PHARMACOLOGICAL AND ELECTROPHYSIOLOGICAL CHARACTERIZATIONS OF DOPAMINERGIC MODULATION OF LONG-TERM POTENTIATION IN THE HIPPOCAMPUS CA1 REGION: IMPLICATIONS FOR ADDICTION

by

MICHAEL STRAMIELLO

(Under the Direction of John J. Wagner)

ABSTRACT

Over the past fifty years, our understanding of dopamine has evolved from a mere acknowledgement of its precursory status in norepinephrine production to the realization that it orchestrates complex modulatory activity in the striatum (reviewed by Onn et al., 2000), the prefrontal cortex (reviewed by Seamans and Yang, 2004), and the hippocampus (reviewed by Lisman and Grace, 2005). Importantly, the hallmark of addictive drugs is their ability to augment dopamine levels in the brain. Because addiction engages the neurophysiological underpinnings of learning and memory, I focused my research on dopaminergic modulation of synaptic plasticity within the hippocampus CA1 region, the brain's gateway for storage and recall of many types of information. Specifically, I investigated the ability of dopamine to modulate long-term potentiation (LTP), a persisting enhancement of communication between neurons that serves as a cellular model for learning and memory.

Using extracellular recording methods, I demonstrated that both $D_{1/5}$ and D_3 agonists enhance LTP via distinct pathways that ultimately converge upon NR2B-NMDARs. I also showed that D_4 receptor activation inhibits $D_{1/5}$-mediated, but not D_3-mediated, enhancement of

LTP. With whole-cell voltage clamp recording technique, I provided evidence that D_3-mediated disinhibition in the stratum radiatum of hippocampus CA1 takes place at extrasynaptic $GABA_A$ receptors innervated by μ-opioid receptor-expressing interneurons. Finally, I addressed the laminar-specificity of cocaine-mediated LTP enhancement in the CA1 region: D_3 receptor activation is required for this effect in stratum radiatum; $D_{1/5}$ receptor activation is required for this effect in stratum oriens. Taken together, my results illustrate the multifaceted nature of dopaminergic modulation of hippocampal LTP and provide numerous pharmacological targets for altering hippocampal output.

INDEX WORDS: cocaine; synaptic plasticity; disinhibition; NR2B-NMDAR; D1/5 receptor; D3 receptor; D4 receptor; Ro256981; SKF38393; PD128907; PD168077; stratum radiatum; stratum oriens

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B.S., University of Georgia, 2005

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2010

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May 2010

DEDICATION

In memory of my dear sister, who would be very proud of me.

ACKNOWLEDGEMENTS

I am indebted to past students of the Wagner Lab and many other researchers whose work served as a foundation for my own. I thank my ARCS sponsors, Kay and Buddy Weiss, as well as the ARCS Foundation and the University of Georgia Graduate School for their support. I am also grateful to Gaylen Edwards and Phil Holmes for their time and teachings. Most of all, I thank John Wagner for his wisdom and guidance.

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CHAPTER ONE

GENERAL INTRODUCTION AND LITERATURE REVIEW

Dopamine has a number of important roles in the central nervous system: it is critical for cognition, initiation of motor movement, and working memory. Its malfunction in these capacities is thought to contribute to schizophrenia, Parkinson's Disease, and attention-deficit hyperactivity disorder (ADHD), respectively. Because of its involvement with the brain's reward pathway, dopaminergic activity has also become a major focus in the study of addiction. Together, these disease states affect tens of millions of U.S. citizens and take an estimated annual toll of over $450 billion on the U.S. economy (Table 1).

TABLE 1.1 Disorders of dopaminergic function: impact within the United States

	U.S. population affected (millions)	Cost to U.S. economy (billions annually- USD)	References
Addiction†	23	366	*a, b, c*
ADHD	4.5	35	*d*
Parkinson's disease	0.5	6	*e*
Schizophrenia	2.5	62	*f, g*

a) U.S. Department of Health and Human Services, 2008
b) Office of National Drug Control Policy, 2004
c) Harwood, 2000
d) Pelham et al., 2007
e) U.S. Department of Health and Human Services, 2006
f) Regier et al., 1993
g) Wu et al., 2005

† Addiction is defined as having a substance use disorder or receiving treatment at a specialty facility within the past year for alcohol or an illicit drug (does not include use of nicotine or caffeine alone). Costs of addiction include those that are crime-related.

Addiction is often described as a "usurpation" or "hijacking" of the neural mechanisms that subserve learning and memory, presumably through neuroadaptations involving dopaminergic neurotransmission. In my efforts to elucidate such changes in the drug-exposed brain, I have focused my efforts on the hippocampus, a structure widely regarded as the brain's epicenter for learning and memory acquisition. Historically, the impact of dopamine acting

within the hippocampus has received little attention, despite that the hippocampus is often credited for its influence on dopaminergic activity within the nucleus accumbens. Recently, however, dopamine has become known as a critical modulator of synaptic plasticity in the hippocampus- here, dopamine can enhance learning (Lisman and Grace, 2005) and is known to play a key role in regulating storage of unpredicted information (Li et al., 2003).

The Hippocampus:

Of all brain slice preparations, the hippocampal slice is perhaps the one most commonly employed (see Table II). The hippocampus is particularly well-suited to slice studies because of its distinct anatomy and well-characterized circuitry, which remains largely intact in transverse sections. Additionally, curvature of the rodent hippocampus allows for transverse slices to be taken from horizontal, coronal, or even sagittal brain sections.

TABLE 1.2 The brain slice preparation

Advantages	Disadvantages
• rapid preparation	• transient ischemia
• mechanical stability	• limited viability
• environmental control	• lack of blood-borne factors
• elimination of the BBB†	• peripheral damage to slice
• general structural integrity	• severed inputs/outputs
• direct visualization	

† Blood brain barrier

Points in table are taken from text of Schurr (1986).

The hippocampus is a bilateral structure located in the medial temporal lobes of the brain. It is known for its importance in declarative memory, novelty detection, and spatial navigation. The significance of the hippocampus is perhaps best illustrated by the well-publicized case of Henry Molaison (aka "HM"), whose hippocampal function was compromised at age twenty-

seven by a surgical procedure intended to alleviate symptoms of epilepsy. The surgery heavily damaged Molaison's hippocampus and entorhinal cortex (EC), rendering him incapable of most long-term memory formation (Corkin et al., 1997).

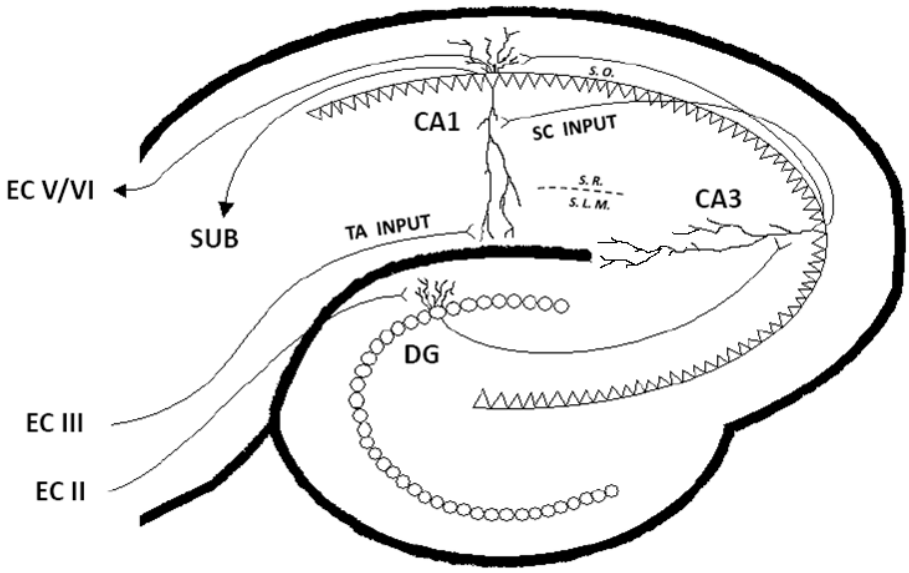

Figure 1.1. The hippocampal slice. The hippocampus receives major inputs from the entorhinal cortex (EC) layers II and III. Input from EC II is processed by the "trisynaptic circuit," first synapsing in the dentate gyrus (DG), and then the CA3 region, before arriving at CA1 pyramidal cells via Schaffer collaterals (SC). Conversely, EC III input is sent directly to CA1, via the temporoammonic (TA) pathway. *S.L.M., stratum lacunosum-moleculare; S.R., stratum radiatum; S.O., stratum oriens; SUB, subiculum.* Image by Michael Stramiello.

The EC acts as the primary input to the hippocampal formation (HF, a collective term referring to the hippocampus proper, the dentate gyrus, and the subiculum). Somata of the hippocampus's principal neurons, pyramidal cells, are densely packed into a layer that spans clearly identifiable regions (CA1-CA3). These regions are interconnected via the "trisynaptic circuit," which processes information in a chiefly unidirectional manner: from layer II entorhinal cortex (EC) to the dentate gyrus (DG) via the perforant pathway, from the DG to CA3 cells via the mossy fibers, and from CA3 to CA1 cells via the Schaffer collaterals (SCs). This SC pathway, which primarily innervates the proximal apical dendritic layer (stratum radiatum) and

the basal dendritic layer (stratum oriens) of CA1, constitutes one of two major glutamatergic inputs to that region. Its counterpart, the temporoammonic (TA) pathway, arrives directly from layer III EC and terminates in the distal apical dendritic layer (stratum lacunosum-moleculare) of CA1, bypassing trisynaptic processing. Although CA1 pyramidal cells provide a major output from the hippocampus proper, conveying information to the subiculum and deep layers of the EC, they must first execute the paramount task of interpreting a combination of trisynaptically-processed and direct cortical signals.

In 2001, Lisman and Otmakhova described a model in which animals acting in familiar settings make predictions using stored firing sequences in the DG and CA3; those signals, carried by SCs to the CA1, are then compared to sensory input arriving directly from the EC via the TA pathway. These authors further suggested that upon mismatch between predicted and actual sensory input, the CA1 indirectly triggers novelty firing in the ventral tegmental area (VTA), a midbrain area that serves as a primary source of forebrain dopamine. The potential importance of such a dopamine-mediated shift in CA1 input responsiveness might facilitate LTP of information being buffered upstream in the trisynaptic circuit (i.e. in the DG and CA3), while dampening possibly disruptive ongoing sensory input arriving directly from the EC via the TA pathway (see Dopaminergic Neurotransmission, below). This is perhaps relevant to addiction, as many drugs of abuse enhance dopamine release and exposure could effectively trigger a shift in dopamine responsiveness that enhances the formation of memories and habits associated with drug-seeking and drug-taking.

Synaptic Plasticity (also see Glutamatergic Neurotransmission, below):

Mechanisms of synaptic plasticity such as long-term potentiation (LTP) and long-term depression (LTD) are widely believed to provide the molecular basis for learning and memory (Abraham, 1999; Malenka and Nicoll, 1997). The former, LTP, is the focus of my studies. There are many studies demonstrating that LTP is a likely mechanism for memory storage *in vivo*. For example, it has recently been shown that inhibition of a PKC isoform necessary for maintenance of LTP can produce a persistent loss of spatial memory in an active place avoidance paradigm (Pastalkova et al., 2006). Also, fear conditioning has been shown to cause LTP-like changes in synaptic function which are required for the formation of fear memories (McKernan and Shinnick-Gallagher, 1997; Rogan et al., 1997).

As the importance of LTP and related cellular phenomena becomes increasingly well-documented, it has also emerged that at least several forms of LTP exist, and differences can be detected depending on the synapse being observed, the developmental stage of the subject, and the distinct tetanus protocol being utilized (Malenka and Bear, 2004). My focus, like that of many others, is on LTP in the mature hippocampus CA1 region. Despite a tremendous amount of research, strikingly few generalizations can be made about exactly which cellular events and molecules are required to mediate this LTP. In part, such uncertainty results because of the wide variety of experimental conditions utilized by LTP researchers. However, it is generally accepted that requirements for LTP induction include glutamate-mediated N-methyl-D-aspartate receptor (NMDAR) activation, increased postsynaptic Ca^{++} concentration, and activity of calcium/calmodulin-dependent protein kinase II (CaMKII) (Malenka and Bear, 2004), while other components such as alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors

(AMPARs) appear to be more important for subsequent LTP expression (Song and Huganir, 2002). Although there remains ambiguity regarding mediators of LTP, much is known about its modulators.

Unlike mediators, modulators are capable of altering LTP but are not necessary for its induction or expression. For example, proteins such as cAMP-dependent protein kinase (PKA), protein kinase C (PKC), Src, brain-derived neurotrophic factor (BDNF), and mitogen-activated protein kinase (MAPK), as well as neurotransmitters such as gamma-aminobutyric acid (GABA), serotonin, and dopamine, are known to play key modulatory roles in LTP (Lynch, 2004; Malenka and Bear, 2004; Otmakhova and Lisman, 1996; Salter and Kalia, 2004; Wang and Arvanov, 1998). In many cases, addictive drugs are capable of modulating of LTP (see Addiction, below), and repeated exposure can contribute to long-lasting neuroadaptations that might be involved in drug-craving and relapse (Thompson et al., 2004).

Addiction:

It is interesting to note that the term "addiction" has itself been the focus of ongoing debate (O'Brien et al., 2006a; O'Brien, et al., 2006b; Erickson & Wilcox, 2006; Fainsinger et al., 2006; Hasin, 2008; Miller 2006; Streltzer et al., 2006). Beginning with the revised third edition of the DSM (American Psychiatric Association, 1987), "addiction" was replaced with "substance use disorders," which encompasses both "substance abuse" and "substance dependence" (Kranzler & Li, 2008). This change in terminology, which by one vote passed the APA revision committee (O'Brien et al., 2006b), was reportedly driven by at least three major concerns: 1) that the term "addiction" would perpetuate stigmatization of people with substance use disorders (O'Brien et al., 2006b), 2) that practitioners might avoid discussing "addiction" with their

patients in fear of damaging their relationship (Kranzler & Li, 2008), and 3) that the term "addiction" had been significantly devalued due to its misuse and overuse (Kranzler & Li, 2008). In recent years, prominent experts such as Charles O'Brien and Nora Volkow have advocated for readoption of "addiction," charging that the change in terminology has led to harm (i.e., confusion among physicians resulting in unnecessary withholding of palliative medications from suffering patients) outweighing the APA committee's original apprehensions. Despite concerns about use of the label "addiction," experts largely agree upon its definition (O'Brien et al., 2006b), which is reflected in the DSM-IV criteria for substance use disorders (Table 4).

TABLE 1.3. Criteria for substance use disorders

Abuse	Dependence
• recurrent use resulting in failure to fulfill major role obligation at work, home, or school	• tolerance (marked increase in dose taken to achieve effects; marked decrease in effects)
• recurrent use in physically hazardous situations	• characteristic withdrawal symptoms; substance taken to avoid or relieve withdrawal
• recurrent substance-related legal problems	• much time/activity to obtain, use, recover
• continued use despite persistent or recurrent social or interpersonal problems caused or exacerbated by effects of the substance	• use continues despite knowledge of adverse consequences (e.g., failure to fulfill role obligation, use when physically hazardous)
	• substance taken in larger amount and for longer period than intended
	• important social, occupational, or recreational activities given up or reduced because of substance use
	• persistent desire or repeated unsuccessful attempts to quit

Points in table are taken from the DSM IV-TR (American Psychiatric Association, 1994)

Like the proteins and neurotransmitters previously listed, drugs of abuse are also capable of modulating LTP in hippocampus CA1(Fujii et al., 1999; Pu et al., 2002; Pyapali et al., 1999;

Thompson et al., 2005). It is perhaps unsurprising then that such drugs are often addictive in humans, particularly in light of the emergent connection between addiction and learning via underlying neural mechanisms. For example, both learning experiences and addictive drugs have been shown to produce similar changes in dendritic morphology, such as a persisting increase in spine density (Moser et al., 1994; Robinson et al., 2001). Drug-related stimuli also activate the same glutamate-rich neuronal circuitry implicated in learning and memory (Wolf et al., 2004), and abused drugs are known to activate signal transduction pathways critical to learning, such as cAMP/PKA/CREB (Berke and Hyman, 2000). Perhaps the most widely recognized link between learning and addiction, however, is the crucial involvement of dopamine in both processes.

Addictive drugs do not all share a common molecular structure or affinity for any specific receptor protein. They do, however, share one fundamental characteristic: they augment neural firing of reward-relevant dopaminergic neurons that arise in the midbrain. These neurons innervate forebrain structures such as the nucleus accumbens, the prefrontal cortex, and the hippocampus (O'Brien and Gardner, 2005), and modulate brain-reward function. While examination of such dopaminergic activity typically focuses on the nucleus accumbens, my research concentrates on the effects of dopamine release in another target of ventral tegmental area (VTA) efferents, the hippocampus, which itself sends glutamatergic efferents to the nucleus accumbens (NAc) and can activate the VTA. Augmentation of dopaminergic activity in the hippocampus may contribute to the maladaptive learning processes intertwined with addiction. Importantly, the hippocampus has already been shown to mediate reinstatement of drug-seeking (Sun & Rebec, 2003; Fuchs et al., 2005; Rogers & See, 2007).

Cocaine:

Cocaine is a robust reinforcer and cessation of its use elicits fewer physical withdrawal symptoms than other addictive drugs, such as heroin or alcohol (Haney, 2009; Leshner, 1997). This makes it a useful agent for behavioral models of addiction, such as those previously employed in our lab (Thompson et al., 2004). Man has made use of cocaine's psychostimulant effects for millennia, beginning in the elevated regions of South America, the native habitat of the coca plant from which cocaine is derived. Traditionally, coca leaves are balled up into "quids" and chewed throughout the workday for energy and contentment, or used to make tea (uses much akin to contemporary uses of tobacco, coffee, or tea). The documented abuse of coca products emerged with the development of extraction/isolation and refinement processes that led to overprescription of cocaine for treatment of various illnesses and increased availability of much more abuse-friendly forms of the drug (powder and tinctures) than the traditional leaf preparations offered. Concerns about cocaine grew with the advent of "crack," a freebase form of cocaine that is smoked for a more rapid onset of effects than may be experienced by sniffing or intravenous injection. Its recreational use is now banned, but cocaine is still employed as a local anesthetic in nearly 200,000 U.S. surgeries per year (mostly in procedures involving the nose and throat) (Gahlinger, 2004). Cocaine exerts its local anesthetic effects via voltage-gated sodium channel blockade and its psychoactive effects via dopamine transporter (DAT) blockade.

Dopaminergic Neurotransmission:

Prior to the mid-1950s, dopamine was regarded merely as a precursor to norepinephrine and epinephrine (Cooper et al., 2004). The discovery of dopamine's distinct, non-uniform distribution in the brain led to its recognition as a neurotransmitter (Strange, 2000), and

subsequent discoveries linked dopaminergic function to various disease states. While it is now often associated with reward-seeking, dopamine is more generally recognized as a critical modulator of working memory (Barch, 2004): optimal dopamine levels are required for proper memory performance, and dopamine-enhancing pharmacotherapy can improve deficiencies in brain function during working memory tasks (Barch, 2004). Pharmacotherapies targeting dopamine neurotransmission have also become cornerstones in treatment of schizophrenia and Parkinson's disease (see Table 4).

TABLE 1.4. Dopaminergic pharmacotherapy for brain disorders

Disorder	Drug	Relevant Action
Addiction	bupropion†	DAT inhibitor
	disulfiram*	DBH inhibitor
ADHD	amphetamine	VMAT inhibitor / DAT reversal
	lisdexamfetamine	(prodrug for amphetamine)
	methylphenidate	DAT inhibitor
Parkinson's disease	apomorphine	D2-like agonist
	bromocriptine	″
	L-DOPA	DA precursor
Schizophrenia	chlorpromazine	D1- , D2-like antagonist
	perphenazine	″
	haloperidol∞	D2-like antagonist
	clozapine∞	″
	olanzapine∞	″
	quetiapine∞	″
	risperidone∞	″
	sulpiride∞	″
	aripiprazole∞	D2-like partial agonist

† Bupropion, a commonly prescribed anti-depressant, is marketed under the trade name "Zyban" as a cessation aid for nicotine use. Its action at DAT is complemented by concurrent blockade of NET and inhibition of nAChR-evoked dopamine overflow (Miller et al., 2002).

* Disulfiram is marketed as a deterrent for alcohol consumption due to its ability to inhibit acetaldehyde dehydrogenase; however, recent research suggests that disulfiram may be an effective treatment for cocaine addiction because of its ability to indirectly enhance dopamine levels and diminish drug craving (Gossop and Carroll, 2006).

∞ These "atypical" antipsychotics also have significant antagonist activity at other receptors, particularly 5-HT2R.

Dopaminergic nuclei can be categorized into three major groups based on the length of their efferents: ultrashort, intermediate-length, or long (see Table 5). The long systems, originating in the VTA and SN, receive a large amount of interest because of their well-documented involvement in the pathogenesis of Parkinson's disease and addiction. Tyrosine hydroxylase within these dopamine-producing cells acts as a rate-limiting enzyme in the synthesis of dopamine, converting tyrosine to L-DOPA (which is well-known for its therapeutic use in the treatment of Parkinson's disease). DOPA decarboxylase converts L-DOPA into dopamine, which is then packaged into vesicles via the vesicular monoamine transporter (VMAT; Nestler et al., 2001) and released into the synapse.

TABLE 1.5. Overview of dopaminergic systems

Category (based on length of efferents)	System	Connections
ultrashort	interplexiform	intra-retina
	periglomular	intra-olfactory bulb
intermediate-length	tuberohypophyseal (tuberoinfundibular)	hypothalamus → pituitary
	periventricular	medulla → hypothalamus
	incertohypothalamic	intra-hypothalamic; hypothalamus → thalamus; intra-periaqueductal grey; intra-zona incerta; zona incerta → hypothalamus
long	mesostriatal	SN / VTA → anteromedial striatum
	mesocortical	VTA / SN → insular and prefrontal cortices
	mesolimbic	VTA / SN → limbic cortices, amygdala, NAc, hippocampus
	mesodiencephalic	SN / VTA → thalamus; VTA / SN → hypothalamus
	mesorhombencephalic	SN → spinal cord; SN & VTA → monoaminergic nuclei; SN / VTA → superior colliculus, reticular formation, periaqueductal grey

Chart adapted from Oades & Halliday (1987); incorporates information from Cooper et al., (2004).

Dopamine transporters (DATs) are responsible for the reuptake of dopamine into dopaminergic neurons following its release into the synapse. Only these neurons express DAT, which is localized to peri- and non-synaptic sites and found at relatively low densities. While DATs represent one clearance mechanism for extracellular dopamine, diffusion is typically the primary force responsible for limiting peak synaptic levels and spillover of DA. DAT does, however, limit the lifetime and sphere of influence of quantal dopamine transients (Cragg & Rice, 2004). The DAT is a protein of key importance in the actions of various psychostimulants (cocaine, amphetamine, and amphetamine-derivatives) and bupropion, an antidepressant and cessation aid for smoking. While the exact actions of amphetamine at the DAT may be indirect, cocaine and bupropion exert their effects largely via direct blockade of dopamine movement through DATs. All of these drugs ultimately enhance the actions of dopamine at its receptors.

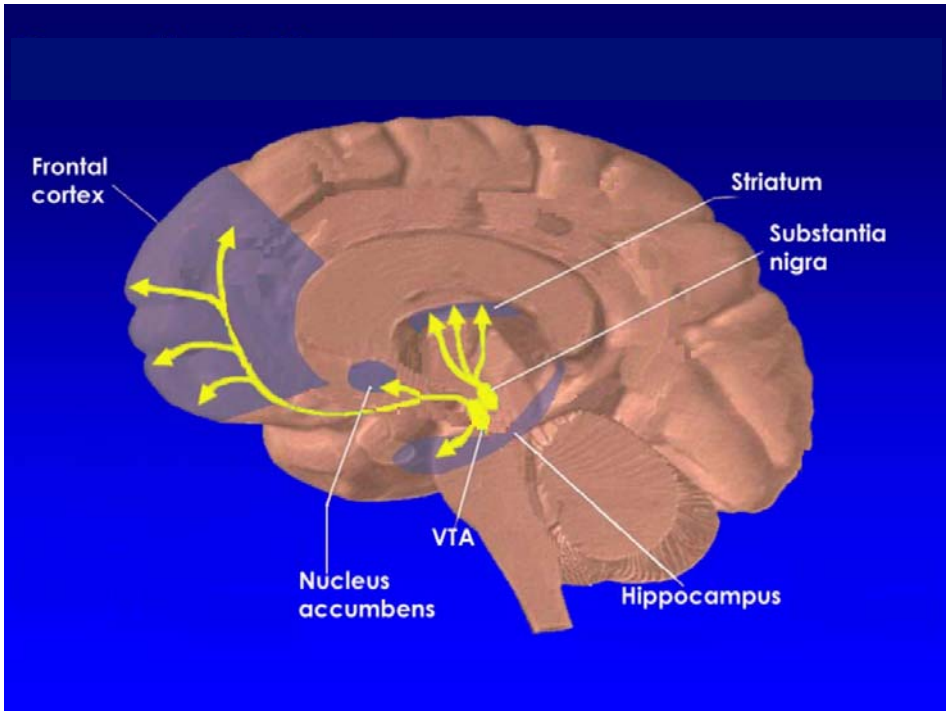

Figure 1.2. Dopamine pathways. Public domain image (courtesy of the NIH/NIDA Image Bank) adapted by Michael Stramiello

Dopamine receptors can be categorized into two major groups: D1-like and D2-like (see Table 6). All are G-protein coupled. D_2 receptors often function as autoreceptors, hence their abundance in brain regions heavily populated by dopaminergic nuclei (SN, VTA, ZI). D_3 receptors are notable because of their exceptionally high affinity for dopamine. Dopamine acting at D_5 receptors has recently been shown to stimulate PI hydrolysis in the hippocampus (which is relatively D_5-enriched). In recent years, the D_5 receptor has received increased attention, but this work remains hindered by a lack of pharmacological agents capable of discriminating between it and D_1 receptors. Caution must be used when selecting D1/5 agents: while the most commonly used ligands (SKF38393 and SKF81297- *aka 6-chloro-PB*) activate both AC and PI hydrolysis when used in appropriate concentrations, several ligands (SKF83822, SKF85174, and SKF82684) activate AC exclusively, and one (SKF838959) is known for its ability to activate PI hydrolysis without stimulating AC (Jin et al., 2003). Additionally, care must be used when interpreting results obtained with "dopaminergic" and "serotonergic" ligands, as currently available compounds typically have high affinity for both dopamine and serotonin receptors.

In the hippocampus, dopamine has been reported to modulate excitatory and inhibitory neurotransmission (Gribkoff and Ashe, 1984; Hsu, 1996), voltage-gated ion channels (Cantrell et al., 1997), and calcium-activated K+ current (Benardo and Prince, 1982). Importantly, there is recent evidence that dopaminergic activity in the hippocampus during anticipation of reward can promote memory formation prior to learning (Adcock et al., 2006), and that spatial novelty facilitates hippocampal LTP via activation of $D_{1/5}$ receptors (Li et al., 2003). Moreover, it has been demonstrated that dopaminergic input from the VTA can gate hippocampal LTP (Lemon and Manahan-Vaughan, 2006). It is this role of dopamine that I investigate in the hippocampus CA1 region.

TABLE 1.6. Overview of dopamine receptors

Family	Receptor	Affinity for Dopamine[a]	Signaling	Influence on ionotropic activity	High/moderate mRNA expression[a]
D1-like	D1	micromolar	AC ↑	NR2A-NMDARs ↓[b]	CP, NAc, OT, Am
	D5	submicromolar	AC ↑, PLC ↑[c]	NR2B-NMDARs ↑[b]	Hi, Th
D2-like	D2	micromolar	AC ↓	—	SN, VTA, ZI, CP, NAc, OT
	D3	nanomolar	AC ↓	$GABA_A$Rs ↓[d, e]	NAc
	D4	submicromolar	AC ↓	—	—

Am, amygdala; CP, caudate putamen; Hi, hippocampus; NAc, nucleus accumbens; OT, olfactory tubercle; SN, substantia nigra; Th, thalamus; VTA, ventral tegmental area; ZI, zona incerta.

a) Cooper, et al., 2003
b) Varela et al., 2009
c) Sahu et al., 2009
d) Chen et al., 2006
e) Swant et al., 2008

While DATs (which are generally considered to be a hallmark of dopaminergic fibers) are yet to be detected in the hippocampus, recent work by Kwon et al. (2009) revealed dopaminergic fibers (positive for tyrosine hydroxylase, negative for dopamine-β-hydroxylase) within the stratum oriens layer of hippocampus CA1. Expression of D_1, D_3, D_4, and D_5 receptors occurs in all dendritic layers of the hippocampus CA1, with D_2 receptors largely confined to the pyramidal cell body layer (Huang et al., 1992; Khan et al., 1998). While the earliest studies investigating dopaminergic neurotransmission in the hippocampus were conducted with dopamine itself (Benardo and Prince, 1982), subsequent research on the role of dopamine in LTP has typically utilized compounds that preferentially bind certain subtypes of the receptor (Frey et al., 1989; Frey et al., 1991; Huang and Kandel, 1995; Mockett et al., 2004; Navakkode et al., 2007; Otmakhova and Lisman, 1996; Swanson-Park et al., 1999).

At the beginning of the 1990s, studies of dopaminergic activity in the hippocampus began to focus on its modulatory effects on synaptic plasticity, particularly its role in late-LTP, a protein-synthesis-dependent form of synaptic plasticity that can persist for many hours *in vitro*. Initial work carried out with D2-like receptor antagonists, and then repeated with $D_{1/5}$-specific antagonists, demonstrated that late LTP in the hippocampus CA1 is dependent upon the activity of endogenous dopamine (Frey et al., 1989; Frey et al., 1990; Frey et al., 1991). A controversy has since developed regarding the putative capacity of dopaminergic agonists to induce this late-phase LTP in the absence of a tetanus event (Frey et al., 1993; Huang and Kandel, 1995; Mockett et al., 2004; Navakkode et al., 2007), an action that is perhaps attributable to $D_{1/5}$ receptor-mediated reversal of long-term depression (LTD; Mockett et al., 2007). Less controversial has been the discovery that $D_{1/5}$ receptor activation can enhance early-LTP in the CA1 region (Otmakhova and Lisman, 1996), a finding consistent with previous work showing that D1/5 antagonism can mitigate early LTP (Frey et al., 1991) and further supported by two ensuing discoveries: first, that via a cAMP/PKA mechanism, D1/5 receptor activation can inhibit depotentiation of LTP by low-frequency stimulation (Otmakhova and Lisman, 1998); and second, that inhibition of cAMP/PKA is capable of decreasing early LTP (Otmakhova et al., 2000).

In 2008, I demonstrated that D1/5-mediated LTP enhancement is dependent on both PKA and Src-family kinases (SFKs) and is reliant upon NR2B-NMDAR activity. More recently, Varela et al. (2009) reported bidirectional modulatory effects of $D_{1/5}$ receptor activation in the CA1 region, showing that depression of synaptic NR2A-NMDAR currents required direct physical interaction between NR2A and the D_1R itself, while potentiation of NR2B-NMDAR currents required G-protein activity. Because the synaptic NR2A/NR2B ratio was greater at TA

inputs than at SC inputs, and NMDAR subunit composition was found to determine the directionality of $D_{1/5}$ modulatory effects, activation of $D_{1/5}$ receptors via amphetamine or SKF81297 ultimately exerted a net shift away from direct cortical excitatory influence of the TA pathway and toward SC-mediated, excitatory influence of the highly processed trisynaptic circuit (Varela et al., 2009) (see Fig 2). These findings are consistent with the previously described Lisman & Otmakhova (2001) model (see The Hippocampus, above), and they demonstrate how dopamine is particularly well-suited to play a critical modulatory role in comparator tasks of the CA1 region. As this role is perhaps imperative for normal hippocampal function and learning, its malfunction may contribute to disease states. For example, Lisman and Otmakhova (2001) described a scenario whereby hyperdopaminergic activity in the CA1 might effectively relegate TA signaling to the extent that the excessive imbalance of TA and SC inputs would manifest pathophysiology (e.g. schizophrenia).

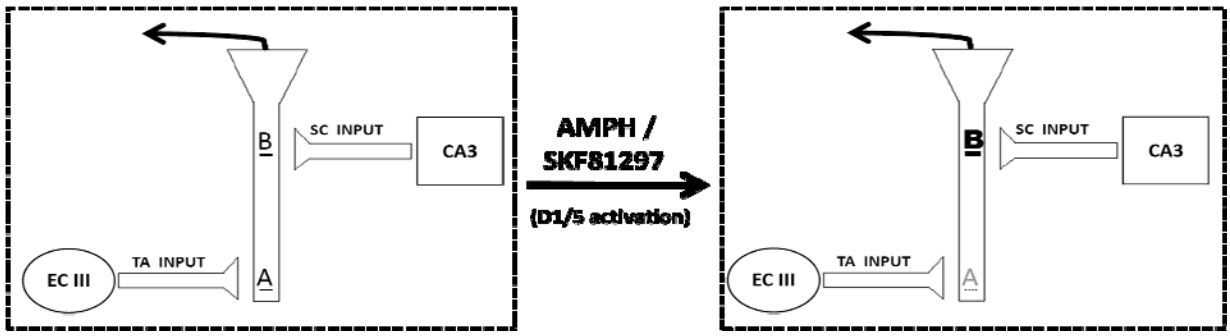

Figure 1.3. The influence of D1/5 activation on CA1 apical dendrites. In the CA1 region, distal apical dendrites receive excitatory input primarily via the TA pathway, and proximal apical dendrites primarily via the SC pathway. The results of Varela et al. (2009) indicate that via D1/5 activation, amphetamine and SKF81297 decrease responsiveness of CA1 cells to TA input (A) and increase their responsiveness to SC input (B). Images by Michael Stramiello.

Glutamatergic Neurotransmission:

There is a growing body of evidence for both direct and indirect interactions between dopamine and glutamate receptors which documents the capacity of dopamine receptors to affect glutamatergic activity by altering NMDAR channel function (Cepeda et al., 1993; Chen and Roche, 2007; Lee et al., 2002; Levine et al., 1996; Snyder et al., 1998), and/or NMDAR distribution (Dunah and Standaert, 2001; Hallett et al., 2006; Kopp et al., 2007). Glutamate is considered to be the brain's primary excitatory neurotransmitter. It is distributed throughout the brain in a fairly uniform manner (Rang et al., 2003). It is typically synthesized from glutamine by glutaminase. Glumate receptors can be divided into ionotropic and metabotropic families. The ionotropic glutamate receptors include widely expressed NMDARs and AMPARs, as well as kainate receptors, which are concentrated in a few specific areas of the brain.

Metabotropic glutamate receptors, mGluR1-8, are also widely expressed in the brain (Cooper et al., 2004). Unlike the ionotropic glutamate receptors, mGluRs do not form an ion channel (though they can regulate ion channels). mGluRs be divided into three major groups: Group I (which includes mGluR1 and mGluR5), Group II (which includes mGluR2 and mGluR3), and Group III, which includes mGluR4, mGluR6, mGluR7, and mGluR8). Group I mGluRs are positively coupled to PI hydrolysis, while Groups II and III are negatively coupled to AC. mGluRs are located both pre- and postsynaptically. Metabotropic glutamate receptors may play an important role in long-term plasticity. Pre-tetanus application of a mGluR antagonist has been demonstrated to block LTP, and agonist application may inhibit adenylyl cyclase or activate PLC, depending on the class of mGluR (Byrne et al., 2004). PLC-mediated Ca release may actually be necessary for LTD in Purkinje cells (Byrne et al., 2004).

NMDARs are postsynaptic and typically contain four subunits: two essential NR1 and two NR2 subunits, with NR3 subunits usually limited to a neuromodulatory role in certain subpopulations (Chen and Roche, 2007). Whereas NR1 is ubiquitous in the brain throughout development, NR2 isoforms are broadly expressed but developmentally regulated. The NR2A subunit is increasingly expressed as animals mature; NR2B expression decreases overall, but remains substantial in the hippocampus and cortex (Chen and Roche, 2007). In the hippocampus, it has been suggested that the NR2A subunit plays a significant role in the generation of LTP, while NR2B, which has a relatively greater affinity for glutamate (Kutsuwada et al., 1992), may be of primary importance to LTD induction (Liu et al., 2004). NMDAR activation requires the binding of glutamate and a co-agonist (D-serine or glycine) in the presence of sufficient depolarization to relieve a voltage-dependent Mg^{++} blockade that prevents cation flow through the receptors under resting conditions. Once activated, NMDARs have a high level of Ca^{++} permeability, and are often implicated in excitotoxicity.

AMPARs, also located postsynaptically, are tetramers consisting of various combinations of four subunits, GluR1-4. GluR2 subunits are noteworthy because they impart a large degree of Ca^{++} impermeability to AMPARs, they act to mitigate AMPAR conductivity, and they confer voltage-dependent inhibition at positive membrane potentials (Nestler et al., 2001). Rather than incorporating removal of a Mg^{++} blockade like NMDARs, AMPAR activation is largely dependent upon structural changes. Each AMPAR subunit contains a ligand-binding core. This is an extracellular, bilobed structure that consists of one stationary and one mobile lobe, separated by a binding cleft. In the presence of glutamate or other agonists, the ligand-binding core of each AMPAR subunit is stabilized in a “closed” state, the agonist enclosed in its binding cleft. Together, these four “closing” subunits result in a screw-like movement that makes the

receptor's ion channel accessible to hydrated ions (Armstrong and Gouaux, 2000), allowing for the influx of Na^+. In the unbound state, AMPAR subunit ligand-binding cores are effectively "open," with their binding clefts expanded, and the receptor's ion channel inaccessible to Na^+. In the presence of CNQX or other antagonists, the ligand-binding core of each AMPAR subunit is stabilized in this "open" state, and the receptor itself is stabilized in an inactive conformation that minimizes accessibility of its ion channel to Na^+ (Figure 1.4).

While NMDARs and AMPARs are widely distributed in the CNS, kainate receptors are concentrated in a few specific areas (Cooper et al., 2004) and can be located in the pre- or postsynaptic membrane. Kainate receptors, also tetramers, consist of various combinations of five available subunits, GluR5-7, KA1, and KA2. KA1 and KA2 are unlikely to function in homomeric states because they lack a functional ion channel, thus they are typically regarded as modulatory subunits for receptors containing GluR5-7 subunits (Nestler et al., 2001). Like AMPARs, kainite receptors have very low Ca^{++} permeability.

Glutamate receptors, especially NMDARs, AMPARS, and mGluRs, have long been implicated in long-term potentiation and long-term depression. While constitutive AMPAR trafficking can be observed in the electrophysiological phenomena of "run-down" and "run-up," regulated trafficking for these receptors might be evident in some forms of LTP and LTD, which are believed to be influenced significantly by activity-dependent modulation of functional AMPAR density in the postsynaptic membrane (Esteban, 2003; Passafaro et al., 2001). The regulated pathway by which many types of AMPARs are trafficked into the synaptic membrane is initiated by NMDAR activation (Malenka et al., 1989). Once nearby conduits provide sufficient depolarization in the presence of glutamate and a co-agonist, NMDAR magnesium

blockade is eliminated, allowing the influx of Na^{+} and Ca^{++} into the cell (Hayashi et al., 2000). One effect of this rise in intracellular Ca^{++} is the subsequent delivery of AMPAR subunits to the synapse, which has been seen with GluR1 in cell cultures (Malinow & Malenka, 2002).

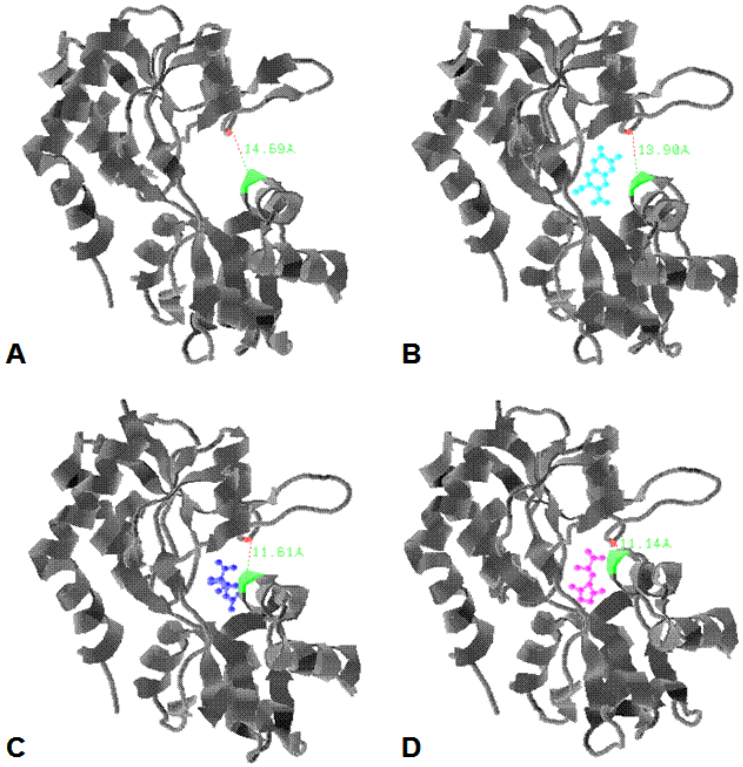

Figure 1.4. Progression of AMPAR GluR2 subunit binding cleft closure (as indicated by distance between Lys449 and Ser531). A. Unbound state (14.69Å) B. Bound antagonist DNQX (13.90Å) C. Bound partial agonist kainate (11.61Å) D. Bound full agonist AMPA (11.14Å). Images by Michael Stramiello using Rasmol and PDB 1FTO, 1FTL, 1FW0, and 1FTM from Armstrong N, Gouaux E. (2000).

This notion of regulated AMPAR trafficking to and from the synaptic plasma membrane has received additional support from light and electron microscopy studies, which provide evidence for the existence of AMPARs in both intracellular stocks and nonsynaptic regions of plasma membrane (Bredt et al., 2003; Lee et al., 2000). Additionally, inhibitors of membrane

fusion can preclude LTP, perhaps in part due to preventing insertion of AMPARs into the synaptic membrane (Luscher et al., 1999). Complementary to these proposed mechanisms for activity-induced increase in synaptic AMPAR density is a less characterized activity-regulated pathway for decreasing AMPAR density at the synapse (Passafaro et al., 2001). Little remains known about this process, but it is likely dependent upon an endocytotic mechanism, as endocytosis inhibitors have been shown to block LTD (Hollmann et al., 1989). This endocytosis might target synaptic AMPARs, as decreases in synaptic AMPAR density have been shown to accompany LTD (Luthi et al., 1999), and LTD can be occluded by induced decreases in surface density of AMPARs (Jin et al., 2005).

Earlier evidence points toward another possible activity-induced pathway for synaptic plasticity in which phosphorylation and dephosphorylation of receptors preexisting in the synaptic membrane contribute to LTP and LTD (Lee et al., 2000). Supporting experiments for these mechanisms demonstrate that during LTP, GluR1 is phosphorylated (Derkach et al., 1999) and cation channel conductance increased (Benke et al., 1998). A corresponding increase in single channel GluR1 conductance has been shown during and as a requirement for LTP (Lledo et al., 1998). Additionally, phosphorylation of NMDAR subunits potentiates ion flux through the receptor complex, evoking a stronger CaMKII response and increasing AMPAR phosphorylation (Byrne et al., 2004). It has been hypothesized that the kinase activity of CaMKII is triggered in response to strong stimuli that result in a large Ca^{++} influx, while weaker stimuli resulting in smaller Ca^{++} influx are more likely to stimulate activity of Protein Phosphatase 1 (PP1; Byrne et al., 2004). A balance between phosphorylation and dephosphorylation is therefore likely, and much like phosphorylation of glutamate receptors can contribute to LTP, dephosphorylation of AMPARs may play a role in LTD (Byrne et al., 2004).

Summary:

Long-term potentiation of glutamatergic neurotransmission is a critical component of the neurophysiological foundation for learning, and addiction is a disease that usurps many aspects of this foundation. Importantly, the hallmark of addictive drugs is their ability to augment dopamine levels in the brain. Drawing on these concepts, the following studies examine dopaminergic modulation of LTP within the hippocampus CA1 region, the brain's gateway for many forms of memory.

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CHAPTER TWO

D1/5 RECEPTOR-MEDIATED ENHANCEMENT OF LTP REQUIRES PKA, SRC FAMILY KINASES, AND NR2B-CONTAINING NMDARS

Stramiello, M., Wagner, J.J. 2008. *Neuropharmacology* 55, 871-877.

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Abstract:

The efficacy of the $D_{1/5}$ agonist SKF38393 (100 nM-60 μM) to increase long-term potentiation (LTP) in the CA1 region was investigated in the rat hippocampal slice preparation. The receptor specificity of this enhancing effect was confirmed using the $D_{1/5}$ antagonist SKF83566 (2 μM). Although the ability of $D_{1/5}$ receptors to increase both the persistence and the early magnitude of LTP has previously been linked to activation of the cAMP/PKA pathway, the subsequent molecular events leading to the enhancement of LTP have not been characterized. In experiments using SKF38393 (20 μM), a requirement for the activation of both protein kinase A (PKA) and Src family tyrosine kinase pathways was demonstrated, as pretreatment with either H89 (10 μM) or PP2 (10 μM) kinase inhibitors prevented the $D_{1/5}$-mediated enhancement of LTP. In addition, NMDA receptors containing the NR2B subunit were identified as a potential downstream target for this signaling pathway, as pretreatment with the selective antagonist Ro 25-6981 (1 μM) also prevented the $D_{1/5}$-mediated enhancement of LTP. The results identify a crucial role for NR2B-containing NMDA receptors in the modulation of LTP by $D_{1/5}$-receptors in the CA1, suggesting that endogenously released dopamine may act through this mechanism as a modulator of hippocampal-dependent learning and memory tasks.

Keywords - hippocampus; synaptic plasticity; neuromodulator; SKF38393; Ro25-6981

Introduction:

Dopamine is recognized as an important modulator of synaptic plasticity in the CA1 region of the hippocampus (Frey et al., 1990; Lisman and Grace, 2005) and dopamine-dependent alterations in synaptic plasticity in this region are involved in hippocampal-dependent learning (Lemon and Manahan-Vaughan, 2006). Although a role for the $D_{1/5}$ subtype of dopamine receptor in enhancing both the duration (Frey et al., 1991) and the initial magnitude (Otmakhova and Lisman, 1996) of long-term potentiation (LTP) has been established, it remains unclear how these effects are mediated. In the case of both the enhanced persistence of LTP (Frey et al., 1993), and the facilitating effects of dopamine agonists on the magnitude of early LTP (Otmakhova and Lisman, 1996), the activation of $D_{1/5}$ receptors has been linked to an increase in cAMP levels. Subsequent steps in this signaling pathway have not been described.

A major portion of the LTP evoked following activation of the Schaffer collateral input to CA1 is dependent upon the activation of N-methyl-D-aspartate receptors (NMDARs, (Collingridge et al., 1983). The contribution of specific NMDAR subunits to the induction of LTP and long-term depression (LTD) has recently been a topic of great interest and ongoing controversy (MacDonald et al., 2006). It has been suggested that the NR2A subunit plays a significant role in the generation of NMDAR-dependent LTP whereas the NR2B subunit may be of primary importance to the induction of NMDAR-dependent LTD (Liu et al., 2004). In this report, we address the question of how the actions of dopamine can alter functional participation of different NMDAR subtypes in the induction of LTP. Aside from any effects on LTP/LTD, it is clear that the activation of $D_{1/5}$ dopamine receptors could impact the activity of NMDARs through a variety of mechanisms encompassing both changes in channel function (Cepeda et al.,

1993; Chen and Roche, 2007; Lee et al., 2002; Levine et al., 1996; Snyder et al., 1998) and/or channel distribution (Dunah and Standaert, 2001; Hallett et al., 2006; Kopp et al., 2007).

In these experiments we have utilized a compressed train, high-frequency stimulation (HFS) protocol that results in the induction of an NMDAR-dependent LTP which is not dependent on the activity of PKA (Woo et al., 2003). This has allowed us to determine, without altering the initial "baseline" level of potentiation observed in the absence of $D_{1/5}$ receptor activation, whether a $D_{1/5}$-mediated increase in PKA activity is involved in the ability of the $D_{1/5}$-selective agonist SKF38393 to enhance the magnitude of LTP. Our findings indicate that dopaminergic activation of PKA promotes the participation of NR2B-containing NMDARs in the induction of LTP in a manner also dependent upon the activation of Src family kinases (SFKs). These results demonstrate a potential link between the gating function of PKA (Blitzer et al., 1995) and a SFK-dependent enhancement of LTP via NR2B-containing NMDARs that can be initiated by $D_{1/5}$ receptor activation in the CA1 region of the hippocampus.

Methods:

1. Extracellular electrophysiology

Hippocampal slices were prepared from male Sprague-Dawley rats (40-90 days old) using an experimental protocol performed in compliance with the University of Georgia Animal Care and Use guidelines. All rats were anesthetized with halothane prior to decapitation. The brain was removed and submerged in ice-cold, oxygenated (95% O_2/ 5% CO_2) dissection artificial cerebrospinal fluid (ACSF) containing (mM): NaCl (120), KCl (3), $MgCl_2$ (4), NaH_2PO_4 (1), $NaHCO_3$ (26), and glucose (10). Horizontal brain slices were cut at a thickness of 500 μM, the hippocampus dissected, and the CA3 region removed. Slices were then perfused with room-temperature, oxygenated (95% O_2/5% CO_2) standard ACSF containing (mM): NaCl (120), KCl (3), $MgCl_2$ (1.5), NaH_2PO_4 (1), $CaCl_2$ (2.5), $NaHCO_3$ (26), and glucose (10) at approximately 1 ml/min. Slices recovered in the recording chamber for one hour at room temperature, and then a second hour at 30°C, the temperature at which recordings were obtained. A bipolar stimulating electrode (Kopf Instruments) was placed on the CA3-side of the CA1 region in the stratum radiatum and a 1.0 MΩ tungsten recording microelectrode (World Precision Instruments) was then positioned in the same layer in CA1.

2. Quantification of synaptic plasticity

Data were digitized at 10 kHz, low-pass filtered at 1 kHz, and analyzed with pCLAMP 9.2 software (Axon Instruments). The initial slope of the population fEPSP was measured by fitting a straight line to a 1 msec window immediately following the fiber volley. A stimulus-response curve was obtained at the beginning of each experiment, with stimulus pulses consisting of a single square wave of 270 μs duration delivered at 40-160 μA. The stimulation

intensity was adjusted to obtain a field EPSP amplitude of 1.0-1.5mV to begin baseline recording, and fEPSPs were elicited by stimulation of the Schaffer collateral-commissural pathway in stratum radiatum once every 60 s (.0167 Hz) for the duration of the experiment. Synaptic responses were normalized by dividing all slopes by the average of the 5 fEPSP slopes obtained from the 5 min prior to tetanization. The tetanization protocol used to induce LTP in all experiments was a standard HFS protocol consisting of 3 trains of 100 Hz/1s administered at 20 s intertrain intervals. Control LTP values were collected periodically throughout the study. Planned comparisons with control were made using unpaired *t*-tests. In reporting our results, n-values indicate first the number of slices, and then the number of animals.

3. Drugs

None of the drugs applied in these experiments significantly altered the baseline fEPSP response at the indicated doses. For all experiments in which either SKF38393 or SKF81297 was tested, application of the $D_{1/5}$ agonist began 30 min prior to induction of LTP, and fEPSP responses were monitored for an additional 30 min post HFS in the continued presence of the drug (Figs 1&5). When antagonists or inhibitors were tested, they were applied for at least 20 min prior to initiating the $D_{1/5}$ agonist co-application, from which point all drugs continued to be applied for the duration of recording (Figs 2-7). 8-Bromo-2,3,4,5-tetrahydro-3-methyl-5-phenyl-1H-3-benzazepin-7-ol hydrobromide (SKF83566), 3-(4-chlorophenyl) 1-(1,1-dimethylethyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (PP2), (αR,βS)-α-(4-Hydroxyphenyl)-β-methyl-4-(phenylmethyl)-1 -piperidinepropanol maleate (Ro25-6981), 6-Chloro-2,3,4,5-tetrahydro-1-phenyl-1H-3-benzazepine hydrobromide (SKF81297), and 3-(4-[4-Chlorophenyl]piperazin-1-yl)-methyl-1H-pyrrolo[2,3-b]pyridine trihydrochloride (L745870) were obtained from Tocris.

N-[2-(p-Bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride (H89) was obtained from Sigma. (±)-1-Phenyl-2,3,4,5-tetrahydro-(1H)-3-benzazepine-7,8-diol hydrochloride (SKF38393) was obtained from both Sigma and Tocris over the course of the study. Drugs were applied by addition to the perfusion reservoir; stocks for both PP2 and SKF81297 were initially dissolved in DMSO, with the final concentrations of this solvent being 0.04% and 0.08%, respectively.

Results:

1. The dopaminergic agonist SKF38393 dose-dependently facilitates LTP

Field EPSP (fEPSP) responses were monitored in s. radiatum layer of the CA1 region and LTP was evoked with HFS using 3 x 100 Hz/1 s trains administered at 20 s intertrain intervals. For a cumulative control set of slices collected throughout the study, the fEPSP slope was increased 70 ± 2% (n = 60 slices, 20 animals) 30 min following LTP induction (Fig 2.1A). In groups of slices treated with the dopamine agonist SKF38393, a dose-dependent modulation of LTP was evident (Fig 2.1B). At a concentration of 100 nM SKF38393 the magnitude of LTP was 80 ± 4%; n = 9, 3. Higher concentrations of SKF38393 significantly enhanced LTP [2 μM: (95 ± 5%; n = 9, 3; $p < 0.01$), 6 μM: (104 ± 3%; n = 9, 3; $p < 0.01$), 20 μM: (112 ± 7%; n = 20, 7; $p < 0.01$), 30 μM: (107 ± 1%; n = 9, 3; $p < 0.01$)]. In contrast, application of 60 μM SKF38393 resulted in an LTP magnitude (72 ± 9%; n = 9, 3) which was not significantly different from the control group.

2. $D_{1/5}$ receptor antagonism prevents enhancement of LTP by SKF38393

Previous studies have indicated that activation of $D_{1/5}$ receptors in the CA1 can enhance the magnitude of LTP measured 30 min post-HFS in both slices (Otmakhova and Lisman, 1996) and in vivo (Lemon and Manahan-Vaughan, 2006). Consistent with these reports, when the $D_{1/5}$ receptor selective antagonist SKF83566 (2 μM) was applied prior to the agonist SKF38393 (20 μM), LTP was no longer enhanced in the presence of the dopaminergic agonist (75 ± 7; n = 12, 4; Fig 2.2B). Applied alone, the antagonist did not significantly affect LTP magnitude (74 ± 8%; n = 18, 6) when compared to the control group, suggesting that basal activation of $D_{1/5}$ receptors

by endogenously released dopamine was not required for expression of the early component of LTP induced using our HFS protocol.

3. Involvement of cAMP-dependent protein kinase

As it has been reported that cAMP and protein kinase A activity can affect the magnitude of early LTP (Blitzer et al., 1995; Otmakhova et al., 2000), and since $D_{1/5}$ receptors are positively coupled to cAMP/PKA, we tested the selective PKA inhibitor H89 in our experiments. When H89 (10 μM) was applied prior to SKF38393 (20 μM), LTP was no longer enhanced in the presence of the dopaminergic agonist (71 ± 5%; n = 9, 3; Fig 2.3B). Applied alone, the PKA inhibitor did not significantly affect LTP magnitude (69 ± 6%; n = 9, 3) when compared to the control group. This result is consistent with the expectations outlined by Woo et al. (2003) for a temporally "compressed" HFS pattern, which was the type of conditioning protocol utilized in our experiments. Taken together, these findings suggest that although the early LTP evoked under our induction conditions was not dependent upon PKA activation, the $D_{1/5}$-mediated enhancement was PKA-dependent.

4. NR2B NMDARs are required for the $D_{1/5}$ enhancement of LTP

One mechanism by which increased PKA activity might be postulated to enhance LTP is via enhanced NMDA receptor activity. Because we were able to prevent $D_{1/5}$-mediated increases in early LTP without affecting the "baseline" magnitude of potentiation, we tested the possibility that NR2B-containing NMDARs were selectively participating in the $D_{1/5}$-mediated enhancement of early LTP, as it has been suggested that these receptors are not required for LTP (Liu et al., 2004). In the presence of the selective NR2B antagonist Ro25-6981 (1 μM) alone,

LTP magnitude was not significantly different from that of the control group (76 ± 5%; $n = 9, 3$; Fig 2.4B). Thus the early LTP evoked using our induction protocol was not dependent upon the participation of NR2B NMDARs. However, prior application of Ro25-6981 prevented the enhancement of LTP by SKF38393 (20 μM), and furthermore, early LTP was significantly decreased (51 ± 2%; $n = 9, 3$; $p < 0.01$) when compared to the control group. Co-application of the $D_{1/5}$ antagonist SKF83566 (2 μM) did not mitigate this decrease, as LTP magnitude in the presence of all three compounds was still significantly reduced (52 ± 6%; $n = 9, 3$; $p < 0.02$). This result suggests that the SKF38393-mediated decrease in LTP magnitude observed in the presence of Ro25-6981 was not mediated by $D_{1/5}$ receptors.

5. NR2B NMDARs are required for the $D_{1/5}$ enhancement of LTP (continued)

In groups of slices treated with a $D_{1/5}$ full agonist, SKF81297 (30 μM), the magnitude of LTP was significantly enhanced (118 ± 6%; $n = 9, 3$; $p < 0.01$; Fig 2.5B) when compared to the control group. LTP was no longer enhanced when NR2B antagonist Ro25-6981 (1 μM) was applied prior the dopaminergic agonist (72 ± 11%; $n = 11, 4$). In addition, slices exposed to both Ro25-6981 and SKF81297 did not show a decrease in LTP magnitude when compared to the control group, in contrast to the results obtained following co-application of Ro25-6981 and SKF38393 in the previous figure. These findings with SKF81297 further suggest that the decrease in LTP observed in the presence of co-applied Ro25-6981 and SKF38393 (Fig 2.4) is not attributable to $D_{1/5}$ receptor activation, and is instead an effect of SKF38393 which is not shared by SKF81297.

6. Activation of D_4 receptors contributes to the descending portion of the SKF38393 dose-response curve

As previously noted (see 3.1 above), application of 60 μM SKF38393 resulted in an LTP magnitude which was not significantly different from the control group. However, when the D_4 receptor selective antagonist L745870 (10 μM) was applied prior to the $D_{1/5}$ agonist SKF38393 (60 μM), LTP was significantly enhanced (105 ± 7; n = 9, 3; $p < 0.01$; Fig 2.6B). The D_4 antagonist alone did not significantly affect LTP magnitude (83 ± 3%; n = 9, 3) when compared to the control group, suggesting that basal activation of D_4 receptors by endogenously released dopamine does not play a significant role in expression of the early component of LTP induced under our conditions.

7. Src-family tyrosine kinase activity is required for the $D_{1/5}$ enhancement of LTP

One example of a functional connection between the cAMP/PKA pathway and the regulation of NR2B-containing NMDARs is via the activation of Src-family tyrosine kinase (Yaka et al., 2003). When the SFK inhibitor PP2 (10 μM) was applied prior to SKF38393 (20 μM), LTP was no longer enhanced in the presence of the dopaminergic agonist (70 ± 7%; n = 15, 5; Fig 2.5B). Applied alone, the SFK inhibitor did not significantly affect LTP magnitude (69 ± 3%; n = 12, 4). These results suggest that although baseline LTP evoked under our conditions did not require SFK activation, the $D_{1/5}$-mediated enhancement of LTP was SFK-dependent.

Discussion:

In this study, we have determined that the dopaminergic agonist SKF38393 is capable of facilitating LTP via activation of $D_{1/5}$ dopamine receptors in a dose-dependent manner that involves NMDA receptors containing the NR2B subunit. We have also provided evidence that this mechanism is mediated by both protein kinase A and Src-family tyrosine kinase activities.

The dopaminergic agonist SKF38393 has been widely used in characterizing the role of $D_{1/5}$ receptors in LTP (Huang and Kandel, 1995; Mockett et al., 2004; Navakkode et al., 2007; Swanson-Park et al., 1999), and we have tested a range of SKF38393 concentrations under our recording conditions. The results demonstrate that relatively low concentrations were capable of significantly elevating LTP above control levels, and this enhancement reached a maximum at a concentration of 20 μM. Unexpectedly, LTP magnitude returned toward the control level when slices were exposed to a higher concentration of this drug, an effect that was possibly due to a nonspecific (i.e. non $D_{1/5}$-mediated) action of SKF38393. In order to confirm that the enhancement of potentiation was in fact mediated via $D_{1/5}$ receptors, we tested SKF38393 in the presence of the $D_{1/5}$ antagonist SKF83566. As this antagonist was able to block the ability of SKF38393 to enhance LTP beyond the control level seen in drug naïve slices, a $D_{1/5}$-specific drug effect was confirmed. The nature of the nonspecific activity of SKF38393 to inhibit LTP was not the focus of these studies, but we note that this compound exhibits binding affinity in the micromolar range for several other receptors (Toll et al., 1998; Van Tol et al., 1991). Some possible contributors to this nonspecific activity could include D_4, $5HT_{1A}$, or $5HT_{2A}$ receptors, as activation of any of these would be expected to inhibit LTP in the CA1 region (Kotecha et al., 2002; Mori et al., 2001; Wang and Arvanov, 1998). Accordingly, our results with D_4 antagonist

L745870 indicate that the negative consequences for LTP following D_4 receptor activation (Kotecha et al., 2002) significantly contributes to the summed effects of 60 μM SKF38393 on LTP magnitude. When the suppressive influence of D_4 receptor activation was blocked, the enhancement of LTP via $D_{1/5}$ receptor activity was restored.

Utilizing the selective PKA inhibitor H89, we determined that the magnitude of early LTP expressed under our conditions was not reliant upon PKA activity. This is consistent with a previous report that specifically tested "compressed" (20 s intertrain interval) vs. "spaced" (5 min intertrain interval) induction protocols and found that early LTP induced by compressed protocols was independent of PKA activity (Woo et al., 2003). Our ability to induce a baseline amount of LTP without the involvement of PKA activity afforded us the opportunity to examine the possible PKA-dependence of the $D_{1/5}$ receptor-mediated enhancement of LTP. As $D_{1/5}$ receptors are positively coupled to adenylate cyclase, we hypothesized that exposing slices to H89 prior to and during application of SKF38393 would impede the drug's ability to enhance LTP. Indeed, under such conditions LTP magnitude was not altered from control levels, supporting the postulate that stimulation of cAMP/PKA is an initial step in the resultant signaling cascade following $D_{1/5}$ receptor activation.

It is clear that there are a number of routes by which G-protein coupled receptors (GPCRs) are capable of modifying NMDAR activity at synapses in the hippocampal CA1 region, and evidence for a positive regulation of NR2B NMDAR activity by pituitary adenylate cyclase activating peptide has been reported (Yaka et al., 2003). Our findings provide additional evidence that GPCR activation (exemplified here by $D_{1/5}$ receptors) has the ability to recruit signaling mechanisms that target NR2B receptor activity. Using the selective antagonist Ro25-

6981, we determined that NR2B-containing NMDA receptor activation does not play a significant role in control LTP under our conditions. This was not unexpected, as it has been previously suggested that the dominant form of NMDAR NR2 subunit involved with LTP is NR2A, while NR2B has been suggested to be more critical in the expression of LTD (Liu et al., 2004). Applying Ro25-6981 to slices prior to and during application of either SKF38393 or the SKF81297 rendered these $D_{1/5}$ agonists incapable of enhancing LTP. In sum, these findings demonstrate that NR2B-containing NMDARs are a key component in the $D_{1/5}$ receptor-mediated neuromodulation of LTP in the hippocampal CA1 region.

It has previously been shown that Src-family kinase activation is involved in the induction of LTP in the CA1 region (Lu et al., 1998). Under our experimental conditions, this appears not to be the case as the SFK inhibitor PP2 had no effect on LTP. However, in order to examine the role of Src-family tyrosine kinase activity in the $D_{1/5}$-mediated enhancement of LTP, we exposed slices to PP2 prior to and during application of SKF38393. In the presence of this inhibitor, D1/5 agonist SKF38393 was unable to significantly increase LTP beyond a control level. This provides evidence for the involvement of SFK activity in mediating the dopaminergic enhancement of LTP. Our findings with PKA and SFK are consistent with a model for G_s-coupled phosphorylation of the NR2B subunit in which both kinase activities participate in GPCR-mediated enhancement of NMDAR activity (Yaka et al., 2003). The model describes a trimolecular signaling complex which regulates the phosphorylation of NR2B-containing NMDARs in CA1 pyramidal cells. In this complex, the receptor for activated C-kinase 1 (RACK1) associates with both the NR2B subunit and SFKs, obstructing their ability to phosphorylate tyrosine residues on the cytoplasmic tail of the NR2B subunit. PKA-mediated phoshorylation of RACK1 causes a dissociation of the protein from the NR2B subunit, thereby

allowing SFK activity to potentiate NMDAR currents. $D_{1/5}$ receptor activation would be expected to initiate this regulatory mechanism via activation of either Gs- or Gq-coupled cascades (Salter and Kalia, 2004).

Although these current investigations are focused on the ability of $D_{1/5}$ activation to enhance the magnitude of early LTP, a number of studies have investigated dopaminergic involvement in the late, protein-synthesis dependent stage of LTP (Frey et al., 1991; Frey et al., 1990; Huang and Kandel, 1995; Matthies et al., 1997; O'Carroll and Morris, 2004; Swanson-Park et al., 1999). In particular, early work revealed that activation of both D2-like and D1-like dopamine receptors during the tetanus event is necessary for the persistence of LTP, and D_1-deficient mice were found to be incapable of expressing late LTP (Frey et al., 1989; Frey et al., 1991; Frey et al., 1990; Matthies et al., 1997). Furthermore, some reports have presented evidence that activation of D1/5 receptors in the absence of a tetanus event is sufficient to induce a potentiation that is similar to late LTP (Huang and Kandel, 1995; Navakkode et al., 2007), although this has not been a consistent observation (Mockett et al., 2004; Otmakhova and Lisman, 1996). Similar to the latter reports, we found no significant effects on baseline synaptic responses with any of the dopaminergic agonists applied during the 30 minute application that preceded the induction of LTP in our experiments. As previously noted (Mockett et al., 2004; Navakkode et al., 2007), there are several methodological considerations that may underlie this discrepancy.

Finally, it is interesting to consider the potential role that endogenously released dopamine may have on the modulation of synaptic plasticity. Recent work from our laboratory has explored the mechanism by which cocaine and the dopamine transporter blocker GBR12935

enhance LTP (Thompson et al., 2005), both of which exert their effect via D_3-receptor activation and the inhibition of inhibitory synaptic input on CA1 pyramidal neurons (Hammad and Wagner, 2006; Swant and Wagner, 2006). Now in this report, we have confirmed the enhancement of LTP via $D_{1/5}$-receptor activation and described a mechanism which directly involves the facilitation of excitatory input on CA1 pyramidal neurons. As the net effects of both of these mechanisms are excitatory, they might be additive or even synergistic, since reducing IPSCs on dendritic shafts would potentially allow increased depolarization and activation of extrasynaptic NR2B-containing NMDARs. Alternatively, D_3 receptor activation could be permissive for the actions of endogenously released dopamine in the CA1 since this receptor subtype has the highest affinity for dopamine, and disinhibition could be a requirement for extrasynaptic NR2B-containing NMDARs to become activated under physiological conditions. Regardless, together our results point toward an increase in dopaminergic activity in the CA1 region of the hippocampus to be working to enhance the synaptic plasticity processes thought to be involved with learning and memory via participation of NR2B-containing NMDARs in synaptic signaling.

Acknowledgements - J.J.W. was funded by National Institute on Drug Abuse (DA 16302).

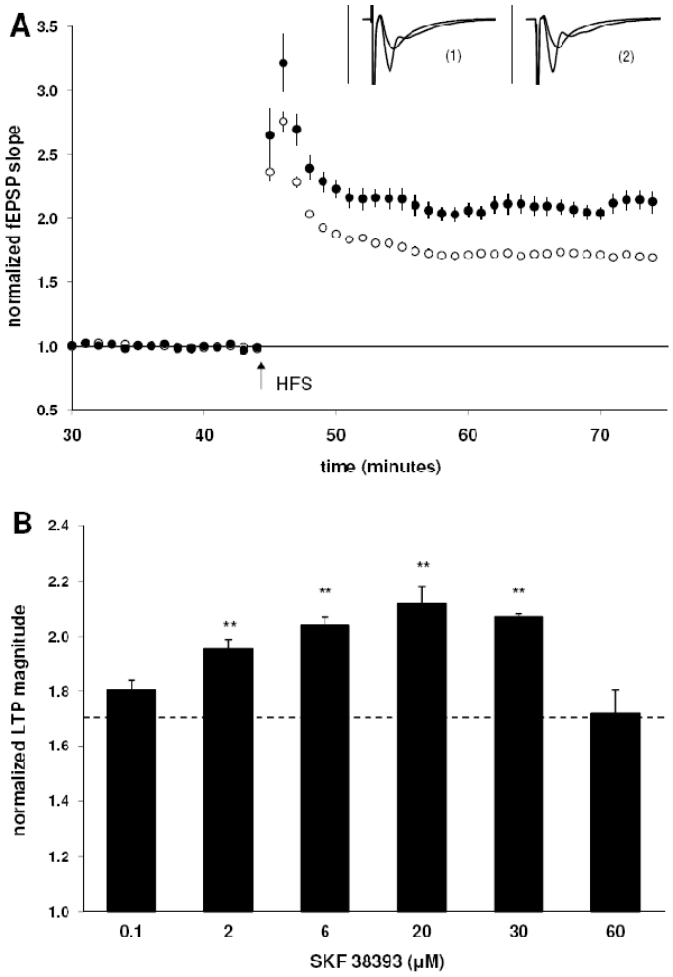

Figure 2.1. The dopaminergic agonist SKF38393 induced a dose-dependent modulation of LTP. **A)** Summary plot of normalized fEPSP slope measurements evoked and recorded in the stratum radiatum layer of the CA1 region. Open circles show responses from control slices; closed circles depict responses from SKF38393-treated slices (20 μM). Insets are 50 ms sweeps averaged from all experiments illustrating the mean fEPSP 1–5 min prior to and 26–30 min post-tetanus (vertical scale bar is 3 mV). The left pair of sweeps (1) is from non-drug, control slices and the right pair (2) is from SKF38393-treated slices. **B)** Summary quantification of LTP magnitude in the presence of increasing concentrations of SKF38393. Control LTP is indicated by the dashed line. LTP magnitude was significantly enhanced at concentrations of 2 μM, 6 μM, 20 μM, and 30 μM relative to control (** $p < 0.01$). Error bars show ± SEM.

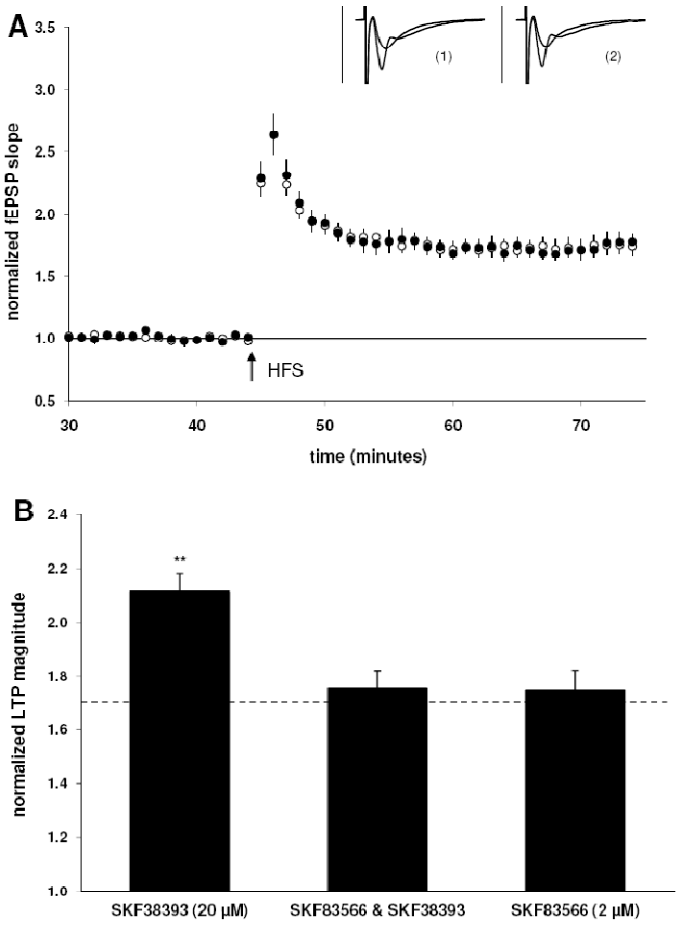

Figure 2.2. Co-application with the $D_{1/5}$ antagonist SKF83566 (2 µM) blocked the SKF38393-mediated enhancement of LTP. **A)** Summary plot of normalized fEPSP measurements. Open circles show normalized fEPSP slope from slices treated with SKF83566 (2 µM) alone; closed circles depict slices treated with both SKF83566 (2 µM) and SKF38393 (20 µM). Insets are 50 ms sweeps averaged from all experiments illustrating the mean fEPSP 1–5 min prior to and 26–30 min post-tetanus (vertical scale bar is 3 mV). The left pair of sweeps (1) is from SKF83566-treated slices and the right pair (2) is from SKF83566 & SKF38393-treated slices. **B)** Summary quantification of LTP magnitude in the presence of the agonist SKF38393 alone (taken from Fig. 1, illustrated for comparison), when co-applied with the antagonist SKF83566, or SKF83566 alone. Significance is relative to the control group (indicated by the dashed line, ** $p < 0.01$). Error bars show ± SEM.

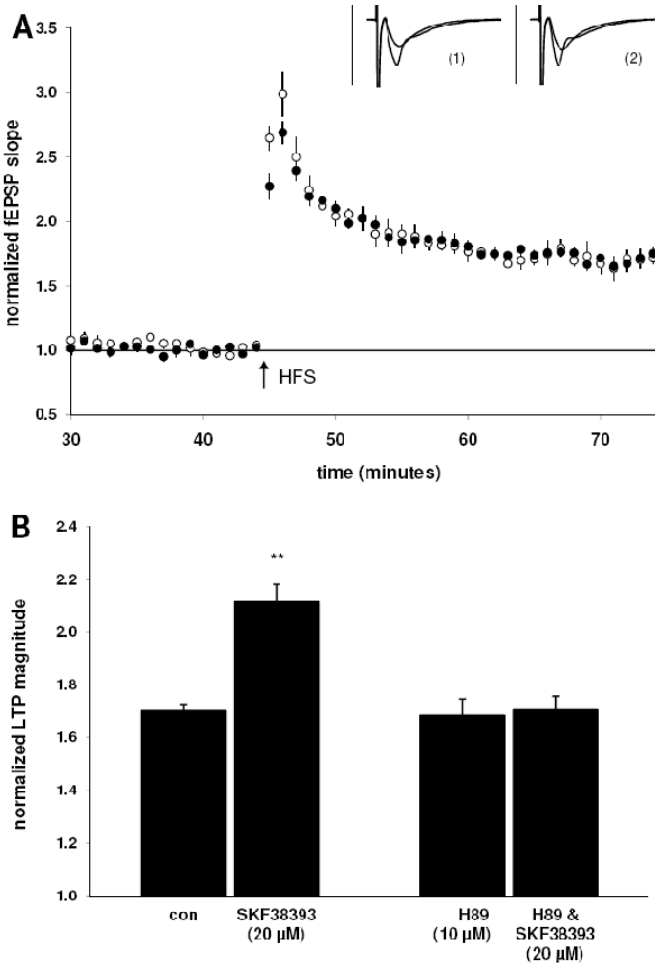

Figure 2.3. Co-application with the PKA inhibitor H89 (10 µM) blocked the SKF38393-mediated enhancement of LTP. **A)** Summary plot of normalized fEPSP measurements. Open circles show normalized fEPSP slope from slices treated with H89 (10 µM); closed circles depict slices treated with both H89 (10 µM) and SKF38393 (20 µM). Insets are 50 ms sweeps averaged from all experiments illustrating the mean fEPSP 1–5 min prior to and 26–30 min post-tetanus (vertical scale bar is 3 mV). The left pair of sweeps (1) is from H89-treated slices and the right pair (2) is from H89 & SKF38393-treated slices. **B)** Summary quantification of LTP magnitude in the presence of H89 alone and when co-applied with SKF38393, as compared to both the control and SKF38393 (20 µM) alone groups taken from Fig. 1 ($^{**}p < 0.01$). Error bars show ± SEM.

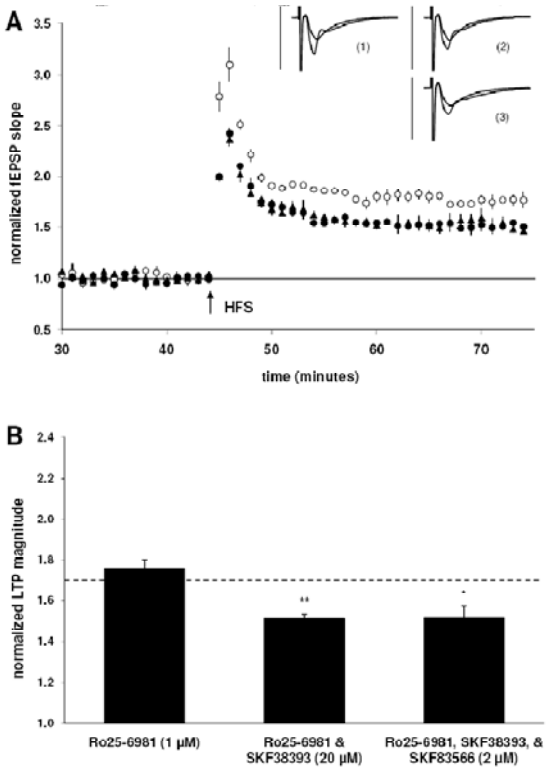

Figure 2.4. Co-application with the NR2B antagonist Ro25-6981 (1 µM) blocked the SKF38393-mediated enhancement of LTP. **A)** Summary plot of normalized fEPSP measurements. Open circles show normalized fEPSP slope from slices treated with Ro25-6981 (1 µM); closed circles depict slices treated with both Ro25-6981 (1 µM) and SKF38393 (20 µM); closed triangles depict slices treated with these drugs in addition to $D_{1/5}$ antagonist SKF83566 (2 µM). Insets are 50 ms sweeps averaged from all experiments illustrating the mean fEPSP 1–5 min prior to and 26–30 min post-tetanus (vertical scale bar is 3 mV). The left pair of sweeps (1) is from Ro25-6981-treated slices, the right upper pair (2) is from Ro25-6981 & SKF38393-treated slices, the right lower pair (3) is from Ro25-6981/SKF83566 & SKF38393-treated slices. **B)** Summary quantification of LTP magnitude in the presence of Ro25-6981 alone, when Ro25-6981 is co-applied with SKF38393, and when both Ro25-6981 and the antagonist SKF83566 are co-applied with SKF38393 (significance is relative to the control group indicated by the dashed line, $*p < 0.05$, $**p < 0.01$). Error bars show ± SEM.

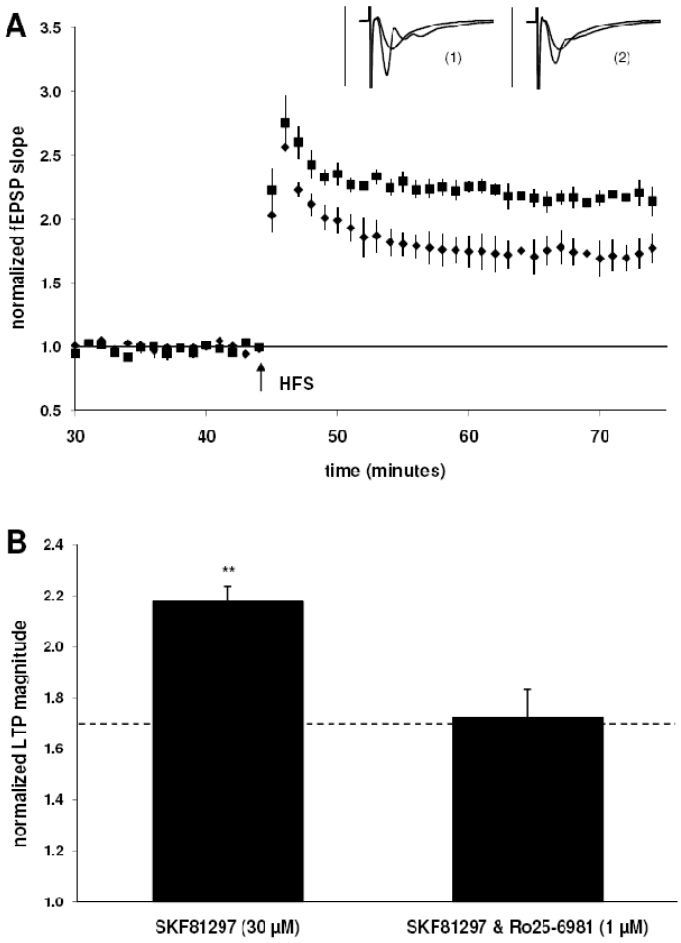

Figure 2.5. Co-application with NR2B antagonist Ro25-6981 (1 µM) also blocked the ability of $D_{1/5}$ full agonist SKF81297 to enhance LTP. **A)** Summary plot of normalized fEPSP measurements. Closed squares depict slices treated with $D_{1/5}$ agonist SKF81297 (30 µM). Closed circles depict slices treated with both Ro25-6981 (1 µM) and SKF81297 (30 µM). Insets are 50 ms sweeps averaged from all experiments illustrating the mean fEPSP 1–5 min prior to and 26–30 min post-tetanus (vertical scale bar is 3 mV). The left pair of sweeps (1) is from SKF81297-treated slices and the right pair (2) is from Ro25-6981 & SKF81297-treated slices. **B)** Summary quantification of LTP magnitude in the presence of SKF81297 alone and when co-applied with Ro25-6981 (significance is relative to the control group indicated by the dashed line, $^{**}p < 0.01$). Error bars show ± SEM.

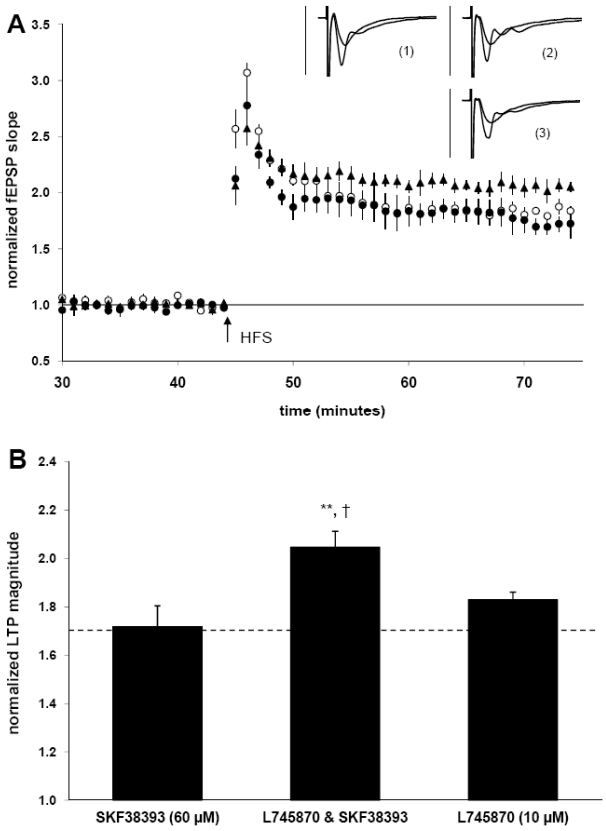

Figure 2.6. Co-application with the D_4 antagonist L745870 (10 µM) enabled 60 µM SKF38393 to enhance LTP. **A)** Summary plot of normalized fEPSP measurements. Open circles show normalized fEPSP slope from slices treated with L745870 (10 µM); closed circles depict slices treated with SKF38393 (60 µM); closed triangles depict slices treated with both L745870 (10 µM) and SKF38393 (60 µM). Insets are 50 ms sweeps averaged from all experiments illustrating the mean fEPSP 1–5 min prior to and 26–30 min post-tetanus (vertical scale bar is 3 mV). The left pair of sweeps (1) is from SKF38393-treated slices, the right upper pair (2) is from L745870 & SKF38393-treated slices, the right lower pair (3) is from L745870-treated slices. **B)** Summary quantification of LTP magnitude in the presence of SKF38393 alone, when L745870 is co-applied with SKF38393, and when L745870 is applied alone (significance relative to the control group indicated by the dashed line, $^{**}p < 0.01$; significance relative to the L745870 group is also indicated, $\dagger p < 0.05$). Error bars show ± SEM.

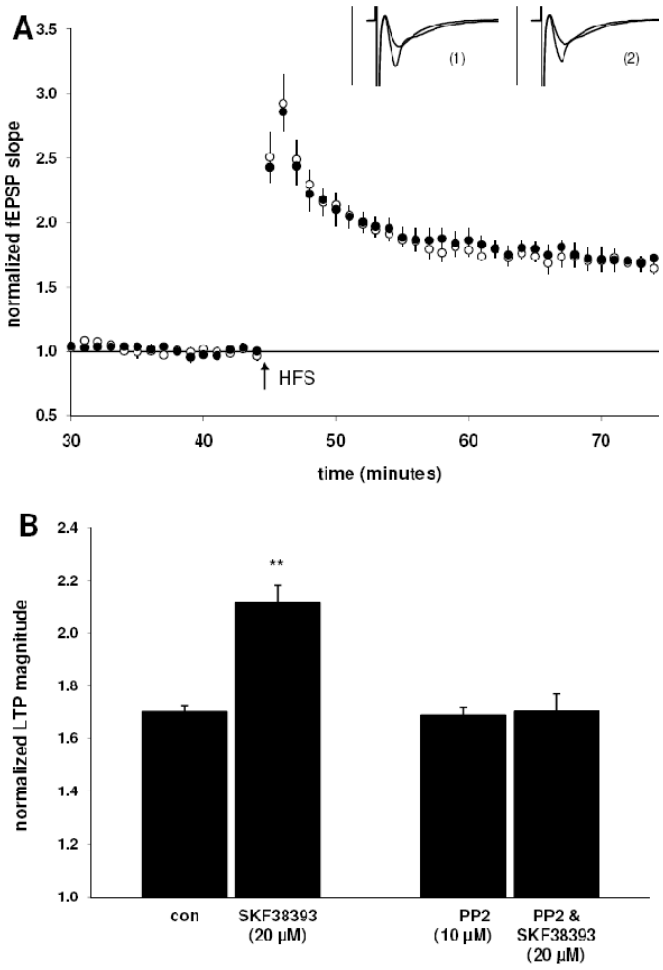

Figure 2.7. Co-application with the Src-family tyrosine kinase inhibitor PP2 (10 µM) blocked the SKF38393-mediated enhancement of LTP. **A)** Summary plot of normalized fEPSP measurements. Open circles show normalized fEPSP slope from slices treated with PP2 (10 µM); closed circles depict slices treated with both PP2 (10 µM) and SKF38393 (20 µM). Insets are 50 ms sweeps averaged from all experiments illustrating the mean fEPSP 1–5 min prior to and 26–30 min post-tetanus (vertical scale bar is 3 mV). The left pair of sweeps (1) is from PP2-treated slices and the right pair (2) is from PP2& SKF38393-treated slices. **B)** Summary quantification of LTP magnitude in the presence of PP2 alone and when co-applied with SKF38393, as compared to the control and SKF38393 (20 µM) alone groups (taken from Fig. 1, illustrated for comparison, $^{**}p < 0.01$). Error bars show ± SEM.

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CHAPTER THREE

MODULATORY INFLUENCES OF D2-LIKE RECEPTORS IN EARLY-PHASE LTP IN THE CA1 REGION OF RAT HIPPOCAMPUS

Stramiello, M. and Wagner, J.J. To be submitted to Journal of Pharmacology and Experimental Therapeutics.

Abstract:

In spite of their strong presence in the hippocampus CA1 region, little is known about the modulatory influence of D2-like dopamine receptors on long-term potentiation. Using the hippocampal slice preparation, I first illustrate that bath application of the D_4 agonist PD168077 (0.3 – 10 μM) does not affect LTP magnitude. However, when pre-applied at 3 μM, this drug provides a complete blockade of $D_{1/5}$-mediated LTP enhancement (SKF38393, 20 μM), a result suggesting that D_4 receptors may be important for "counterbalancing" $D_{1/5}$-mediated LTP enhancement in the stratum radiatum layer of CA1. In addition, I expand upon the previous characterization of D_3-mediated LTP enhancement (Swant et al., 2008) using the D_3 agonist PD128907 (0.1 μM – 3 μM). Utilizing the subunit-specific NMDAR antagonist Ro25-6981 (1 μM), I demonstrate that D_3-, like $D_{1/5}$-mediated LTP enhancement, requires NR2B-NMDAR participation. Unlike the $D_{1/5}$ modulation, however, prior application of PD168077 (3 μM) does not prevent D_3-mediated LTP enhancement. These results advance the understanding of the importance of NR2B-NMDARs in LTP, and suggest that dopamine acts to modulate hippocampal LTP through multiple mechanisms.

Keywords - D4 receptor; D3 receptor; PD168077; PD128907; NR2B; Ro25-6981

Introduction:

Dopamine receptors are grouped into two families, "D1-like" and "D2-like," nomenclature that can be traced back to 1979 (Kebabian and Calne, 1979; Kebabian 1992). While numerous subtypes of dopamine receptors have since been discovered in each family, the D1-like and D2-like division remains relevant, as currently known subtypes of the dopamine receptor each fit into one of these two major categories when considering their structural and pharmacological characteristics (Missale et al., 1998). Additionally, biochemical evidence upholds the separation: D1-like receptors (D_1 and D_5) are recognized for their ability to stimulate adenylate cyclase via G_s, while D2-like receptors (D_2, D_3, and D_4) are known to inhibit adenylate cyclase via G_i. All of these receptor subtypes have been detected in the hippocampus (Huang et al., 1992; Khan et al., 1998; Khan et al., 2000).

Despite its relatively low concentration when compared to other monoamine neurotransmitters present in the hippocampus (Hortnagl et al., 1991; Verhage et al., 1992), dopamine is increasingly recognized for its critical influence on long-term potentiation (LTP) expressed in the CA1 region. Initial studies demonstrated the importance of D2-like receptors in this capacity, especially as they pertain to late-phase LTP (Frey et al., 1989; Frey et al., 1990). Nevertheless, attention quickly turned to the modulatory role of dopaminergic function on the induction and expression of early-phase LTP, a field in which effects mediated by D1-like receptors have been the dominant focus for nearly twenty years. Recently, however, my lab reported that D_3 activation can enhance the magnitude of early-phase LTP recorded in stratum radiatum- establishing that D2-like receptors are also relevant to the discussion of early-phase LTP.

In this series of experiments, I further illustrate the ability of two D2-like receptors, D_3 and D_4, to modulate early-phase LTP recorded in the stratum radiatum layer of the CA1 region. Using the hippocampal slice preparation and extracellular recording technique, I demonstrate that application of a D_4 receptor agonist can inhibit $D_{1/5}$-mediated LTP enhancement without exerting any discernible effect of its own on LTP magnitude when compared to the control level. I also provide additional characterization of D_3-mediated LTP enhancement, showing evidence for the necessary role of NR2B-NMDAR activity, a commonality shared with $D_{1/5}$-mediated LTP enhancement. Together, these findings suggest that mechanisms involving D2-like dopamine receptors may play a significant role in modulation of early-phase LTP.

Methods:

1. Extracellular electrophysiology

Hippocampal slices were prepared from male Sprague-Dawley rats (40-90 days old) using an experimental protocol performed in compliance with the University of Georgia Animal Care and Use guidelines. All rats were anesthetized with halothane prior to decapitation. The brain was removed and submerged in ice-cold, oxygenated (95% O_2/ 5% CO_2) dissection artificial cerebrospinal fluid (ACSF) containing (mM): NaCl (120), KCl (3), $MgCl_2$ (4), NaH_2PO_4 (1), $NaHCO_3$ (26), and glucose (10). Horizontal brain slices were cut at a thickness of 500 μM, the hippocampus dissected, and the CA3 region removed. Slices were then perfused with room-temperature, oxygenated (95% O_2/5% CO_2) standard ACSF containing (mM): NaCl (120), KCl (3), $MgCl_2$ (1.5), NaH_2PO_4 (1), $CaCl_2$ (2.5), $NaHCO_3$ (26), and glucose (10) at approximately 1 ml/min. Slices recovered in the recording chamber for one hour at room temperature, and then a second hour at 30°C, the temperature at which recordings were obtained. A bipolar stimulating electrode (Kopf Instruments) was placed on the CA3-side of the CA1 region in the stratum radiatum and a 1.0 MΩ tungsten recording microelectrode (World Precision Instruments) was then positioned in the same layer in CA1.

2. Quantification of synaptic plasticity

Data were digitized at 10 kHz, low-pass filtered at 1 kHz, and analyzed with pCLAMP 9.2 software (Axon Instruments). The initial slope of the population fEPSP was measured by fitting a straight line to a 1 msec window immediately following the fiber volley. A stimulus-response curve was obtained at the beginning of each experiment, with stimulus pulses consisting of a single square wave of 270 μs duration delivered at 40-160 μA. The stimulation

intensity was adjusted to obtain a field EPSP amplitude of 1.0-1.5mV to begin baseline recording, and fEPSPs were elicited by stimulation of the Schaffer collateral-commissural pathway in stratum radiatum once every 60 s (.0167 Hz) for the duration of the experiment. Synaptic responses were normalized by dividing all slopes by the average of the 5 fEPSP slopes obtained from the 5 min prior to tetanization. The tetanization protocol used to induce LTP in all experiments was a standard HFS protocol consisting of 3 trains of 100 Hz/1s administered at 20 s intertrain intervals. Control LTP values were collected periodically throughout the study. Planned comparisons with control were made using unpaired *t*-tests. In reporting the results, n-values indicate first the number of slices, and then the number of animals.

3. Drugs

Except for PD168077 at the 10μM dose (see Results 3.1.), none of the drug applications in these experiments significantly altered baseline fEPSP responses. For all experiments in which either SKF38393 ($D_{1/5}$ agonist) or PD128907 (D_3 agonist) was tested, its application began 30 min prior to induction of LTP, and fEPSP responses were monitored for an additional 30 min post HFS in the continued presence of the drug. In experiments utilizing co-application of PD168077 (D_4 agonist) or Ro25-6981 (NR2B-NMDAR antagonist) with SKF38393 or PD128907, the former two drugs were applied for at least 20 min prior to initiating the SKF38393 or PD128907 co-application, from which point all drugs continued to be applied for the duration of recording.

(αR,βS)-α-(4-Hydroxyphenyl)-β-methyl-4-(phenylmethyl)-1-piperidinepropanol maleate (Ro25-6981), (4aR,10bR)-3,4a,4,10b-Tetrahydro-4-propyl-2H,5H-[1]benzopyrano-[4,3-b]-1,4-oxazin-9-ol hydrochloride (PD128907), and N-(Methyl-4-(2-cyanophenyl)piperazinyl-3-

methylbenzamide maleate (PD168077) were obtained from Tocris. (±)-1-Phenyl-2,3,4,5-tetrahydro-(1H)-3-benzazepine-7,8-diol hydrochloride (SKF38393) was obtained from both Sigma and Tocris over the course of the study. Drugs were applied by addition to the perfusion reservoir; stocks for PD168077 were initially dissolved in DMSO (Sigma), with the final concentration of this solvent being 0.05%.

Results:

1. Dopamine D_4 receptor activation alone does not affect LTP magnitude

Field EPSP (fEPSP) responses were monitored in stratum radiatum layer of the CA1 region and LTP was evoked with HFS using 3 x 100 Hz/1 s trains administered at 20 s intertrain intervals. For a cumulative control set of slices collected during this time ("$control_{D4}$ group"), the fEPSP slope was increased 64 ± 3% (n = 44 slices, 13 animals) 30 min following LTP induction (Fig 1A). In slices treated with the following concentrations of dopamine D_4 receptor agonist PD168077, no significant drug affect on LTP magnitude was observed (Fig 3.1B): [300 nM: (68 ± 4%; n = 9, 3), 3 μM: (67 ± 4%; n = 13, 5), 10 μM: (55 ± 4%; n = 15, 4)]. However, when applied at 10 μM or higher, PD168077 did have a significant inhibitory effect on pre-tetanus, baseline fEPSPs (15 ± 4%; n = 14, 5; $p < 0.01$; data not shown). As this drug has micromolar affinity for D_2 receptors (Glase et al., 1997), this inhibitory effect may be mediated by nonspecific inhibitory activity of PD168077 (Hsu, 1996).

2. Dopamine D_4 receptor activation prevents $D_{1/5}$-mediated LTP enhancement

It is now well-established that activation of $D_{1/5}$ receptors enhances early LTP evoked in the hippocampus CA1 region (Otmakhova & Lisman, 1996). Here, I reconfirm $D_{1/5}$-mediated LTP enhancement (relative to the $control_{D4}$ group) using the agonist SKF38393 (20 μM; 87 ± 4%; n = 9, 3; $p < 0.01$; Fig 3.2B). This is a robust effect that I know to be mediated by PKA, Src family kinases (SFKs), and NR2B-NMDARs (Stramiello & Wagner, 2008). My past experimentation (2008) also pointed toward a potential modulatory role for D_4 receptors in early LTP, based upon data obtained from slices exposed to the D_4 antagonist L745870 (10 μM). In the current study, I utilize PD168077 (3 μM) to show that although D_4 agonism does not alter

LTP magnitude (Fig 3.1B) relative to the control level, it is capable of exerting a complete blockade on $D_{1/5}$-mediated LTP enhancement (66 ± 3%; n = 15, 5; Fig 3.2B).

3. Dopamine D_3 receptor activation dose-dependently facilitates LTP

While my more recent work has focused on $D_{1/5}$-mediated LTP enhancement, I have also demonstrated that D_3 receptor activation is capable of enhancing LTP (Swant et al., 2008). Here, I provide a dose-response curve for LTP evoked in the presence of the D3 agonist PD128907, demonstrating that this drug is capable of enhancing LTP even at submicromolar levels of bath application. For a cumulative control set of slices collected during this time ("$control_{D3}$ group"), the fEPSP slope was increased 56 ± 3% (n = 34 slices, 13 animals) 30 min following LTP induction (Fig 3.3A). PD128907 significantly enhanced LTP at the following concentrations: [100 nM: (68 ± 6%; n = 12, 4; $p < 0.05$), 300 nM: (73 ± 4%; n = 12, 4; $p < 0.001$), 1 μM: (83 ± 5%; n = 16, 6; $p < 0.001$), 3 μM: (76 ± 4%; n = 9, 4; $p < 0.001$)].

4. D_3-mediated enhancement of LTP requires NR2B NMDARs; is not inhibited by D_4 activation

My prior research has demonstrated that the NR2B-NMDAR agonist Ro25-6981 (1 μM) does not alter LTP magnitude relative to control levels (Stramiello & Wagner, 2008). This drug was, however, capable of preventing $D_{1/5}$-mediated LTP enhancement. Here, I show that NR2B-NMDARs also contribute to D_3-mediated LTP enhancement. When slices were pretreated with Ro25-6981 (1 μM), application of PD128907 (1 μM) was no longer capable of enhancing LTP (53 ± 4%; n = 10, 4; Fig 3.4B) relative to the $control_{D3}$ group. In contrast to $D_{1/5}$-mediated effects, however, co-application of a D_4 agonist did not block D_3-mediated LTP enhancement (73 ± 4%; n = 12, 4; $p < 0.01$; Fig 3.4B) relative to the control level.

Discussion:

I have described modulation of early-phase LTP by the two subtypes of D2-like receptors that are prevalent in the stratum radiatum layer of the hippocampus CA1 region. My primary findings are twofold. First, activation of D_4 receptors inhibits $D_{1/5}$-mediated enhancement of LTP at Schaffer collateral synapses. This inhibition occurs without decreasing LTP beyond the control level and application of the D_4 agonist alone has no apparent effects on LTP magnitude. Second, like its $D_{1/5}$-mediated counterpart, D_3-mediated LTP enhancement necessitates participation of NR2B-NMDARs. This requirement builds upon my previous characterization of the mechanism underlying D_3-facilitated LTP and also underscores the importance of NR2B-NMDARs as critical regulatory subunits in long-term plasticity, but not necessarily determinants of its polarity (Liu et al., 2004).

The D_4 receptor is heavily expressed in the CA1 region and found at very low levels elsewhere in the hippocampus proper (Defagot et al., 1997; Khan et al., 1998). Yet, despite the foremost prevalence of LTP studies in this region (Malenka and Bear, 2004), a PubMed search for "D_4 and dopamine and LTP" generates only two results, both recent publications from the same group of researchers. Under their experimental conditions, ErbB receptor-mediated D_4 activation was shown to rapidly produce a complete depotentiation of LTP in the CA1 (Kwon et al., 2008; Neddens et al., 2009). The authors speculated that D_4 receptors might function to "counterbalance pro-LTP effects of D1/D5Rs," noting that $D_{1/5}$ receptor activation has been shown to inhibit depotentiation in the CA1 (Otmakhova & Lisman, 1998).

In a previous report (Stramiello & Wagner, 2008), I demonstrated that high-dose application of the $D_{1/5}$ agonist SKF38393 (60 μM) failed to exhibit LTP enhancement that was seen with lower dose applications of the drug (concentrations ranging from 0.1 – 30 μM). Due to

known affinity of SKF38393 for several other receptors, particularly D_4, I investigated the possibility that high-dose application of this drug was eliciting an opposing influence on LTP expression via nonspecific binding activity. Thus, I employed the D_4 receptor antagonist L745870 (10 μM, which had no effect of its own on LTP magnitude) to show that this drug, when co-applied with SKF38393 (60 μM), fully restored the $D_{1/5}$ agonist's ability to increase LTP. Curiously, this so-called opposing influence had prevented a $D_{1/5}$-mediated LTP enhancement without decreasing LTP below the control level. Because of its capacity to prevent an important functional change associated with $D_{1/5}$-induced synaptic modification (without causing any discernible impairment in normal plasticity), I was motivated to confirm and further examine the actions of D_4 receptor activation. To accomplish this, I utilized the agonist PD168077, a drug which exhibits several hundredfold selectivity for D_4 receptors over D_2 and D_3 receptors (Glase et al., 1997).

Due to a historical lack of ligands showing selectivity for individual subtypes of dopamine receptor (Enguehard-Gueiffier & Gueiffier, 2006), the physiological properties of D2-like dopamine receptors have not in many cases been well-distinguished from one another. I thus tested a range of PD168077 concentrations to determine the ability of D_4 activation to influence LTP under my recording conditions. At doses of up to 10 μM, PD168077 showed no effect on LTP magnitude. Because D_4 signaling is known to inhibit AC, and many forms of LTP depend upon PKA activity (Woo et al., 2003), I note that LTP evoked using my "compressed" (20 s intertrain intervals) induction protocol is not PKA-dependent (Woo et al., 2003; Stramiello & Wagner, 2008).

It is well-established that activation of $D_{1/5}$ receptors increases the magnitude of evoked LTP (Otmakhova & Lisman, 1996; Swanson-Park, et al., 1999; Stramiello & Wagner, 2008).

Using SKF38393 (20 μM), I confirmed my ability to reproduce this drug effect, and then demonstrated that pre-application of PD168077 (3 μM) was capable of completely blocking $D_{1/5}$-mediated LTP enhancement, resulting in a magnitude of potentiation indistinguishable from the control level. This D_4-induced check on the LTP-increasing effects of $D_{1/5}$ activation provides direct evidence of the D_4-$D_{1/5}$ LTP "counterbalance" in CA1 that has been suggested by Kwon et al. (2009) regarding the reversal of potentiation. An underlying mechanism for this D_4-induced offset of $D_{1/5}$-mediated LTP enhancement is yet to be determined, but it likely involves D_4-signaled inhibition of AC, as the $D_{1/5}$ effect is reliant upon PKA activity (Stramiello & Wagner, 2008). Interestingly, this presumed D_4 inhibition of cAMP production did not occlude D_3-mediated LTP enhancement, which likely results from endocytosis of $GABA_A$Rs mediated by a D_3-induced inhibition of the AC/cAMP/PKA pathway (Swant et al., 2008).

Having previously shown that the D_3 receptor agonist PD128907 (1 μM) enhances LTP recorded in the stratum radiatum of CA1, I tested several concentrations of this drug to confirm the optimal dose for maximizing its effect on LTP. I then demonstrated that, although D_3- and $D_{1/5}$-mediated LTP enhancement are not equally susceptible to blockade via D_4 activation, both require NR2B-NMDAR participation. Potential differences in the physical location of relevant receptors provide the most parsimonious explanation for reconciling between D_3- and $D_{1/5}$-mediated LTP enhancement: 1) shared NR2B-NMDAR-dependence and 2) differences in sensitivity to D_4 signaling. The rationale for this assertion is outlined below.

NR2B-NMDARs exist in the postsynaptic density (PSD; Yaka et al., 2002), but are most concentrated extrasynaptically (outside the dendritic spine) and perisynaptically (in the spine, but outside the postsynaptic density (Kohr, 2006). D_3 receptors are primarily extrasynaptic (Diaz et al., 2000), and disrupt PKA signaling to stimulate endocytosis of $GABA_A$Rs (Swant et al., 2008),

which are typically located along the dendritic shaft. Activation of D3 receptors might therefore reduce inhibition in the surrounding area, facilitating the spread of depolarization that is required for the activation of NR2B-NMDARs that would otherwise remain latent during LTP induction (perhaps at nearby extra- and perisynaptic pools). In contrast, $D_{1/5}$ receptors are located on dendritic spines (Huang et al., 1992; Levey et al., 1993). Via PKA and SFKs, $D_{1/5}$ receptors enhance the activity of NR2B-NMDARs (Stramiello & Wagner, 2008) - a series of events likely occurring at the PSD-associated scaffolding protein RACK1 (Yaka et al., 2003). Thus, although both D_3- and $D_{1/5}$-mediated pathways both ultimately enhance NR2B-NMDAR activity (possibly at difference receptor pools), the varying proximities of D_3 and $D_{1/5}$ receptors to D_4 might result in different levels of susceptibility to D_4-mediated disruption of cAMP signaling. Although it has been determined that CA1 pyramidal cells express D_4 receptors (Khan et al., 1998), their exact position has not yet been described. Our findings would be consistent with their presence in dendritic spines, near $D_{1/5}$.

Hippocampal dopamine has been shown to strongly impact various forms of learning and memory, including inhibitory avoidance (Bernabeu et al., 1997) and spatial navigation (Gasbarri et al., 1996; also see review by Lisman & Grace, 1995). Thus, it is important to understand the various ways in which this neurotransmitter affects hippocampal LTP. In this work, I elucidated such contributions from two D2-like dopamine receptors which are prevalent in the stratum radiatum of CA1. Interestingly, one dopamine receptor subtype, D_3, elicits an effect also caused by $D_{1/5}$ activity—enhancement of LTP via NR2B-NMDARs. The apparent cooperation between these receptor subtypes underscores the significance of dopaminergically-enhanced LTP in the CA1 region, as well as the modulatory importance of NR2B-NMDARs to LTP in general. The other dopamine receptor subtype, D_4, is capable of preventing $D_{1/5}$-mediated LTP enhancement.

This observation is of potential therapeutic interest, as there is mounting evidence that the D_4 receptor is important in the pathophysiology of addiction (reviewed by Lauzon and Laviolette, 2010) and perhaps in offsetting $D_{1/5}$-mediated behaviors related to drug-exposure [e.g., $D_{1/5}$-mediated, amphetamine/methamphetamine-induced locomotor activity (Hall et al., 2009), to which $D_4^{-/-}$ animals exhibit supersensitivity (Rubinstein et al., 1997)]. Although controversial, a potential role for D_4 receptors in schizophrenia and/or the therapeutic profile of antipsychotics is also possible (Wilson et al., 1998; Oak et al., 2000; Wong and Van Tol, 2004; Kapur et al., 2006). Future studies of D_4 receptor function and expression in the CA1 region, as well as increased availability of highly selective ligands, will surely shed light on the therapeutic relevance of this largely understudied receptor.

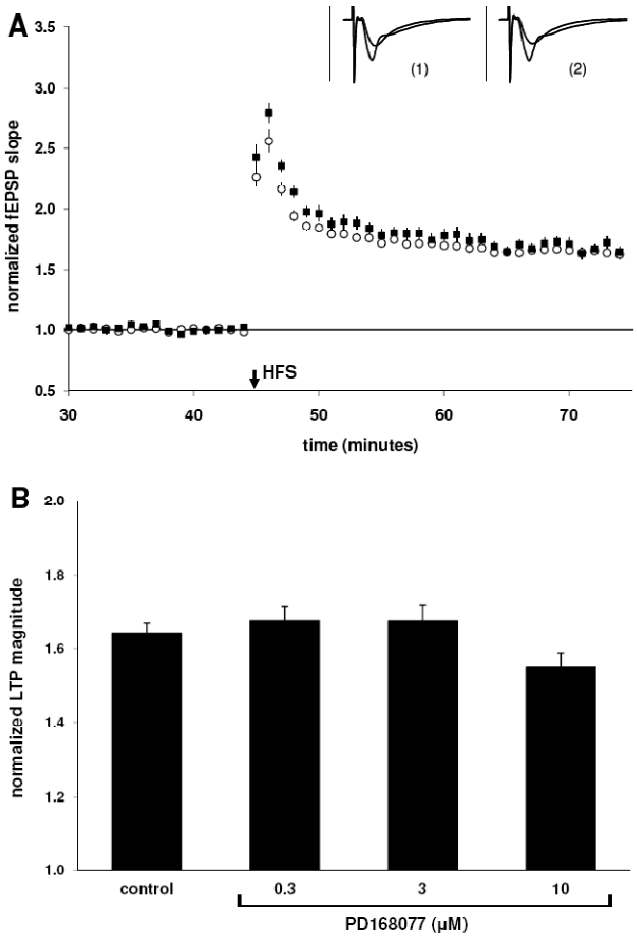

Figure 3.1. The D_4 dopamine receptor agonist PD168077 did not affect LTP magnitude. **A)** Summary plot of normalized fEPSP slope measurements evoked and recorded in the stratum radiatum layer of the CA1 region. Open circles show responses from control slices; closed circles depict responses from PD168077-treated slices (3 μM). Insets are 50 ms sweeps averaged from all experiments illustrating the mean fEPSP 1–5 min prior to and 26–30 min post-tetanus (vertical scale bar is 3 mV). The left pair of sweeps (1) is from non-drug, control slices and the right pair (2) is from PD168077-treated slices. **B)** Summary quantification of LTP magnitude in the presence of increasing concentrations of PD168077. LTP magnitude was not significantly altered at concentrations of 0.3 μM, 3 μM, or 10 μM relative to control. Error bars show ± SEM.

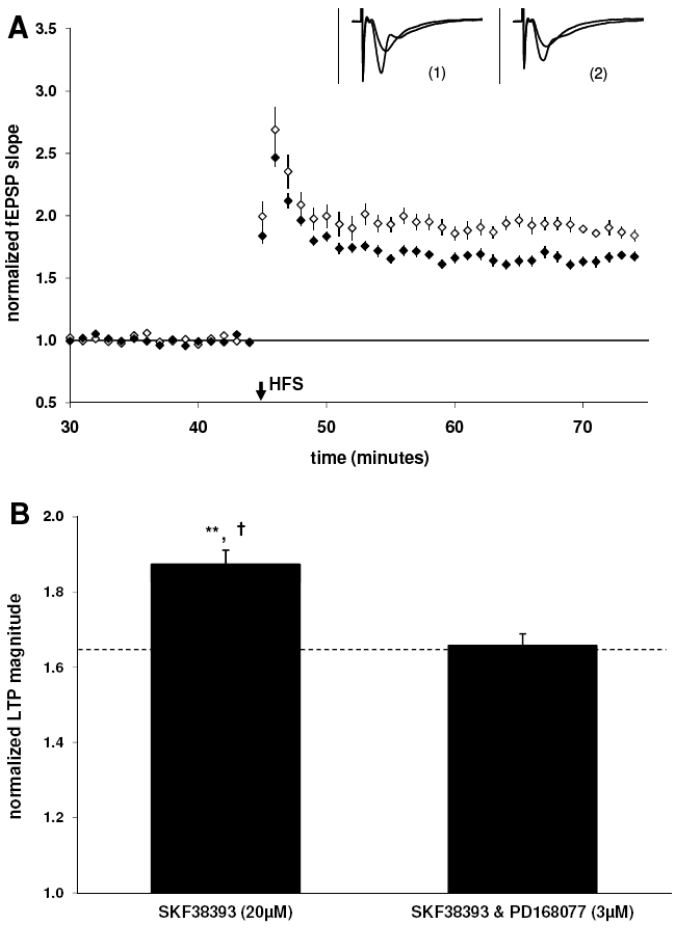

Figure 3.2. PD168077 blocked the ability of $D_{1/5}$ agonist SKF38393 (20 μM) to enhance LTP. **A)** Summary plot of normalized fEPSP measurements. Open diamonds depict slices treated with $D_{1/5}$ agonist SKF38393 (20 μM). Closed diamonds depict slices treated with both PD168077 (3 μM) and SKF38393 (20 μM). Insets are 50 ms sweeps averaged from all experiments illustrating the mean fEPSP 1–5 min prior to and 26–30 min post-tetanus (vertical scale bar is 3 mV). The left pair of sweeps (1) is from SKF38393-treated slices and the right pair (2) is from PD168077 & SKF38393-treated slices. **B)** Summary quantification of LTP magnitude in the presence of SKF38393 alone and when co-applied with PD168077 (significance relative to the control group indicated by the dashed line, $^{**}p < 0.01$; significance relative to the SKF38393 & PD168077 co-application group is also indicated, $\dagger p < 0.01$). Error bars show ± SEM.

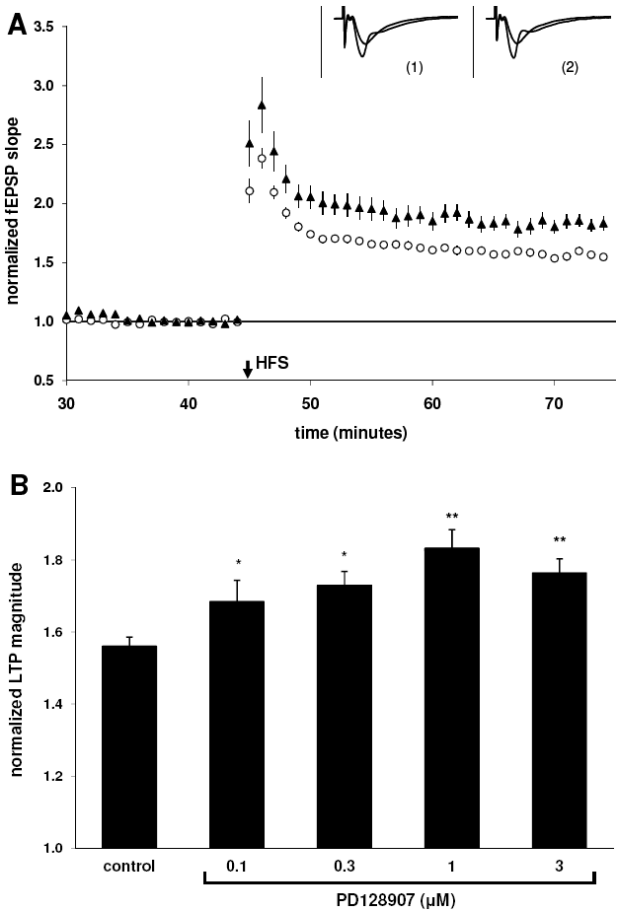

Figure 3.3. The D_3 dopamine receptor agonist PD128907 induced a dose-dependent enhancement of LTP. **A)** Summary plot of normalized fEPSP slope measurements evoked and recorded in the stratum radiatum layer of the CA1 region. Open circles show responses from control slices; closed triangles depict responses from PD128907-treated slices (1 μM). Insets are 50 ms sweeps averaged from all experiments illustrating the mean fEPSP 1–5 min prior to and 26–30 min post-tetanus (vertical scale bar is 3 mV). The left pair of sweeps (1) is from non-drug, control slices and the right pair (2) is from PD128907-treated slices. **B)** Summary quantification of LTP magnitude in the presence of increasing concentrations of PD128907. Control LTP is indicated by the dashed line. LTP magnitude was significantly enhanced at concentrations of 0.1 μM, 0.3 μM, 1 μM, and 3 μM relative to control (*$p < 0.05$, **$p < 0.01$). Error bars show ± SEM.

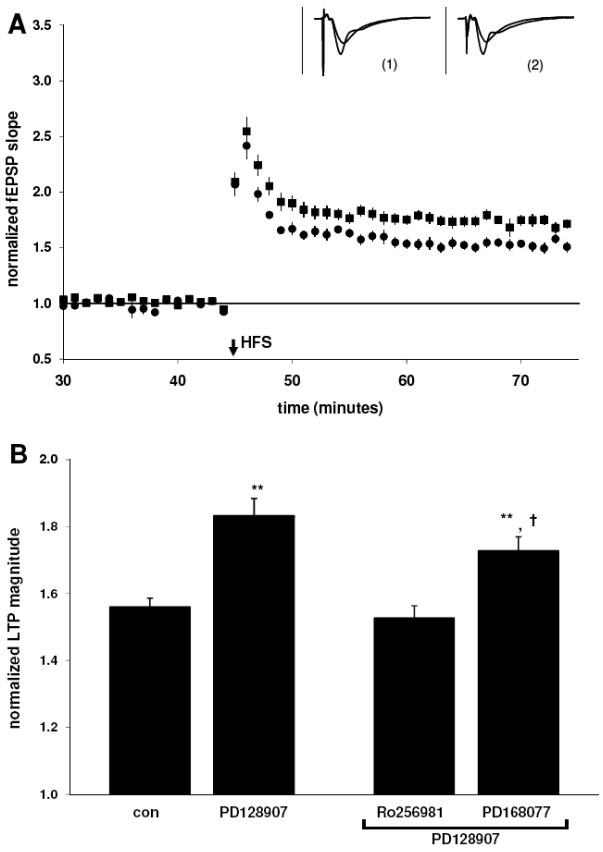

Figure 3.4. Co-application with the NR2B NMDAR antagonist Ro25-6981 (1 μM), but not D_4 agonist PD168077, inhibited PD128907-mediated enhancement of LTP. **A)** Summary plot of normalized fEPSP measurements. Closed circles show normalized fEPSP slope from slices treated with both PD128907 (1 μM) and Ro256981 (1 μM); closed squares depict slices treated with both PD128907 (1 μM) and PD168077 (3 μM). Insets are 50 ms sweeps averaged from all experiments illustrating the mean fEPSP 1–5 min prior to and 26–30 min post-tetanus (vertical scale bar is 3 mV). The left pair of sweeps (1) is from PD128907 & Ro256981-treated slices and the right pair (2) is from PD128907 & PD168077-treated slices. **B)** Summary quantification of LTP magnitude in the presence of PD128907 when co-applied with Ro25-6981 and PD168077, as compared to the control and PD128907 (1μM) alone groups (taken from Fig. 3, illustrated for comparison; significance relative to the control group, $^{**}p < 0.01$; significance relative to the PD128907 & Ro25-6981-treated group, $\dagger p < 0.01$). Error bars show ± SEM.

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CHAPTER FOUR

COCAINE ENHANCEMENT OF LONG-TERM POTENTIATION IN THE CA1 REGION OF RAT HIPPOCAMPUS: LAMINA-SPECIFIC MECHANISMS OF ACTION

Stramiello, M., Wagner, J.J. 2010. *Synapse (in press)*.

Abstract:

There is an expanding body of work characterizing dopaminergic modulation of synaptic plasticity in the hippocampus CA1 region, an area known to be involved in learning and memory. However, in vitro studies to date have focused almost exclusively on the proximal and distal apical dendritic layers (strata radiatum and lacunosum moleculare, respectively). In this report we establish that dopaminergic activity can enhance long-term potentiation (LTP) in the basal dendritic layer (stratum oriens) of CA1 in the rat hippocampal slice preparation. Application of the $D_{1/5}$ agonist SKF38393 (20μM) significantly increased the magnitude of basal LTP of the fEPSP response following high-frequency stimulation of the Schaffer collateral/commissural inputs in the stratum oriens layer. In addition, endogenous dopamine activity facilitated by the presence of cocaine (6μM) was also capable of enhancing the magnitude of basal LTP. Prior application of the $D_{1/5}$ antagonist SKF83566 (2μM) prevented this effect of cocaine, indicating that endogenously released dopamine was exerting its LTP-enhancing effect in stratum oriens via activation of $D_{1/5}$ receptors. This final result stands in contrast with the previously characterized effects of cocaine on apical LTP in the stratum radiatum, which instead have been shown to require D_3 receptor activation. These observations demonstrate that dopaminergic mechanisms resulting in the enhancement of hippocampal LTP are lamina specific at Schaffer collateral/commissural synapses in the CA1 region.

Keywords - synaptic plasticity; stratum oriens; D_1 receptor; SKF38393

Introduction:

The hippocampus has been identified as a brain region involved with reinstatement of drug-seeking behavior in rats previously allowed to self-administer cocaine (Vorel et al., 2001). As a preclinical model, rodent self-administration is argued to have relevance for investigating the relapse of drug-taking in human addiction to drugs of abuse (Epstein et al., 2006). More specifically, the dorsal hippocampus is required for context-induced reinstatement of cocaine-seeking (Fuchs et al., 2005) and the ventral hippocampus is known to mediate both cue- and drug-primed reinstatement behavior (Rogers and See, 2007; Sun and Rebec, 2003). These findings emphasize the relevance of investigating the actions of cocaine in the hippocampus, which, as a brain structure known to be involved with learning and memory, may therefore be a key site of interest for investigating the "memories of addiction" as they may relate to the relapse of drug-taking behavior.

Prior studies have demonstrated that physiologically relevant concentrations of cocaine can increase the magnitude of long-term potentiation (LTP) in the CA1 region of rat hippocampal slices (Thompson et al., 2005). As LTP is thought to be one potential mechanism for information processing and storage in the brain that contributes to experience-dependent modification of neural circuitry (Malenka and Bear, 2004), cocaine-mediated enhancement of LTP may be an important component of the neuroadaptations that occur following drug exposure (Thompson et al., 2004), in addition to direct cocaine-induced potentiation itself (Ungless et al., 2001). Underlying its enhancement of LTP is cocaine's action as an inhibitor of the dopamine transporter (DAT) and a subsequent increase in the activity of endogenous dopamine (Thompson et al., 2005). This effect of cocaine is mediated by the D_3 subtype of dopamine receptor (Swant and Wagner, 2006). Interestingly, D_3 receptor activation decreased evoked IPSCs and enhanced

LTP specifically at apical synapses in the stratum radiatum layer, and no such effects were observed at basal synapses in the stratum oriens layer of CA1 (Swant et al., 2008).

This last observation, along with the known ability of D1/5 agonists (Otmakhova and Lisman, 1996) and dopamine transporter inhibitors (Thompson et al., 2005) to enhance LTP in the stratum radiatum, prompted us to further investigate the actions of cocaine and dopaminergic modulation of LTP in the basal dendritic compartment of CA1. Additionally, a recent report has described the presence of dopaminergic fibers in the stratum oriens (Kwon et al., 2008), further suggesting that LTP at basal dendrites may likewise be influenced by endogenously released dopamine.

Methods:

1. Extracellular electrophysiology

Hippocampal slices were prepared from male Sprague-Dawley rats (40-90 days old) as previously described (Stramiello and Wagner, 2008). A bipolar stimulating electrode (Kopf Instruments) was placed on the CA3-side of the CA1 region in the stratum oriens, and a 1.0 MΩ tungsten recording microelectrode (World Precision Instruments) was then positioned in the same layer in CA1. Data were digitized at 10 kHz, low-pass filtered at 1 kHz, and analyzed with pCLAMP 9.2 software (Axon Instruments). The initial slope of the population fEPSP was measured by fitting a straight line to a 1 msec window immediately following the fiber volley. A stimulus-response curve was obtained at the beginning of each experiment, with stimulus pulses consisting of a single square wave of 270 μs duration delivered at 40-160 μA. The stimulation intensity was adjusted to obtain a field EPSP of approximately 1mV in amplitude to begin baseline recording, and fEPSPs were elicited by stimulation of the Schaffer collateral-commissural pathway in stratum oriens once every 60 s (.0167 Hz) for the duration of the experiment.

2. Quantification of synaptic plasticity

Synaptic responses were normalized by dividing all slopes by the average of the 5 fEPSP slopes obtained from the 5 min prior to tetanization. The tetanization protocol used to induce LTP in all experiments was a standard HFS protocol consisting of 3 trains of 100 Hz/1s administered at 20 s intertrain intervals. LTP measurements were calculated by averaging fEPSP slope values from 26-30 min following HFS administration. Slices exhibiting LTP values greater or less than 2 times the standard deviation of the mean of the respective test groups were

discarded from the analysis as outliers (2 of 56 slices). A one-way ANOVA followed by Dunnet's post hoc test was used to evaluate the group data reported in Fig. 2B. In reporting our results, n-values indicate first the number of slices, and then the number of animals. Drugs were bath-applied for at least 30 min prior to tetanus, and remained present for the duration of recording. None of the applied compounds had significant effects on the baseline fEPSP response during the wash-in period (range 0-7% change/30 min).

3. Drugs

(±)-1-Phenyl-2,3,4,5-tetrahydro-(1H)-3-benzazepine-7,8-diol hydrochloride (SKF38393) and 8-Bromo-2,3,4,5-tetrahydro-3-methyl-5-phenyl-1*H*-3-benzazepin-7-ol hydrobromide (SKF83566) were obtained from Tocris, and cocaine hydrochloride was obtained from NIDA (RTI).

Results / Discussion:

Afferent input to the hippocampus CA1 region arrives via two major pathways. One, the temporoammonic pathway, arises in layer III of the entorhinal cortex and synapses directly on distal apical dendrites of stratum lacunosum moleculare. The other pathway, consisting of Schaffer collateral/commissural fibers from the CA3 region, synapses on both apical dendrites in stratum radiatum and basal dendrites in stratum oriens. Several reports from the last twenty years have demonstrated that exogenously applied dopaminergic agonists and antagonists can modulate the magnitude and/or persistence of LTP in the stratum radiatum layer of the CA1 region of the hippocampus (Frey et al., 1990; Frey et al., 1991; Huang and Kandel, 1995; Otmakhova and Lisman, 1996; Swanson-Park et al., 1999; Swant and Wagner, 2006), and endogenously released dopamine is thought to serve an important role for novelty detection and long-term memory formation (Li et al., 2003; Lisman and Grace, 2005). However, in contrast to this apical LTP, the study of dopaminergic modulation of basal LTP within stratum oriens synapses of CA1 has received minimal attention.

Compared to apical LTP in stratum radiatum, basal LTP evoked in stratum oriens has previously been characterized in vivo as exhibiting a lower threshold and higher amplitude (Kaibara and Leung, 1993), suggesting that there may be differences in how neuromodulators influence synaptic plasticity processes in these lamina. Indeed, recent evidence indicates that cholinergic tone from the basal forebrain hippocampal input can selectively enhance LTP in the stratum oriens (Doralp and Leung, 2008; Ovsepian, 2008). The physiological relevance of the differential distribution of LTP across basal and apical dendritic compartments is not known, but the behavioral state of the animal can alter the propensity for LTP recorded in the stratum oriens

(Leung et al., 2003), thus the ratio of basal/apical potentiation may be a salient factor in the processing of information through the CA1 region of the hippocampus.

An exception to the relative lack of information concerning dopaminergic modulation of LTP at basal synapses in the CA1 is our recent report that D_3 activation has no effect on LTP in the stratum oriens, despite its efficacy for enhancing LTP at apical dendritic synapses of the stratum radiatum (Swant et al., 2008; see Appendix A). As evidence indicates that dopamine is present at a higher concentration in stratum oriens relative to the stratum radiatum (Gasbarri et al., 1994; Kwon et al., 2008), it was expected that basal LTP would be subject to dopaminergic modulation. In the current report we have added to the characterization of dopamine actions in the CA1 to now also include effects of $D_{1/5}$ receptor activation and monoamine transport blockade on basal LTP in the stratum oriens. LTP measured 25-30 minutes following high frequency stimulation (100Hz/1 sec *3) in the control group measured 87 ± 6% (n = 12, 5; Fig. 4.1A). We found that both the $D_{1/5}$ agonist SKF38393 (20μM; 134 ± 10%; n = 16, 7; $p < 0.01$; Fig. 4.1B) and the indirect dopamine agonist cocaine (6μM; 121 ± 8%; n = 16, 8; $p < 0.05$; Fig. 4.2A) were each capable of enhancing basal LTP. Additionally, the effects of cocaine were blocked by prior application of the D1/5 antagonist SKF83566 (2μM; 94 ± 6%; n = 10, 5; Fig. 4.2B), indicating that cocaine exerted its LTP-enhancing effect via $D_{1/5}$ receptors in stratum oriens.

Of the five known subtypes of dopamine receptors, D_2 receptors appear to be expressed at very low levels in the CA1 (Khan et al., 1998) and D_4 receptors have been shown to inhibit NMDA receptor activity in the CA1 (Kotecha et al., 2002). Consequently, our work to date concerning the dopaminergic enhancement of LTP has focused on receptors of the $D_{1/5}$ and D_3 subtypes. These studies have demonstrated that activation of either dopamine receptor subtype

(as well as DAT blockade) can enhance apical LTP (Table 4.1). As these receptors are also present in the stratum oriens, we sought to determine whether DA receptor activation would also be effective in enhancing LTP at the Schaffer collateral synapses of the basal dendrites. The data presented in Fig. 1 illustrates the capacity of an exogenously applied $D_{1/5}$ agonist to enhance basal LTP, much as we have observed for apical LTP in the stratum radiatum (Stramiello and Wagner, 2008).

With respect to the role of endogenously released dopamine, we have previously shown that cocaine (5-10 μM) is capable of enhancing apical LTP in the stratum radiatum, an effect that was blocked by coapplication of the $D_{2\text{-like}}$ antagonist eticlopride (Thompson et al., 2005). Further investigation with the DAT-specific compound GBR12935 (1 μM) showed that this effect in the stratum radiatum is dependent upon activation of the D_3 receptors (Swant and Wagner, 2006), which likely enhances apical LTP via an increase in $GABA_A$ receptor endocytosis (Swant et al., 2008). In contrast, $D_{1/5}$ receptor activation enhances apical LTP following enhancement of NR2B-containing NMDA receptor activity (Stramiello and Wagner, 2008). In the former scenario, a D_3-mediated decrease in protein kinase A activity occurs whereas in the latter, a $D_{1/5}$-mediated increase in protein kinase A activity occurs-the net effect of either resulting in a facilitation of LTP. As it is known that dopamine has a relatively high affinity for D_3 receptors in comparison to $D_{1/5}$ receptors (Missale et al., 1998; Sokoloff et al., 1992), and that endogenous DA transmitter is scarce in the stratum radiatum, it is reasonable that DAT blockers such as cocaine and GBR12935 exert their effects via D_3 receptors in this layer where action at a distance is required. The relative abundance and proximity of DA in stratum oriens (Gasbarri et al., 1994; Kwon et al., 2008) would be one explanation for why cocaine can

exert its neuromodulatory effect on basal LTP via $D_{1/5}$ receptor activation in this layer (Fig. 4.2), despite the lower affinity of dopamine for these receptors.

In this study, we have demonstrated that either direct $D_{1/5}$ receptor activation or increased activity of endogenously released dopamine is capable of enhancing basal LTP in the stratum oriens of CA1 pyramidal neurons. A summary of findings to date (Table 4.1) illustrates that the enhancement of LTP at Schaffer collateral synapses via endogenous dopamine release in the hippocampus can exhibit laminar specificity in the CA1 region with respect to the dopamine receptor subtype being activated. Alterations in the balance of synaptic plasticity between apical and basal dendritic compartments of the CA1 region by dopaminergic systems may underlie both physiological (learning & memory) activity and pathophysiological (e.g. schizophrenia, addiction) disease states related to hippocampal function.

TABLE 4.1. Dopaminergic enhancement of LTP at Schaffer collateral/commissural inputs to CA1

	$D_{1/5}$	D_3	cocaine	references
s. oriens	*	— [a]	*	*a)* Swant et al. (2008) *b)* Otmakhova & Lisman (1996)
s. radiatum	*[b]	*[c]	*[d]	*c)* Swant & Wagner (2006) *d)* Thompson et al. (2005)

(*) significance relative to control LTP measured in the same layer.

Acknowledgements – J.J.W. was funded by National Institute on Drug Abuse (DA 16302).

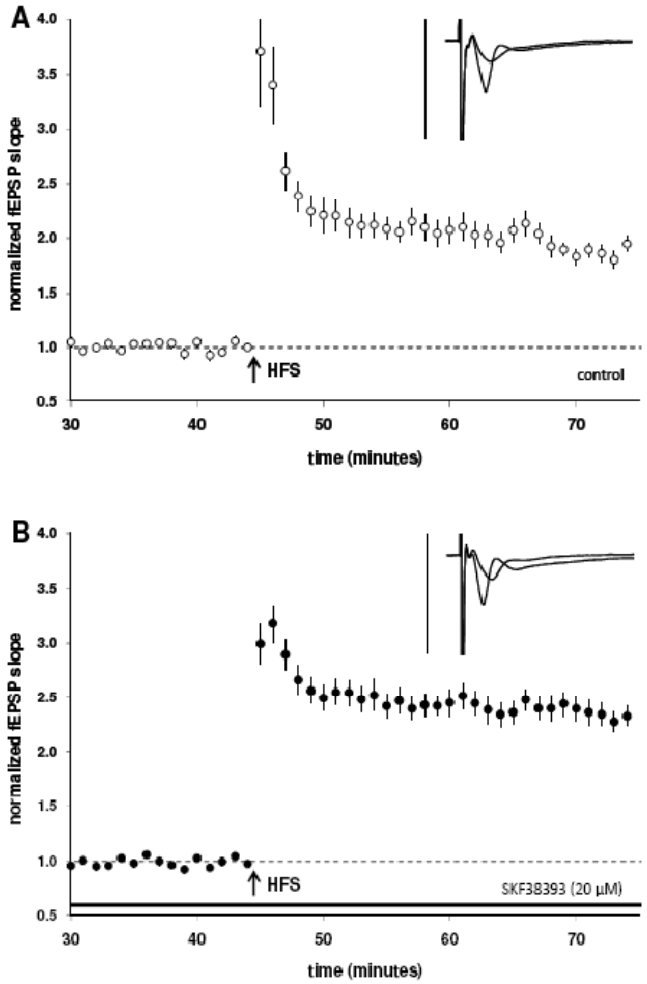

Figure 4.1. Comparison of CA1 basal LTP in controls with a group of slices treated with SKF38393 (20 µM). Insets are 50 ms sweeps averaged from all experiments illustrating the mean fEPSP 1-5 min prior to and 26-30 min post-HFS (vertical scale bar is 3 mV). **A)** Summary plot of normalized fEPSP slope measurements evoked and recorded in the stratum radiatum layer of the CA1 region. **B)** SKF38393 (20 µM) is capable of significantly enhancing LTP in the stratum oriens.

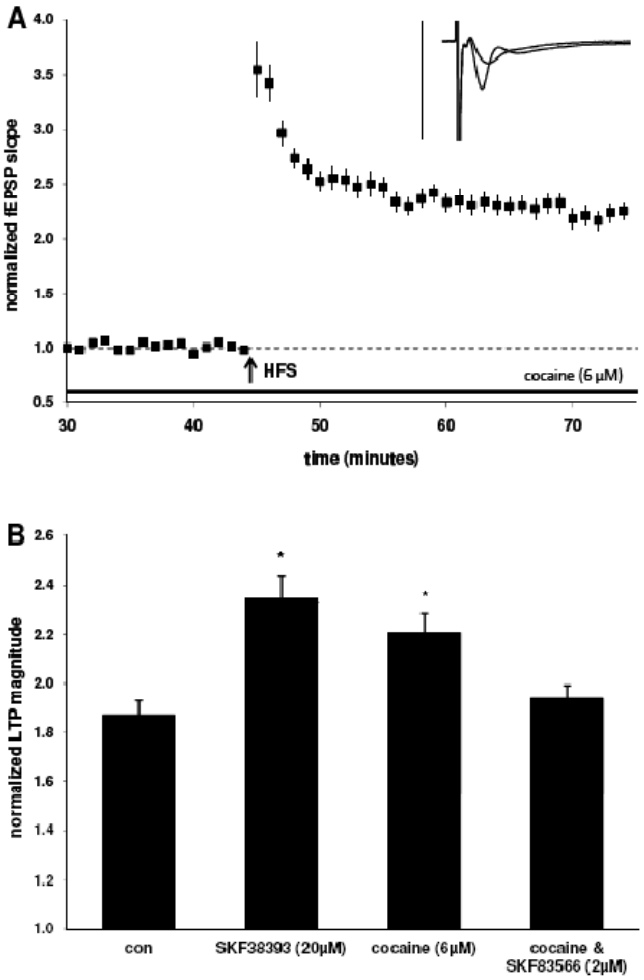

Figure 4.2. Cocaine enhanced basal LTP via $D_{1/5}$ receptor activation. **A)** Cocaine (6 µM) is also capable of significantly enhancing LTP in the stratum oriens. Insets are 50 ms sweeps averaged from all experiments illustrating the mean fEPSP 1-5 min prior to and 26-30 min post-tetanus (vertical scale bar is 3 mV). **B)** LTP magnitude in slices treated with either SKF38393 or cocaine was significantly enhanced when compared to the control group (*, $p<0.05$, one-way ANOVA/Dunnett's), while coapplication of D1/5 antagonist SKF83566 (2 µM) was able to prevent cocaine-mediated LTP enhancement, resulting in LTP which was similar in magnitude to the control level. Bars represent LTP measured at 26-30 minutes post-HFS.

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CHAPTER FIVE

CHARACTERIZATION OF D3-MEDIATED DISINHIBITION IN STRATUM RADIATUM OF THE HIPPOCAMPUS CA1 REGION

Stramiello, M. and Wagner, J.J. To be submitted to Journal of Neurophysiology.

Abstract:

Previous work from the Wagner Lab elucidated the disinhibitory effect of D_3 dopamine receptor activation in the CA1 region of rat hippocampus, an area critically involved in learning and memory. In this report I utilize the whole-cell voltage clamp recording technique to provide additional details of this disinhibition within the hippocampal slice preparation. Application of the D_3 agonist PD128907 (1 μM) significantly decreased the magnitude of IPSC responses stimulated from within the stratum radiatum layer. Prior application of the $D_{1/5}$ agonist SKF38393 (20 μM) or SKF81297 (10 μM) was capable of blocking this effect, consistent with its dependence upon disruption of PKA signaling. The mGluR agonist DHPG (25 μM) was also able to prevent a D_3-induced decrease in IPSC amplitude through a postsynaptic mechanism. Using the μOR agonist DAMGO (1 μM), I demonstrated that the disinhibition resulted from a suppression of input from PV^+ cells, a major class of interneuron innervating the stratum radiatum layer of CA1. Pharmacokinetic changes in IPSCs recorded in the presence of DAMGO support the proposal by Swant et al., (2008) that this disinhibitory effect of D_3 activation occurs at extrasynaptic receptor pools.

Keywords - $D_{1/5}$ receptor; D_3 receptor; PD128907; SKF91297; SKF38393; DAMGO; DHPG; mGluR; extrasynaptic

Introduction:

In the hippocampal CA1 region, long-term potentiation (LTP) has long been a model for the cellular foundations of learning and memory (Selig et al., 1996). Although LTP involves a net strengthening of excitatory output, it is important to consider that a concurrent decrease of inhibition can play a key role in the manifestation of LTP (Stelzer et al., 1993). Indeed, disinhibition has recently been demonstrated to be capable of mediating LTP independently of a classical induction protocol (Ormond & Woodin, 2009). Its effects can also serve a modulatory role. Such modulation is especially pertinent with regard to the effects of cocaine in the hippocampus, as DAT blockade enhances hippocampal LTP via a D_3-mediated disinhibitory mechanism (Swant & Wagner, 2006; Swant et al., 2008).

Inhibitory neurotransmission in the dendritic layers of the CA1 region is driven by well over a dozen types of interneurons (Klausberger, 2009). Although knowledge of these cells is evolving at a rapid pace (Freund & Katona, 2007), a large portion of these known cell types may be divided into two major categories based on their expression of either parvalbumin (PV, a calcium binding protein) or cholecystokinin (CCK, a neuropeptide). Additionally, immunoreactivity studies of the hippocampus have shown that cannabinoid receptor type 1 (CB_1) is primarily localized on CCK^+ cells (Tsou et al., 1999), while μ-opioid receptors (μORs) are absent from CCK^+ cells and primarily expressed by PV^+ interneurons (Drake & Milner, 2002).

Previous work has shown that D_3 dopamine receptor activation can, by stimulating $GABA_AR$ endocytosis, decrease CA1 pyramidal cell IPSCs evoked via stratum radiatum stimulation. Evidence for PKA involvement in this mechanism was provided and it was hypothesized that D_3 receptors preferentially affected extrasynaptic pools of $GABA_ARs$ (Swant et al., 2008). In the current study, I sought to further characterize D_3-mediated inhibition of

IPSCs by determining the class of interneuron input being modulated and identifying additional aspects of the postsynaptic signaling involved. Using the hippocampal slice preparation and whole-cell voltage clamp recording technique, I replicated the D_3 effect and found that it was subject to inhibition by several different ligand pre-treatments, one of which implicates PV^+ interneuron input. Additionally, kinetic observations supported the theory that D_3-induced endocytosis occurs preferentially at extrasynaptic $GABA_A$Rs. Together, these results provide a clearer picture of the way in which dopamine mediates disinhibition in the CA1, ultimately acting to enhance the net excitability at Schaffer collateral inputs.

Methods:

1. Hippocampal slice preparation

Hippocampal slices were prepared from male Sprague-Dawley rats (28-56 days old) using an experimental protocol performed in compliance with the University of Georgia Animal Care and Use guidelines. All rats were anesthetized with halothane prior to decapitation. The brain was removed and submerged in ice-cold, oxygenated (95% O_2/ 5% CO_2) dissection artificial cerebrospinal fluid (ACSF) containing (mM): NaCl (120), KCl (3), $MgCl_2$ (4), NaH_2PO_4 (1), $NaHCO_3$ (26), and glucose (10). Horizontal brain slices were cut at a thickness of 500 μM, the hippocampus dissected, and the CA3 region removed. Slices were then perfused with room-temperature, oxygenated (95% O_2/5% CO_2) standard ACSF containing (mM): NaCl (120), KCl (3), $MgCl_2$ (1.5), NaH_2PO_4 (1), $CaCl_2$ (2.5), $NaHCO_3$ (26), and glucose (10) at approximately 2 ml/min. Slices were placed in the recording chamber and allowed to recover for one hour at room temperature and then a second hour at 30°C, the temperature at which recordings were obtained.

2. Whole-cell electrophysiology

Stimulating and recording pipettes were positioned in the slice using a motorized micromanipulator (Sutter) and microscope (Nikon). A bipolar tungsten stimulating electrode (Kopf Instruments), connected to a constant current stimulus isolation unit (WPI), was placed in stratum radiatum of the CA3-side of the slice in order to stimulate Schaffer collaterals. Subsequently, the recording pipette [ranging from 2 – 4 MΩ; pulled from 1.5-mm thin-wall borosilicate glass tubing (WPI) on a Sutter P-97 flaming/brown type micropipette puller] was positioned outside the surface of the stratum pyramidale in the CA1 region. While using an ez-

gSEAL 100A (NeoBiosystems) to supply it with 100 mmHg of positive pressure, the recording pipette was then advanced into the stratum pyramidale until a cell was struck (as indicated by an increase in resistance at the tip of the recording pipette). Seals were formed by ceasing application of positive pressure in the recording pipette and then applying slight suction of varying intensities between 10 – 40 mmHg. Slight negative holding current was also applied as necessary to help form a seal. Once seals stabilized at or above 150 MΩ, or if spontaneous break-in occurred, suction was immediately ceased and holding current set to -60 mV. When needed for break-in, progressively increasing manual suction was applied to the recording pipette. Synaptic currents, as well as holding current and input resistance, were monitored in voltage-clamp mode using an Axopatch 200B amplifier (Axon Instruments). Data, digitized at 10 kHz and low-pass filtered at 1 kHz, were acquired and analyzed using pCLAMP 9.2 (Molecular Devices).

3. Evoked monosynaptic IPSCs (eIPSCs)

Intracellular recording solution consisted of: 145 mM Cs-$MeSO_4$, 2 mM $MgCl_2$, 2 mM EGTA, 0.2 mM $CaCl_2$, 2 mM Mg-ATP, 5 mM QX-314, 0.5 mM Mg-GTP, and 2 mM HEPES, pH 7.2. IPSCs were pharmacologically isolated using bath-applied CNQX (10 μM) and DL-AP5 (50 μM). eIPSCs were recorded every 30 s at a holding potential of approximately 0 mV. Input resistance was monitored once per minute using a negative 5-10 mV step, and cells were discounted if input resistance changed 15% or more during drug application. For all experiments, the Schaffer collaterals received paired (500 ms interval between pulses) stimulation once every 30 s (0.033 Hz). Before beginning each experiment, a stimulus-response curve was obtained, with stimulus pulses (delivered at 25-200 μA) consisting of a single square wave of 300 μs

duration. The stimulus intensity was adjusted to maximize eIPSC amplitude. During baseline recording, duration of stimuli alternated between 300 μs and 200 μs (data collected via the latter is not shown in this report).

The peak amplitude of the monosynaptic IPSC response was used for statistical analysis (data 1-5 min before vs. 26-30 min after drug application). Control LTP values were collected periodically throughout the study. Planned comparisons with control were made using unpaired Student's *t*-tests. In reporting my results, n-values indicate the number of cells.

4. Drugs

(+)-(4aR,10bR)-3,4a,4,10b-Tetrahydro-4-propyl-2H,5H-[1]benzopyrano-[4,3-b]-1,4-oxazin-9-ol hydrochloride (PD128907), N-(Methyl-4-(2-cyanophenyl)piperazinyl-3-methylbenzamide maleate (PD168077), (±)-1-Phenyl-2,3,4,5-tetrahydro-(1H)-3-benzazepine-7,8-diol hydrochloride (SKF38393), [D-Ala2, NMe-Phe4, Gly-ol5]-enkephalin (DAMGO), and (S)-3,5-Dihydroxyphenylglycine (DHPG) were obtained from Tocris. Dimethyl sulfoxide (DMSO) and (±)-6-Chloro-2,3,4,5-tetrahydro-1-phenyl-1H-3-benzazepine hydrobromide (SKF81297) were obtained from Sigma. Drugs were applied by addition to the perfusion reservoir; stocks for SKF81297 and PD168077 were initially dissolved in DMSO, with the final concentration of this solvent being 0.05%. For experiments in which two drugs were tested, application of the first began at least 15 min prior to, and continued throughout, application of the second. Each drug, once applied, remained present for the duration of recording.

Results:

1. D_3 dopamine agonist PD128907 (1 μM) decreases IPSCs, an effect that can be inhibited by $D_{1/5}$ agonist pre-application

Monosynaptic IPSCs were evoked by stimulation in the stratum radiatum of the hippocampus CA1 region. Bath application of the D_3 agonist PD128907 (1 μM) significantly decreased IPSC amplitude (-18 ± 3%; $n = 6$; $p < 0.05$) at 30 min following its application, whereas $D_{1/5}$ agonists SKF38393 (20 μM) and SKF81297 (20 μM) had no significant effect when applied alone (+13 ± 7%; $n = 8$; Fig 5.1A). Pretreatment with a $D_{1/5}$ agonist, however, was capable of blocking a significant D_3-mediated effect (-1 ± 3%; $n = 4$; Fig 1B). Because $D_{1/5}$ receptor activation would be expected to stimulate AC (Kebabian & Calne, 1979), this result is consistent with the notion that an inhibition of the AC/cAMP/PKA pathway is required for the D_3 receptor to exert its effects on the evoked IPSC.

2. mGluR agonist DHPG (25 μM) decreases IPSCs and blocks the effect of D_3 agonism on inhibitory transmission

Bath application of the mGluR receptor agonist DHPG (25 μM) significantly decreased IPSC amplitude (-52 ± 9%; $n = 4$; $p < 0.05$; Fig 5.2A). A similar effect due to endocannabinoid-mediated retrograde inhibition of CCK^+ cells has been previously reported (Varma et al., 2001; Maejima et al., 2001); however, my data showed no evidence of altered presynaptic activity (see Results 3.4). This inhibitory effect was likely caused by mGluR-stimulated phospholipase C (PLC) activity (see Discussion), which is known to inhibit IPSCs in the CA1 (Tanaka et al., 1997). When DHPG was applied prior to PD128907 (1 μM), the latter drug no longer significantly affected IPSC magnitude (+4 ± 3%; $n = 3$; Fig 5.2B).

3. μOR agonist DAMGO (1 μM) decreases IPSCs and blocks the effect of D_3 agonism on inhibitory transmission

Bath application of the μOR agonist DAMGO (1 μM) significantly decreased IPSC amplitude (-66 ± 7%; $n = 4$; $p < 0.01$; Fig 5.3A). When DAMGO was applied prior to PD128907 (1 μM), the latter drug no longer affected IPSC magnitude (+1 ± 6%; $n = 6$; Fig 5.3B). Coupled with paired-pulse data recorded from these cells (see Results 3.4) this observation links D_3-mediated inhibition to $GABA_A$Rs innervated by μOR-expressing interneurons.

4. DAMGO inhibits paired-pulse depression of IPSCs; DHPG has no effect

Paired pulse depression (PPD) of IPSCs in the CA1 of adult rat hippocampal slices is predominantly mediated by $GABA_B$Rs (Pitler and Alger, 1994) and reflects presynaptic changes in quantal size and release probability (Chen et al., 2004). Two representative summary sweeps were generated by averaging from all cells data 1-5 min pre-DAMGO and data 26-30 min post-DAMGO (1μM) application (Fig 5.4A). "Δ % of pre-drug PPD" was obtained through the following steps: 1) for individual cells, sweeps 1-5 min pre-drug application were averaged together; 2) for each of these averaged sweeps, the peak amplitude of pulse 2 was divided by the peak amplitude of pulse 1, and the quotient was subtracted from 1.00 ("% pre-drug PPD"); 3) for individual cells, the previous two steps were repeated using sweeps 26-30 min post-drug application ("% post-drug PPD"); 4) the "% pre-drug PPD" was subtracted from the "% post-drug PPD." As demonstrated in Fig 5.4A, DAMGO significantly reduced PPD of IPSCs (Δ % pre-drug PPD: -31 ± 8%; $n = 5$; $p < 0.05$; Fig 5.4B), indicating that its ability to decrease IPSC amplitude occurred via a presynaptic mechanism. Cells exposed to DHPG did not exhibit a

decrease in PPD (Δ % pre-drug PPD: 1 ± 4; $n = 3$; Fig 5.4B), suggesting that this drug's ability to decrease IPSC amplitude resulted from a postsynaptic change.

5. DAMGO decreases half decay time of IPSC amplitude, while DHPG has no effect

Although both DAMGO (1 μM) and DHPG (25 μM) significantly decreased IPSC amplitude (Figs 5.2A and 5.3A), my data demonstrates that these two drugs had distinct effects on IPSCs. Notably, cells exhibiting a half decay time ($t_{1/2}$) of 78 ± 4 ms ($n = 4$) prior to drug exposure showed a significant reduction in $t_{1/2}$ within 30 min of DAMGO application (31 ± 4 ms; $n = 4$; $p < 0.05$; Fig 5.5A), while DHPG had no significant effect on $t_{1/2}$. Because the slower kinetics of extrasynaptic $GABA_A$Rs a are reflected in a slower IPSC decay time (Banks and Pearce, 2000), DAMGO's affect on $t_{1/2}$ suggests that inhibition of PV^+ interneuron input disproportionately reduces the contribution of extrasynaptic $GABA_A$Rs to evoked IPSCs.

Discussion:

In this study, I have investigated the capability of D_3 receptor activation to decrease IPSCs evoked by stimulation of the stratum radiatum layer in the CA1 region of the rat hippocampus. This effect was susceptible to inhibition by activation of either $D_{1/5}$ receptors and or mGluRs, suggesting the involvement of PKA- and PLC-signaling, respectively. Additionally, my results indicated that PV^+ cells make a disproportionately large contribution to extrasynaptic $GABA_AR$ activity, a finding consistent with my hypothesis that extrasynaptic $GABA_ARs$ are the primary target of D_3-mediated endocytosis. The underlying rationale for these conclusions is described below.

Previous work from my lab has shown that either endogenously released dopamine (facilitated by DAT blockade) or a bath-applied D_3 agonist can inhibit IPSCs evoked in the stratum radiatum, causing net excitation (via disinhibition) at Schaffer collateral synapses (Hammad & Wagner, 2006; Swant et al., 2008). In these studies, I replicated D_3-mediated IPSC inhibition using the highly selective D_3 agonist PD128907 (1 μM). I also examined IPSCs in the presence of the $D_{1/5}$ agonist SKF38393 (20 μM) or SKF81297 (10 μM). Although there was a slight trend toward increased IPSC amplitude after $D_{1/5}$ activation, I saw no significant effects. Interestingly, pre-treatment with a $D_{1/5}$ agonist resulted in blockade of D_3-mediated IPSC inhibition, an effect that may be attributed to $D_{1/5}$-stimulated cAMP production (Kebabian & Calne, 1979; Otmakhova & Lisman, 1996), as manipulation of cAMP levels has been shown to prevent D_3-mediated inhibition of IPSCs (Chen et al., 2006; Swant et al., 2008).

Because of the previously described dichotomy in CB_1 vs. μOR expression, it is possible to distinguish between the contributions PV^+ and CCK^+ interneuron classes using pharmacological manipulation. Acting upon CA1 pyramidal cells, mGluR agonists stimulate

PLC activity (Cohen et al., 1998) and drive endocannabinoid (eCB) release (Varma et al., 2001). Through retrograde signaling, the latter effect has been shown to suppress pyramidal cell IPSCs in a CB_1-dependent manner (Varma et al., 2001). Because CCK^+ interneurons are selectively inhibited by CB_1 activation (Glickfeld et al., 2008), I sought to selectively (albeit indirectly) determine the involvement of CCK^+ cells in D_3-mediated disinhibition via bath application of the hydrophilic mGluR agonist DHPG (25 μM). The anticipated reduction of IPSC amplitude was observed, but unexpectedly, paired-pulse depression (PPD) remained unchanged- an indication that the observed IPSC reduction was not presynaptically mediated. One postsynaptic explanation for the observed decrease in IPSC amplitude is PLC signaling. PLC is abundant in adult rat hippocampal pyramidal cells (Yamada et al., 1991) and mGluR activation is coupled to PLC activation in the hippocampal CA1 region (Cohen et al., 1998). Additionally, PLC activity has previously been shown to suppress IPSCs in the CA1 region (Tanaka et al., 1997). Consistent with this, the phosphorylation process necessary for maintaining $GABA_AR$ conductance is known to be disrupted by a counteractive process that is calcium-dependent (Stelzer et al., 1988). Since only the $mGluR_5$ subtype of mGluR is expressed by CA1 pyramidal cells (Lujan et al., 1996), my results point toward a suppressive, PLC-mediated signaling cascade initiated by $mGluR_5$ activation. In the presence of DHPG (1 μM), no further reduction in IPSCs was elicited by PD128907 (1 μM) exposure. This suggests that the subset of $GABA_ARs$ being endocytosed upon D_3 activation is among those which are subject to PLC-mediated inhibition.

PV^+ cells are the primary μOR-expressing interneuron in the stratum radiatum (Drake et al., 2002; Drake et al., 2006). In order to determine the contribution of PV^+ cells to D_3-mediated disinhibition, I utilized the μOR agonist DAMGO (1 μM). DAMGO application caused a rapid and drastic reduction in the magnitude of recorded IPSCs. PPD was nearly eliminated by

DAMGO, confirming the drug's presynaptic site of action. Pretreatment with DAMGO completely blocked any inhibitory effect of PD128907 (1 μM) on IPSCs, indicating that D_3-mediated $GABA_AR$ endocytosis occurs at receptor pools receiving PV^+ cell innervation. Since D_3 receptors are absent from the pyramidal cell body layer (Khan et al., 1998), and immunohistochemical evidence indicates that PV^+ cells synapse the dendritic shafts in the CA1 region (Matyas et al., 2004), it is likely that this endocytosis takes place along the dendritic shaft. Because DAMGO not only reduced peak amplitude of IPSCs, but also their $t_{1/2}$ value, my results suggest that reduction in PV^+ cell input (and thus D_3-mediated endocytosis) disproportionately reduces GABA signaling at extrasynaptic receptor pools. This result is consistent with a hypothesis offered in our previous work (Swant et al., 2008), and also with the known extrasynaptic localization of D_3 receptors (Diaz et al., 2000).

In conclusion, I have demonstrated that D_3-mediated disinhibition in the stratum radiatum of hippocampus CA1 takes place at receptors associated with PV^+ cell innervation, which appear to be disproportionately extrasynaptic. This disinhibition can be blocked by $D_{1/5}$ or mGluR activation. These results advance current knowledge of D_3-mediated disinhibition in the CA1 stratum radiatum layer and provide potential mechanisms for its regulation. Future study of this effect is merited, as dopaminergic disinhibition in the CA1 region has important implications (e.g. a significant modulatory impact on LTP) that likely contribute to long-lasting changes in both behavior and sensory perception (Lisman & Otmakhova, 2001).

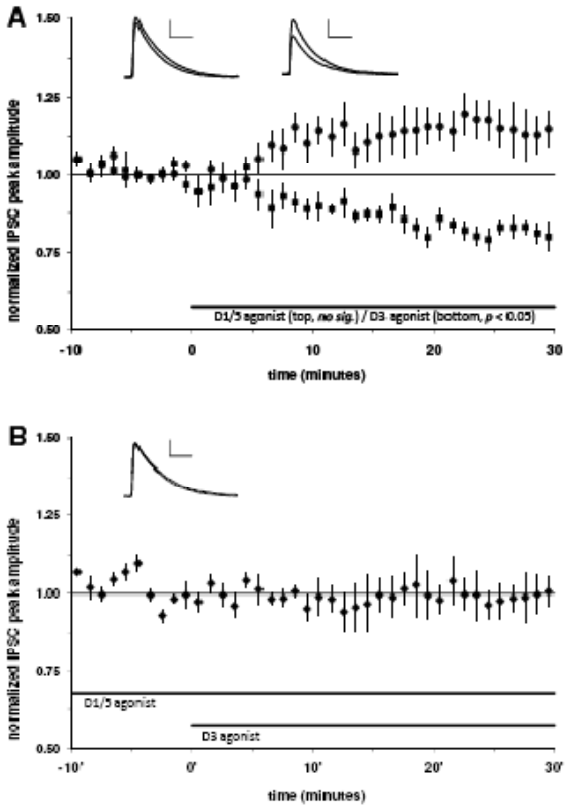

Figure 5.1. $D_{1/5}$ receptor activation blocked D_3-mediated inhibition of IPSCs. Summary plots of normalized peak IPSC amplitudes recorded from CA1 pyramidal cells as a function of time. Drug application began at t = 0; where multiple drugs were used, t′ = 0 denotes the initiation of terminal drug application. Insets are averaged sweeps from representative experiments illustrating relative IPSC amplitudes 0-5 min before and 25-30 min after terminal drug application. Stimulus artifacts have been truncated for clarity. Error bars show ± SEM. The scale bar insets are: 200 pA vertical and 100 ms horizontal (A-left, $D_{1/5}$ agonist); 125 pA vertical and 100 ms horizontal (A-right, D_3 agonist); 225 pA vertical and 100 ms horizontal (B). **A)** IPSC peak amplitude was significantly decreased by the D3 agonist PD128907 (1 μM; $n = 7$; $p < 0.05$), but not D1/5 agonist SKF38393 (20 μM) or SKF81297 (10 μM) (combined, $n = 8$). **B)** When cells were pretreated with this dose of either D1/5 agonist, PD128907 (1 μM) was no longer capable of decreasing IPSC amplitude ($n = 4$).

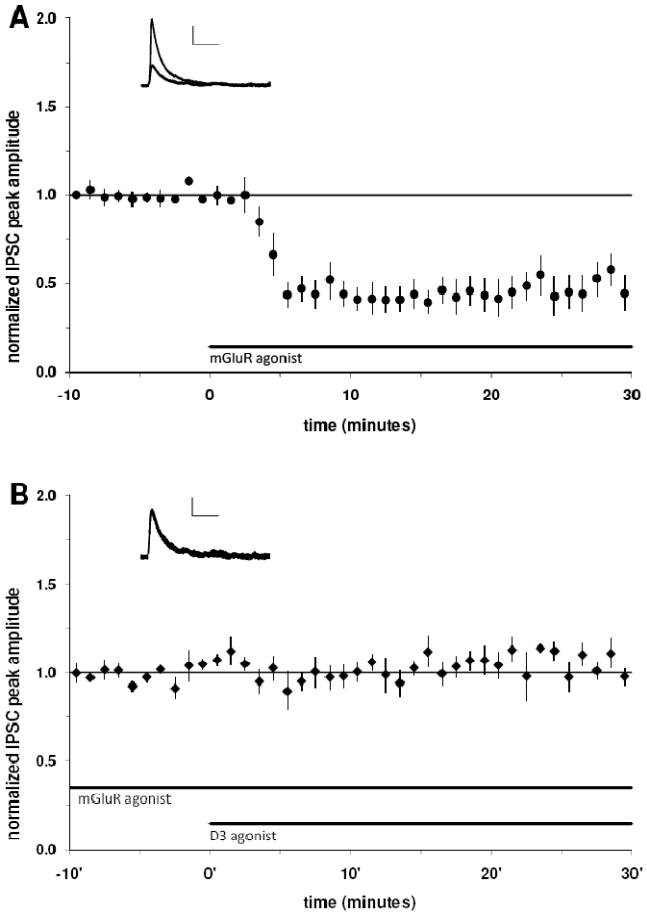

Figure 5.2. Metabotropic glutamate receptor (mGluR) activation inhibited IPSCs and blocked D_3-mediated inhibition of IPSCs. Summary plots of normalized peak IPSC amplitudes recorded from CA1 pyramidal cells as a function of time. Drug application began at t = 0; where multiple drugs were used, t′ = 0 denotes the initiation of terminal drug application. Insets are averaged sweeps from representative experiments illustrating relative IPSC amplitudes 0-5 min before and 25-30 min after terminal drug application. Error bars show ± SEM. Stimulus artifacts have been truncated for clarity. The scale bar insets are: 225 pA vertical and 100 ms horizontal (A); 10 pA vertical and 100 ms horizontal (B). **A)** IPSC peak amplitude was significantly decreased by the mGluR agonist DHPG (25 μM; $n = 4$; $p < 0.05$). **B)** When cells were pretreated with DHPG (25 μM), the D3 agonist PD128907 (1 μM) was no longer capable of decreasing IPSC amplitude ($n = 3$).

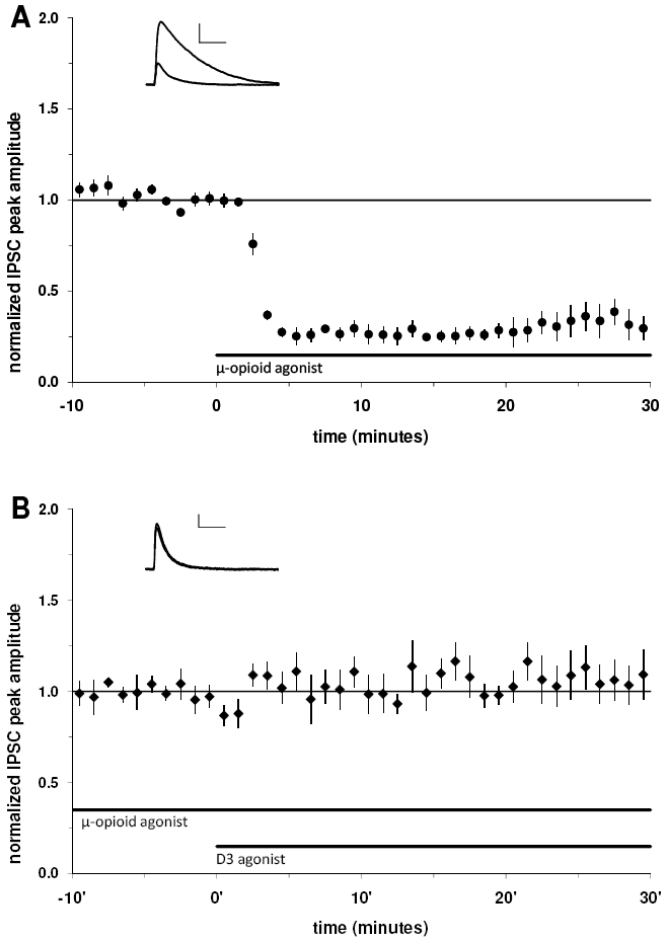

Figure 5.3. μ-opioid receptor activation inhibited IPSCs and blocked D_3-mediated inhibition of IPSCs. Summary plots of normalized peak IPSC amplitudes recorded from CA1 pyramidal cells as a function of time. Drug application began at t = 0; where multiple drugs were used, t′ = 0 denotes the initiation of terminal drug application. Insets are averaged sweeps from representative experiments illustrating relative IPSC amplitudes 0-5 min before and 25-30 min after terminal drug application. Error bars show ± SEM. Stimulus artifacts have been truncated for clarity. The scale bar insets are: 150 pA vertical and 100 ms horizontal (A); 50 pA vertical and 100 ms horizontal (B). **A)** IPSC peak amplitude was significantly decreased by the μ-opioid agonist DAMGO (1 μM; $n = 4$; $p < 0.01$). **B)** When cells were pretreated with DAMGO (1 μM), the D3 agonist PD128907 (1 μM) was no longer capable of decreasing IPSC amplitude ($n = 6$).

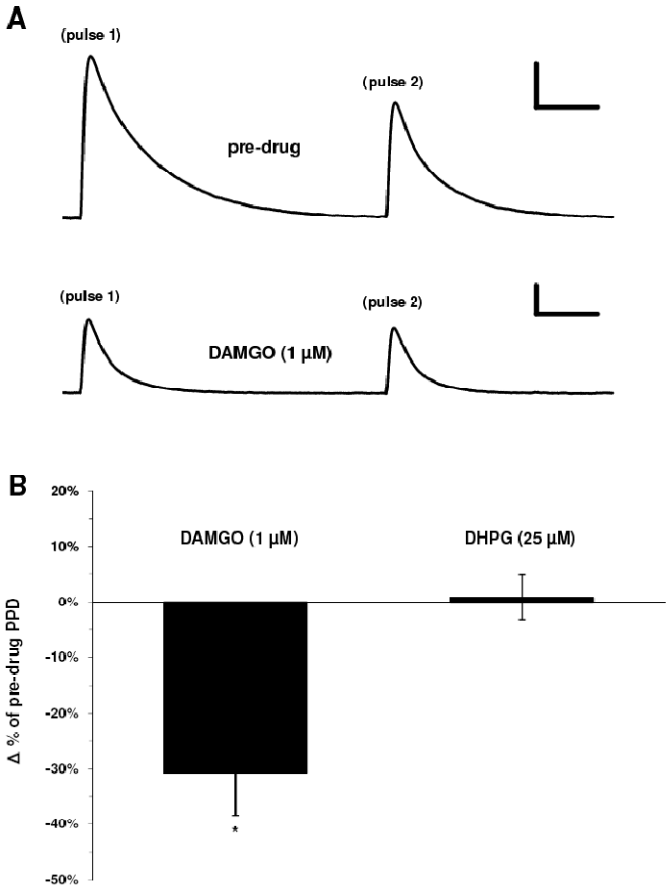

Figure 5.4. μ-opioid receptor agonist DAMGO significantly reduced paired-pulse depression (PPD) of IPSCs. **A)** Averaged sweeps (n = 4 cells from lower panel) depicting PPD of IPSCs pre- and post-DAMGO (1 μM) exposure. Interpulse interval was 500 ms. The top sweep represents paired IPSCs recorded prior to drug application. The bottom sweep represents paired IPSCs recorded 25-30 min following DAMGO application. Stimulus artifacts have been truncated for clarity. The top scale bar insets are: 150 pA vertical and 100 ms horizontal (top); 100 pA vertical and 100 ms horizontal (bottom). **B)** Summary quantification of Δ % PPD resulting from DAMGO application (* $p < 0.05$). Cells instead exposed to DHPG exhibited no such change in PPD of IPSCs. Error bars show ± SEM.

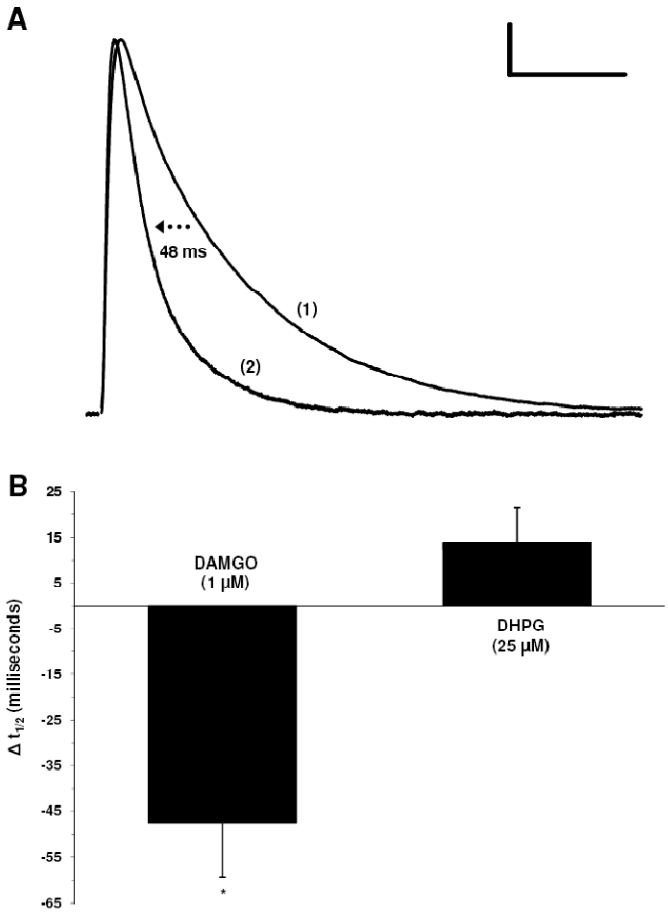

Figure 5.5. μ-opioid receptor agonist DAMGO significantly reduced half decay time ($t_{1/2}$) of IPSCs. **A)** Averaged sweeps (n = 4 cells from lower panel) depicting IPSCs pre- and post-DAMGO exposure. The outer sweep (1) represents IPSCs recorded prior to drug application. The inner sweep (2), which has been scaled up for comparison, represents IPSCs recorded 25-30 min following DAMGO (1 μM) application. Height of the dotted arrow marks 50% decay from peak amplitude, and its direction reflects the 48 s reduction in latency of this value following exposure to DAMGO. Stimulus artifacts have been truncated for clarity. The scale bar inset is 100 pA vertical and 100 ms horizontal. **B)** Summary quantification of change in $t_{1/2}$ resulting from DAMGO application (*$p < 0.05$). Cells instead exposed to DHPG exhibited no such change in $t_{1/2}$ of IPSCs. Error bars show ± SEM.

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CHAPTER SIX

SUMMARY AND CONCLUSIONS

Over the past fifty years, our understanding of dopamine has evolved from mere acknowledgement of its precursory status in norepinephrine production to the realization that it orchestrates complex modulatory activity in the striatum (reviewed by Onn et al., 2000), the prefrontal cortex (reviewed by Seamans and Yang, 2004), and the hippocampus (Lisman and Grace, 2005). The results presented in this dissertation focus on dopaminergic modulation within the hippocampus CA1 region. Pyramidal cells of the CA1 are vital because they are integrative structures that manage both incoming sensory input from the sensory cortex and previously processed information from the dentate (by way of the CA3). Proper assimilation of these signals is of paramount importance to cognition and behavior. Because of the persistent nature of these changes, it is important to understand the way in which dopamine alters CA1 pyramidal cell function.

Earlier work from the Wagner Lab showed that cocaine exposure via either i.p. injection or self-administration enhances long-term potentiation in the CA1 region (Thompson et al., 2002; Thompson et al., 2004). Although $D_{1/5}$ receptor activation is known to increase the magnitude of LTP (Otmakhova and Lisman, 1996; Swanson-Park et al., 1999), cocaine-mediated LTP enhancement occurred via DAT-blockade-facilitated activation at D2-like receptors (Thompson et al., 2005) of the D_3 subtype (Swant & Wagner, 2006). However, these modulatory actions are not uniform among lamina of the CA1 region, as I recently reported that D_3-mediated effects underlying this change in LTP (i.e., D_3-induced endocytosis of $GABA_A$Rs) occurs in the proximal apical dendrites (stratum radiatum) but not the basal dendrites (Swant et al., 2008; LTP results in Appendix A). The primary objective of my research was to further dissect the

modulatory actions of dopamine upon LTP in the CA1. This was accomplished through a combination of extracellular and whole-cell voltage clamp electrophysiology experiments, the results of which will be briefly summarized in the text below.

Hippocampal LTP Enhancement via Dual Dopaminergic Pathways:

With extracellular LTP experiments, I have confirmed the ability of both $D_{1/5}$ and D_3 agonists to enhance LTP via distinct mechanisms that ultimately engage NR2B-NMDARs. The signaling mechanism for $D_{1/5}$-mediated LTP enhancement required PKA and SFK activity and was blocked when slices were pre-treated with a D_4 agonist, despite that the D4 agonist has no effect on LTP when applied alone. In contrast, D_3-mediated LTP enhancement acts via disinhibition and is not susceptible to D_4 blockade. Like the $D_{1/5}$-mediated mechanism, D_3 activation ultimately enhances LTP by enhancing NR2B-NMDAR activity. My whole-cell studies sought to better characterize the disinhibitory actions enabling D_3-mediated LTP enhancement. To do this, I examined D_3-induced suppression of pyramidal cell IPSCs. The results confirmed a role for PKA inhibition in this process, demonstrating that PV^+ cell inputs are the target of D_3-mediated disinhibition (Figure 6.1).

Laminar-Specificity of Pathways:

I also addressed the lamina-specific nature of dopaminergic modulation in the CA1. Using cocaine, I demonstrated that although DAT blockade has been shown to enhance LTP via a D_3-mediated mechanism in stratum radiatum (Swant et al., 2008; see appendix A), it acts via the $D_{1/5}$-mediated pathway in the stratum oriens. There are a number of possible explanations for this laminar difference. For example, there is evidence that higher levels of dopamine exist in the

stratum oriens (basal dendrites) than in the stratum radiatum (proximal apical dendrites; Gasbarri et al., 1994; Kwon et al., 2008). Because $D_{1/5}$ receptors have a relatively low affinity for dopamine, its increased concentration in the oriens might account for the greater relevance of $D_{1/5}$-mediated LTP enhancement compared to that in stratum radiatum. Alternatively, disparities in D_4 localization in apical versus basal dendrites may come into play. For instance, if cocaine-enhanced dopamine levels in the stratum radiatum were sufficient to activate $D_{1/5}$ receptors, it seems likely that co-activation of D_4 receptors (which, like D_3, have a greater affinity for dopamine than $D_{1/5}$ receptors) would also occur, preventing $D_{1/5}$-mediated LTP enhancement.

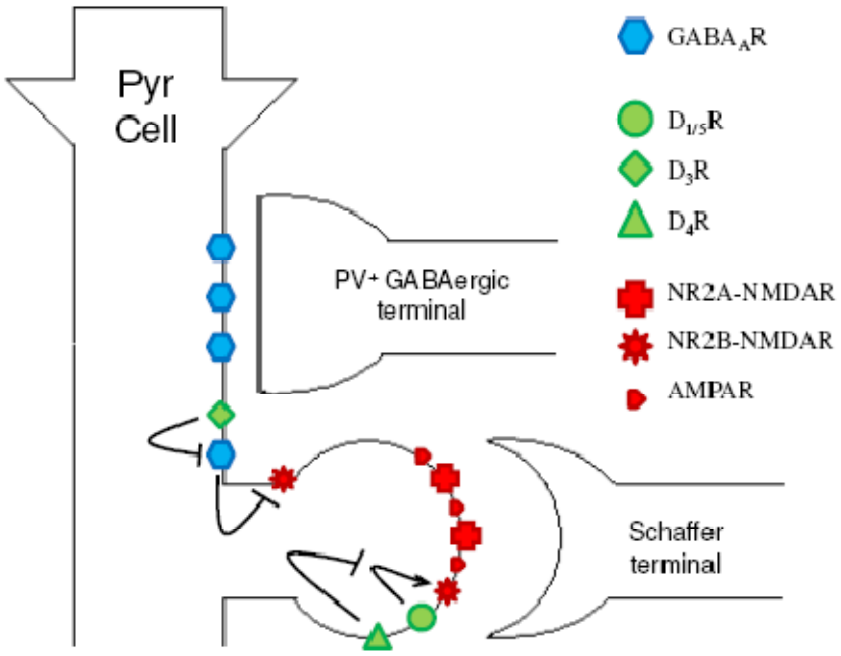

Figure 6.1. Model for dopaminergic modulation of NR2B-dependent LTP enhancement in the stratum radiatum layer of hippocampus CA1. Pyramidal cells receive glutamatergic input from Schaffer terminals and GABAergic input from PV^+ interneurons. D_3-mediated disinhibition and $D_{1/5}$-mediated PKA/SFK activation can both enhance LTP via NR2B-dependent mechanisms. D_4 is capable of preventing the $D_{1/5}$ mechanism, but not the D_3 mechanism. Image by Michael Stramiello.

Discussion:

In sum, the studies presented here have furthered the current understanding of dopaminergic modulatory mechanisms in the hippocampus CA1 region. Because this region is critical for novelty detection and the encoding/retrieval of memories (Lisman & Otmakhova, 2001; Li et al., 2003; Lisman & Grace, 2005), the ability of dopamine to alter function in a persisting manner is likely to have important consequences on a behavioral/systems level, and its malfunction may contribute to pathological states. Schizophrenia, for example, has been hypothesized to involve hyperdopaminergic function in the hippocampus (Krieckhaus et al., 1992; Lisman & Otmakhova, 2001), and it is possible that anti-D2-like receptor-mediated palliative effects of antipsychotics may involve mitigation of LTP enhancement in the stratum radiatum, or perhaps a rebalancing of apical vs. basal processing within CA1 pyramidal cells. With respect to addiction, which is often described as a "hijacking" of the neural foundations for learning and memory, dopaminergic modulation of hippocampal LTP is especially pertinent and likely to be an important contributor to the storage and recall of maladaptive memories associated with addictive behaviors.

TABLE 6.1. Index of pharmacological agents

Target	Agonist	Antagonist / Inhibitor
AMPAR		CNQX
$D_{1/5}R$	SKF38393 SKF81297	SKF83566
D_3R	PD128907	
D_4R	PD168077	L745870
DAT		cocaine
mGluR	DHPG	
μOR	DAMGO	
NMDAR		DL-AP5
NR2B-NMDAR		Ro-256981
PKA		H-89
$vgNa^+$ channel		QX314
tyrosine kinases		PP2†

† PP2 exhibits a high degree of specificity for Src-family tyrosine kinases.

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APPENDIX A

LTP IN THE STRATUM RADIATUM IS SELECTIVELY ENHANCED BY D3 ACTIVATION

Excerpt from Swant J, Stramiello M, and Wagner JJ. 2008. *Hippocampus* 18(5), 492-502.

LTP Evoked/Recorded in the Stratum Radiatum is Selectively Enhanced by PD128907

Given the selective actions of PD128907 described earlier [Swant et al., 2008], we were interested in testing the possibility that LTP evoked and recorded from the stratum radiatum layer might be differentially affected by D3 receptor activation as compared with LTP evoked and recorded in the stratum oriens of the CA1 region. The fEPSP was measured in either the stratum radiatum or the stratum oriens of the CA1 in response to stimulation of the glutamatergic Schaffer collaterals (Fig. 6). In the stratum radiatum, comparison of stimulus response curves between control and PD128907 treated slices revealed no significant difference in baseline fEPSP responsiveness (data not shown). The normalized magnitude of LTP in control slices 30 min post-tetanus was 1.68 ± 0.03 n = 11(4) (n values = number of slices (number of animals)). LTP magnitude 30 min post-tetanus in slices treated with PD128907 (1 μM) was significantly increased to 2.02 ± 0.05 n = 15(5) (Fig. 6A). In contrast, D3 receptor activation had no effect on the magnitude of LTP measured in the stratum oriens, as control LTP was 1.91 ± 0.08 n = 22(7) and PD128907 LTP was 1.94 ± 0.07 n = 20(7) (Fig. 6B). Thus the ability of the D3 receptor agonist to differentially enhance LTP in the stratum radiatum also correlates with its ability to significantly reduce the IPSCs evoked in the same layer in the CA1 region of the hippocampus [see Chapter 5, Fig 1A of this dissertation].

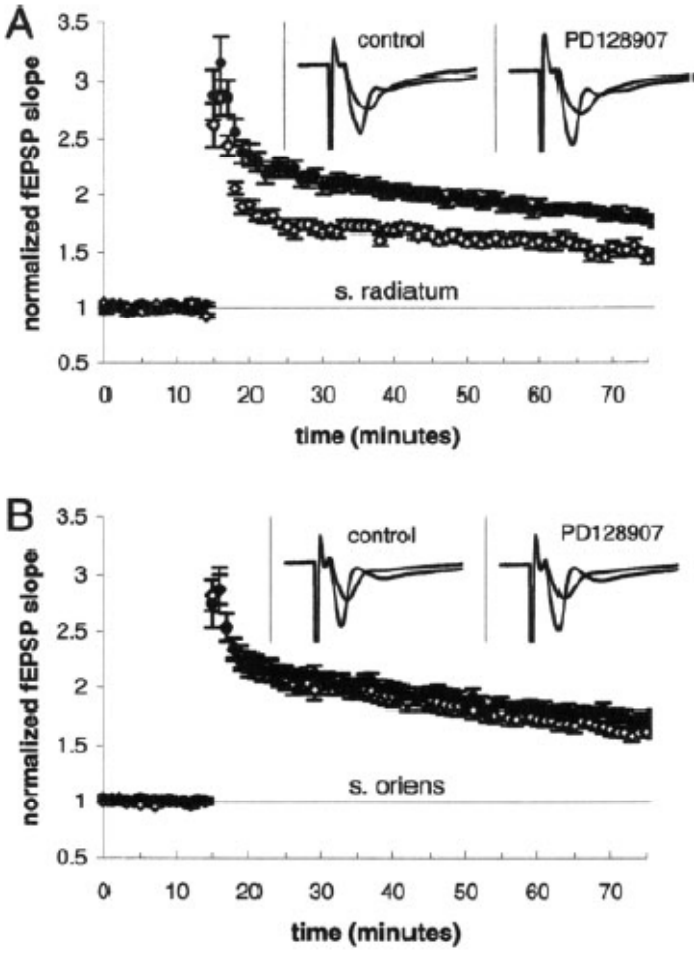

Figure A.6. LTP in the stratum radiatum was selectively enhanced by D3 activation. Summary plots of normalized fEPSP slope measurements recorded in the CA1 region of the hippocampus. The closed circles are from PD128907 (1 μM) treated slices, the open circles show results from control slices (error bars are ± sem). Three 100 Hz/1 s stimulus trains separated by 20 s were used to tetanize the slices at time t = 45 min. Insets are 30 μs group average sweeps illustrating the fEPSP 0–5 min prior to and 25–30 min post-tetanus (vertical scale bar is 3 mV). A: fEPSPs are evoked and recorded from the stratum radiatum layer in CA1. The magnitude of LTP was significantly enhanced in the presence of the D3 agonist. Control n = 11 slices, PD128907 n = 15 slices. B: fEPSPs are evoked and recorded from the stratum oriens layer in CA1. No significant effect on LTP magnitude was observed. Control n = 22 slices, PD128907 n = 20 slices.