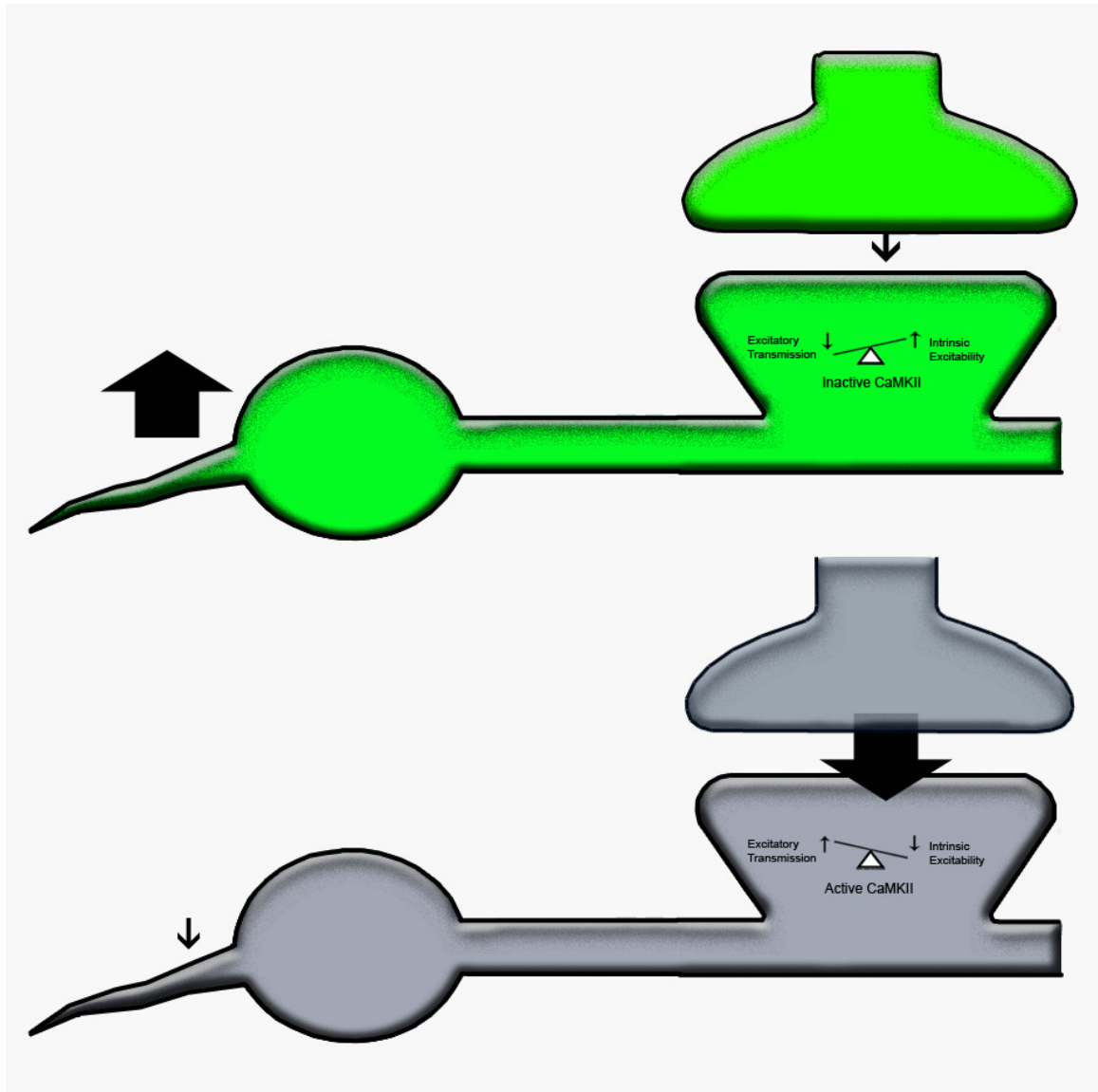
Figure 26. CaMKII Rheostat: Effects of CaMKII Inhibition on Glutamatergic Synaptic Transmission and Intrinsic Excitability in the Dorsal Lateral Striatum. Active CaMKII (grey, control MSN) enhances synaptic transmission and decreases intrinsic excitability. Inhibition of CaMKII (green, EAC3I-containing MSN) leads to a decrease in the number of synapses and an increase in intrinsic excitability.

Differing experimental approaches could be used to examine if inhibition of CaMKII leads to an increase in silent synapse number. Traditionally, silent synapses have been observed using minimal stimulation techniques setting the stimulation intensity at a threshold where no AMPAR-mediated EPSCs are observed, but at positive potentials where NMDAR are activated, NMDAR-mediated currents are observed (Isaac et al., 1995). Normally the failure rate is then compared before and after some manipulation, like the induction of LTP. These experiments, while potentially interesting, have caveats. They are dependent on the stimulation intensity and location of the microstimulator to remain the same as well as the health of the slice. If the number of fibers recruited is altered slightly by changes in the stimulator location or slice then the number of synaptic failures can be altered dramatically. Additionally, because CaMKII is inhibited genetically we do not have a pre versus post comparison to measure synaptic failure rates which is crucial for interpreting results from these experiments. Another approach to determine the presence of silent synapses is the CV-NMDAR/ CV-AMPAR ratio. This technique has been used with success in the ventral striatum to detect silent synapses following repeated cocaine administration (Huang et al., 2009; Brown et al., 2011). Decreases in this ratio are associated with a silent synapse phenotype and we did observe decreases in this ratio when comparing CaMKII-inhibited MSNs versus non-inhibited MSNs. Yet the underlying effect was driven by an increase in the ratio in the non-inhibited MSNs versus all controls, suggesting that non-inhibited MSN may compensate for the loss of CaMKII in neighboring MSNs.

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CaMKII's Role in MSN Morphology

We did not observe any changes in dendritic spine density with CaMKII inhibition. This suggests that CaMKII is potentially not necessary for the formation of new synapses, but instead may control the maturation of striatal synaptic connections. Indeed, previous studies have shown the importance of phosphorylation CaMKII at the T286 site to promote dendritic spine growth, even with a mutation rendering the kinase catalytically dead (Pi et al., 2010b). Yet the direction of synaptic strength alteration depends on the phosphorylation state of the CaMKII T305/306 site (Pi et al., 2010b). Other studies in the hippocampus have shown that CaMKII inhibition can block the increase in dendritic spine size or Wnt7a-mediated spine growth (Matsuzaki et al., 2004; Okamoto et al., 2009; Ciani et al., 2011b). Prior studies have shown that βCaMKII in cultured hippocampal neurons modulate dendritic length as well as synapse number (Fink et al., 2003). While we did not encounter a change in spine density, we observed a significant decrease in dendritic length and complexity. The decrease in dendritic length combined with unaltered spine density may account for the decrease in total synapse number we propose. However, it is not clear whether distal synapses are even sampled under whole-cell voltage clamp due to cable filtering properties previously reported (Williams and Mitchell, 2008). The aforementioned study examined layer 5 pyramidal neurons; we would expect that dendritic filtering would be even greater in MSNs with narrow dendrites and numerous spines. The CaMKII-mediated decrease in dendritic length and complexity may suggest developmental maturation deficits. An interesting

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candidate includes BDNF signaling via TrkB receptors. Mice lacking cortical

BDNF show altered dendritic morphology including decreases in dendritic length

(Baquet et al., 2004). Additionally, signaling through the TrkB receptor has been

shown to activate CaMKII (Hasbi et al., 2009; Minichiello, 2009).

Importance of MSN-specific Inhibition of CaMKII

It is important to consider the localization of CaMKII when interpreting

these results. CaMKII is highly expressed in MSNs (Erondu and Kennedy,

1985), but is also expressed in pyramidal neurons that form the presynaptic

terminal on MSN dendritic spines and shafts (Liu and Jones, 1996). Additionally,

CaMKII is expressed in dopamine neurons which densely innervate MSNs in the

striatum, modulating glutamatergic drive (Fog et al., 2006). CaMKII activation

has been shown to modulate both pre and postsynaptic activity. Presynaptically,

CaMKII has been shown to modulate the trafficking of synaptic vesicles via

phosphorylation of synapsin (Llinas et al., 1985; Stefani et al., 1997; Chi et al.,

2001), P/Q type calcium channels (Elgersma et al., 2002; Jiang et al., 2008), and

voltage-gated sodium channels (Wagner et al., 2006). Additionally, CaMKII has

been shown to modulate catecholamine synthesis (Atkinson et al., 1987) and

dopamine transporter function (Fog et al., 2006; Binda et al., 2008). Thus, we

have utilized a cell specific approach where CaMKII is selectively inhibited only in

dorsal striatal MSNs, eliminating cofounding interpretations on the locus of

CaMKII inhibition and observed effects.

Striatal CaMKII's Regulation of Cortical and/or Thalamic Excitatory

Transmission

MSNs are innervated by both cortical and thalamic glutamatergic inputs and differences in the release probability have been observed between these two inputs. Our measures of sEPSC and mEPSC frequency are likely comprised of both cortical and thalamic-mediated glutamate release. Our data do not rule out the possibility that inhibition of CaMKII may have a greater influence on one of these excitatory inputs over the other. Previous studies have separated cortical versus thalamic inputs with horizontal oblique slices maintaining both cortical and thalamic innervation and stimulating evoked EPSCs in layer 5 of the cortex or at the level of the reticular nucleus preserving some of the parafascicular-centromedian thalamic pathway (Smeal et al., 2007; Ding et al., 2008). Alternatively, the expression of channelrhodopsin-2 in motor cortex or in thalamic nuclei projecting to the dorsal striatum would be a more selective way to separate these two inputs (Osakada et al., 2011). The use of modified rabies viruses carrying channelrhodopsin-2 which in a retrograde manner infects projecting brain areas will help delineate the role of these separate pathways. In both cases the measured EPSC is evoked by electrical stimulation or blue light, respectively. However, our main phenotype in CaMKII inhibited MSNs is a reduction in spontaneous or miniature EPSC frequency which involves spontaneous release of synaptic vesicles. One experimental approach to select a pathway and record spontaneous release-like events involves swapping aCSF calcium for strontium. Strontium, a divalent cation like calcium, promotes

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asynchronous release of synaptic vesicles, by lingering strontium levels in the presynaptic terminal. In strontium aCSF individual pathways could be electrically or optogenetically stimulated. Following the initial large evoked EPSC miniature like release events occurring due to asynchronous release can be analyzed. However, only the amplitude measure of these events is of interest because the frequency of these events is altered by strontium. Thus this makes the separation of cortical and thalamic spontaneous or miniature EPSC frequency difficult.

CaMKII Inhibition in Direct and Indirect Pathway MSNs

There are two canonical MSN output pathways in the dorsal striatum. The direct pathway MSNs are labeled by D1R expression and projections to the substania nigra pars reticulata. The indirect pathway MSNs are labeled with D2R expression and projections to the globus pallidus. These two pathways are thought to oppose one another with the direct pathway promoting movement and the indirect pathway inhibiting movement (Bateup et al., 2010; Kravitz et al., 2010). Using BAC transgenic mice to label direct and indirect pathways notable differences in basal synaptic transmission, plasticity and excitability have been observed (Kreitzer and Malenka, 2007, 2008; Grueter et al., 2010; Andre et al., 2011). The EAC3I CaMKII inhibitory peptide was expressed in both direct and indirect pathway neurons evidenced by the presence of EGFP axon terminals seen in the substantial nigra pars reticulata and globus pallidus, respectively. However, we do not know if there is a bias in the percentage of EAC3I containing

MSNs in direct or indirect pathway. We did not observe a bimodal distribution in our sEPSC frequency data, suggesting a global loss of functional synaptic connections in both pathways. It will be interesting to look at the effects of CaMKII inhibition in either direct or indirect pathway neurons. Retrograde microspheres deposited in target regions or breeding the EAC3I to mCherry expressing BAC transgenics will allow for identification of these two pathways.

Some evidence for distinct CaMKII signaling effects on direct or indirect pathway MSNs has already been observed. CaMKII is known to interact with the M4R, a GPCR linked to Gi/o signaling which is enriched in direct pathway MSNs (Yasuda et al., 1993; Hersch et al., 1994). The phosphorylation of M4R by CaMKII can potentiate M4R signaling and controls the behavioral sensitivity to dopamine (Guo et al., 2010). I might hypothesize that inhibition of MSN CaMKII may alter M4R function or signaling in direct pathway MSNs.

It is difficult to predict how the decrease in numbers of excitatory synapses potentially on direct and indirect pathway MSNs would alter striatal output. Decreased excitatory drive to MSNs should decrease the release of GABA in the globus pallidus and substantia nigra. Yet we observed increased intrinsic excitability possibly countering these effects potentially as compensation to the loss in synapse number. Also interesting is the high level of expression of the CaMKII inhibitor in MSN presynaptic axon terminals in the SN_R and GP. The presynaptic effects of CaMKII inhibition on MSN GABA release have not yet been studied.

Other Modes of CaMKII Inhibition

Utilizing the EAC3I mouse was a selective and cell-specific approach to inhibit CaMKII. However, doxycycline mediated delay of transgene expression contained some caveats. First as reported previously, silencing the transgene from birth and expressing the transgene later in adulthood led to significantly reduced levels of EAC3I expression compared to leaving the transgene on from birth (Bejar et al., 2002). This fact made it difficult to interpret whether the expression levels or time course of CaMKII inhibition led to the observed phenotype. Additionally, there was a lag time of 2-3 weeks for maximal expression or complete inhibition of EAC3I transgene expression when starting or stopping the doxycycline diet, clouding the temporal control of transgene expression (Bejar et al., 2002). Other options for inhibiting CaMKII that have had previous success include viral expression of EAC3I in adult tissue (Zou and Cline, 1999; Li et al., 2010; Swaminathan et al., 2011), infusion of CaMKII inhibitory peptides via a patch pipette (Otmakhov et al., 1997; Shiells and Falk, 2000; Sergeant et al., 2005), or the CaMKII T286A knock in mouse (Giese et al., 1998; Ohno et al., 2006; Kimura et al., 2008; Sametsky et al., 2009). Viral expression or patch pipette inclusion of CaMKII inhibitors may avoid issues with compensation seen with prolonged CaMKII inhibition in genetic models and may yield differing results (Bejar et al., 2002).

Potential Downstream Substrates of Striatal CaMKII

There are numerous downstream substrates of CaMKII that could play a role in our observed decreased sEPSC frequency phenotype. One potential downstream substrate of CaMKII is the GluA1 subunit of the AMPAR. CaMKII regulates the trafficking of synaptic GluA1 incorporation in the hippocampus (Lee et al., 2000; Esteban et al., 2003). The decreased sEPSC frequency phenotype in the EAC3I mice is mimicked in the GluA1KO mice suggesting a common mechanism. Moreover, CaMKIIα mRNA and protein levels are reduced in GluA1 KO animals (Zaitseva et al., 2003; Zhou et al., 2009). Another potential candidate is SAPAP3; a striatally enriched scaffolding protein, which can be phosphorylated by CaMKII. Intriguingly, SAPAP3 KO mice exhibit a similar electrophysiological phenotype as our EAC3I mice (Chen et al., 2011b; Wan et al., 2011). With tens to hundreds of potential CaMKII target substrates at the synapse from receptors like the GluN2B subunit of the NMDA receptor (Omkumar et al., 1996), to scaffolding molecules like SAP90/97, PSD-95 and Homer 1b (Yoshimura et al., 2000; Yoshimura et al., 2002), as well as vanilloid receptors (Jung et al., 2004) it will be interesting to see which molecules underlie our observed effects. Further study is needed to identify other downstream MSN proteins regulated by CaMKII.

Potential Effects of CaMKII on Inhibitory Synaptic Transmission in the

Striatum

While we have extensively isolated our studies to excitatory transmission in the dorsal striatum the potential effects of CaMKII inhibition on GABAergic transmission in the striatum remain unexplored. MSNs receive local inhibitory connections from other neighboring MSNs as well as GABAergic interneuron innervation. Indeed, while little is known of CaMKII's role in inhibitory transmission a recent study has suggested the active CaMKII can phosphorylate GABA$_A$Rs and enhance inhibitory synaptic transmission in the hippocampus (Houston et al., 2009). Thus, it will be of interest to determine if our observed effects are specific to excitatory transmission or generalize to inhibitory transmission as well.

MSN CaMKII Modulation of Intrinsic Excitability

We have observed that inhibition of CaMKII in dorsal striatal MSNs leads to an increase in intrinsic excitability. CaMKII-inhibited MSNs have more depolarized resting membrane potentials, increased membrane resistance, decreased rheobase current injection, and increased firing frequency. This fits with data in the hippocampus where an autonomously active form of CaMKII has been shown to suppress neuronal excitability by increasing cell-surface expression of A-type K+ channel, Kv 4.2, via phosphorylation (Varga et al., 2004). CaMKII inhibition in medial vestibular nucleus neurons increased intrinsic excitability via a reduction in BK-type calcium activated potassium currents

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(Nelson et al., 2005). While both of these channels are expressed in the striatum

and may modulate firing frequency additional mechanisms are likely responsible

for change input resistance and resting membrane potential (Schwanstecher and

Panten, 1994; Varga et al., 2000; Martin et al., 2004; Vacher et al., 2006). One

potential candidate underlying these excitability changes are the Kir2.1/2.3

channels which are highly expressed in the striatum and when downregulated

show a similar phenotype to the EAC3I increased excitability (Cazorla et al.,

2012). The increase MSN intrinsic excitability we see in the EAC3I mice would

drive greater release of GABA in both the globus pallidus and in the substantia

nigra pars reticulata. We did not note a bimodal distribution in the excitability

data suggesting that the CaMKII-mediated increase in excitability occurs in both

direct and indirect pathway MSNs. It would be interesting to examine the effect

of enhanced MSN excitability on downstream basal ganglia nuclei. With the

robust expression of CaMKII inhibitory peptide in the presynaptic terminals in

these regions it would be interesting to look for potential circuit wide changes and

the effects of presynaptic inhibition of MSN CaMKII.

Protracted CaMKII Inhibition: Regulation or Compensation

Opposing regulation of excitatory transmission and excitability suggests

that CaMKII serves as a molecular fulcrum counterbalancing decreases in

synaptic transmission with increases in excitatory output (Figure 26). CaMKII

would act as a check on runaway plasticity by compensating for alterations in

synaptic transmission or excitability. It will be important to determine if

alterations in extrinsic excitability are compensations for the decreased excitatory drive or vice versa. We know that the CaMKII-mediated decrease in sEPSC frequency is seen as early as 3 weeks, so it will be of great interest to see if the increase in excitability is seen early in development. Plotting the time course of the development of this effect will offer a better understanding of when these changes occur and if one precedes the other.

Effects of Early Postnatal GluN2B Deletion in Excitatory Synaptic Transmission in MSNs

We observed an increase in sEPSC amplitude in GluN2B KO animals compared to controls with no change in sEPSC frequency. These AMPAR-mediate sEPSCs data suggests an increase in postsynaptic AMPAR number and/or function. While at first seemingly contradictory, the loss of GluN1 in the CA1 region of the hippocampus late embryonically actually increases AMPAR-EPSCs and enhances the number of functional synapses (Grooms et al., 2006; Ultanir et al., 2007; Adesnik et al., 2008). This process is through to involve a homeostatic-like mechanism (Lu et al., 2011a). These data suggest that NMDARs actually oppose AMPAR trafficking to the synapse. Further postnatal deletion of GluN1, or both GluN2A and GluN2B leads to an increase in AMPAR-EPSCs (Gray et al., 2011). The AMPAR-mediated potentiation seen after the deletion of GluN1 requires the GluA2 subunit (Lu et al., 2011a). In agreement with these findings we saw no alteration in AMPAR EPSC current-voltage relationships, a measure of GluA2 content of AMPARs. This suggests the

AMPARs at the synapse in the GluN2B KO animals are GluA2-containing. In all, it seems that the loss of GluN2B or the indirect loss of GluN1 seen in these GluN2B Kos leads to an increase in GluA2-containing AMPAR number and/or function. Like previous reports with GluN2B deletion in the hippocampus (Gray et al., 2011), we did not see any alteration in PPR, suggesting the probability of release at these synapse is unaltered. These findings are seen despite the major loss of GluN2B we observed in the cortex, a major excitatory input to MSNs.

The deletion of GluN2B in the hippocampus leads to increased mEPSC frequency with no change in mEPSC amplitude, while the deletion of GluN2A leads to the opposite an increase in amplitude, but no change in frequency. Deletion of both subunits leads to an increase in frequency and amplitude. (Gray et al., 2011). We find that GluN2B deletion in MSNs in our mice leads to the opposite phenotype. This is in line with the deletion of GluN2B in dissociated cortical cultures where increases in mEPSC amplitude were observed (Hall et al., 2007).   This may suggest that GluN2B regulation of AMPA-mediated synaptic transmission is different in the striatum than the hippocampus.   Yet this early deletion of GluN2B contrasts to the removal in the adult hippocampus where no alterations in sEPSC frequency or amplitude are observed (von Engelhardt et al., 2008). It would be interesting to look at MSN mEPSC frequency and amplitude in GluN2A KO mice.

While we did not see a decrease in sEPSC frequency or observe changes in PPR, we did observe a significant reduction in dendritic spine density in

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GluN2B KO MSNs. These observations fit with numerous other reports that the deletion of GluN2B reduces spine density (Akashi et al., 2009; Espinosa et al., 2009; Gambrill and Barria, 2011). The deletion of GluN1 does not affect dendritic spine density, suggesting that the loss of GluN2B is underlying this morphological change (Adesnik et al., 2008). The loss of spines in the GluN2B KO MSNs may be due to an premature expression of GluN2A receptors (Gambrill and Barria, 2011). The divergence in morphology data and sEPSC frequency data suggest that the loss of spines is presumably from a loss of silent synapses or synapses that lack AMPARs. The increase in AMPAR/NMDAR data fits with the increase in sEPSC amplitude or a decrease in the NMDAR-mediated component. The loss of silent synapses would remove NMDAR-only containing receptors. The increase in CV-NMDAR/ CV-AMPAR ratio data we observed suggests that indeed the number of silent synapses is reduced in GluN2B KO MSNs.

Effects of Adult GluN2B Deletion on Glutamatergic Transmission in Striatal MSNs

We took advantage of the tet-OFF genetic system to delay the removal of GluN2B (after P45) to look at developmental and adult roles of this subunit. We found that while sEPSC amplitude and AMPAR/NMDAR ratios were increased like in the early deletion condition, but sEPSC frequency was significantly reduced. This would suggest a decrease in the probability of release or the number of synapses. We would like to look at PPR and dendritic spine density at

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this time point to further understand the mechanisms underlying the decrease in sEPSC frequency. These data differ from the deletion of GluN2B in the hippocampus which did not alter mEPSC frequency or amplitude. However, knock down of GluN2B is associated with a decrease in mEPSC frequency (Gambrill and Barria, 2011). This suggests that deletion of GluN2B in striatum in early adolescence versus adulthood has different effects on basal glutamatergic transmission.

Effects of GluN2B Deletion on Behavior

Alterations in synaptic transmission and dendritic spine number were associated with selective striatal-based behavior disruptions. GluN2BKO animals showed a hyperlocomotive phenotype with an escalating locomotor response to the open field compared to within session habituation observed in wildtype littermates. Previous reports using NMDA antagonists as well as GluN2B-preferring antagonists such as Ro 25-6981 at higher doses produced hyperlocomotion (Chaperon et al., 2003; Kiselycznyk et al., 2011). Additionally, in mice in which the GluN2B subunit was genetically replaced by the GluN2A subunit exhibited a hyperlocomotive phenotype (Wang et al., 2011). However, in our case when we delayed the deletion of GluN2B until P45 the hyperlocomotive phenotype was rescued. This suggests that early removal of GluN2B in development leads to this phenotype. The loss of GluN2B in adulthood does not significantly alter locomotor activity. It is known that levels of GluN2B are high in early postnatal development and then start to decline as GluN2A levels rise

(Monyer et al., 1994; Sheng et al., 1994). Additionally, the ability to induce plasticity varies with age and experience (Kirkwood et al., 1996; Quinlan et al., 1999; Yashiro and Philpot, 2008). In the first postnatal week, a time corresponding to the progressive enrichment of GluN2A subunits at the synapse, the efficacy of LTP induction is reduced at thalamocortical synapses (Crair and Malenka, 1995; Isaac et al., 1997; Lu et al., 2001). Interestingly experience dependent exchange of GluN2B for GluN2A subunits in the visual cortex (Quinlan et al., 1999) correlates with an elevated LTP induction threshold (Kirkwood et al., 1996). Together these data suggest that experience driven insertion of GluN2A increases the threshold for LTP induction. Many of our early GluN2B deletion effects could be driven by the unchecked GluN2A expression earlier than normal in development. The delayed removal of GluN2B may allow for proper synaptogenesis and synaptic maturation allowing for a rescue of locomotor behavior.

We observed that deletion of GluN2B early in postnatal development lead to a decrease in performance on the accelerating rotarod, especially in later trials, but it did not disrupt overall motor learning. Surprisingly, postponing the deletion of GluN2B until P45 led to a more severe deficit in rotarod performance in GluN2BKO mice. GluN2BKO mice did not show any significant motor learning over repeated trials compared to littermate controls, suggesting an important role for GluN2B in motor learning in the striatum. The enhanced deficit seen when the deletion of GluN2B is postponed until adulthood, suggests that compensation prevents serious motor deficits after early deletion, but disrupting a system which

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has utilized GluN2B signaling previously may lead to more serious deficits. In

contrast to these data, a study using GluN2A or GluN2B antagonists injected into

the dorsal striatum suggested that while GluN2A was crucial for motor learning

on the accelerating rotarod, GluN2B was not (Lemay-Clermont et al., 2011). This

discrepancy may be accounted for by issues with selectivity with the GluN2A

antagonist, NVP-AAM077 or the greater reliance on the loss of GluN1 subunit

also seen in our studies. Rotarod learning, especially the late component, seems

to involve an increase in NMDAR-mediated synaptic transmission and is

associated with an upregulation of GluN1 (Yin et al., 2009; D'Amours et al.,

2011). Another study showed that disrupting the interaction between CaMKII and

the GluN2B subunit did not disrupt rotarod or locomotor activity, suggesting that

the actual loss of the GluN2B or GluN1 receptor plays the crucial role (Halt et al.,

2012).

In summary, I have shown that both CaMKII and GluN2B are important for

modulating glutamatergic synaptic transmission in the striatum and striatal-based

behaviors. Importantly, CaMKII can bind the GluN2B subunit of the NMDAR and

this binding is necessary for LTP and memory formation (Barria and Malinow,

2005; Zhou et al., 2007; Foster et al., 2010). Conversely, GluN2A subunit does

not interact strongly with CaMKII making the GluN2B/CaMKII an important

complex for plasticity and learning and memory (Strack et al., 2000). These

molecules/complexes are good candidates in development for the regulation of

synaptogenesis, synaptic maturation, and synaptic dynamics. Indeed the

expression of GluN2A acts as a synapse stabilizer where the expression of GluN2B is associated with dynamic synapses (Gambrill and Barria, 2011).

Implications for Striatal CaMKII and GluN2B subunit in Parkinson's Disease and Addiction

Previous studies have linked striatal CaMKII and GluN2B to Parkinson's disease and addiction models. The active form of CaMKII is elevated following dopamine depletion (Picconi et al., 2004b; Brown et al., 2005) and striatal CaMKII inhibition rescues deficits in synaptic plasticity and motor behavior found in models of Parkinson's disease (Picconi et al., 2004b). Striatal CaMKII is essential for the motivational effects of reward cues on goal-directed behaviors (Wiltgen et al., 2007), as well as curbing D1R-mediated cocaine hyperlocomotion (Stein and Hell, 2010), and modulating excitability following chronic cocaine administration (Kourrich et al., 2012). Additionally, both acute and chronic amphetamine administration can modulate CaMKII levels in the striatum (Choe and Wang, 2002; Greenstein et al., 2007). GluN2B levels are decreased in animal models of Parkinson's disease (Oh et al., 1999; Dunah et al., 2000; Hallett et al., 2005) and antagonists of the GluN2B-containing receptor have shown antiparkinsonian actions in both rodents and monkeys (Nash et al., 2000; Steece-Collier et al., 2000; Nash et al., 2004). Various lines of evidence have shown that drug-induced craving is accompanied by a significant upregulation of GluN2B subunit expression (Ma et al., 2006; Ghasemzadeh et al., 2009; Schumann and Yaka, 2009) and blocking GluN2B-containing NMDAR receptors

in the striatum can inhibit drug craving and reinstatement (Ma et al., 2006; Ma et al., 2007). In all, these studies suggest that CaMKII or GluN2B inhibitors may represent potential useful approaches to treat Parkinson's disease symptomology and potentially disrupt or reset the maladaptive alterations in glutamatergic transmission seen in addiction.

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