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Effects of Locus Coeruleus Lesion on α-Adrenoceptor Mediated Long-Term Depression at CA3-CA1 Synapses in Rat Hippocampus

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EFFECTS OF LOCUS COERULEUS LESION ON α1-ADRENOCEPTOR MEDIATED LONG-TERM DEPRESSION AT CA3-CA1 SYNAPSES IN RAT HIPPOCAMPUS

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EFFECTS OF LOCUS COERULEUS LESION ON α1-ADRENOCEPTOR MEDIATED LONG-TERM DEPRESSION AT CA3-CA-1 SYNAPSES IN RAT HIPPOCAMPUS

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ABSTRACT

Our laboratory has recently characterized an NMDA receptor (NMDAR)-dependent form of long-term depression (LTD) at CA3-CA1 synapses that is mediated by selective activation of α1-adrenoceptors (α1-ARs). Norepinephrine (NE) modulation of synaptic plasticity is thought to relevant to many forms of learning and memory, specifically those dependent upon hippocampal function. The first goal of this study was to examine the effects of α1-AR activation by a selective α1-AR agonist and to determine whether this same form of LTD can also be induced by accumulation of endogenous NE. Accordingly, α1-AR LTD was reliably induced via the selective α1-AR agonist phenylephrine as well as by endogenously released NE accumulated extracellularly via norepinephrine transporter (NET) and monoamine oxidase (MAO) inhibition.

The second goal of this study was to examine the effects of NA degeneration on α1-AR LTD. The hippocampus receives its sole NE input from the locus coeruleus (LC); therefore, the NE-specific neurotoxin DSP-4 was used to selectively target this system and cause significant NE degeneration in hippocampus. Following an 85% decrease in NE content, α1-AR LTD was induced by selective α1-AR activation via phenylephrine and endogenous NE via inhibition of NET and MAO that was not different from animals with intact NE innervation. Thus, these data confer that despite significant decreases in NE input to hippocampus, the mechanisms necessary for the induction of α1-AR LTD

remain functional. Furthermore, α1-AR activation could be a viable therapeutic target for pharmacological intervention in diseases and disorders where malfunctions in NE neurotransmission occur.

Keywords: hippocampus, norepinephrine, LTD, α1-AR, locus coeruleus

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α1-AR	α1 adrenergic receptor
α1-AR LTD	α1 adrenergic receptor long-term depression
α2-AR	α2 adrenergic receptor
aCSF	artificial cerebrospinal fluid
ACh	Acetylcholine
AD	Alzheimer's disease
ADHD	Attention deficit hyperactivity disorder
AMPAR	2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl)proprionic acid receptor
Atmx	atomoxetine
β-AR	β adrenergic receptor
Ca^{2+}	calcium
Clor	clorglyine
CA3-CA1	*Cornu Ammonis* areas 3 and 1
BNST	bed nucleus of the stria terminalis

DMSO	dimethylsulfoxide
DβH	dopamine β-hydroxylase
DSP-4	*N*-(2-chloroethyl)-*N*-ethyl-2-bromobenzylamine
ERK	extracellular signal related kinase
fEPSP	field excitatory postsynaptic potential
$GABA_A$	gamma-aminobutyric acid type A
GPCR	G-protein coupled receptor
Hz	hertz
KO	knockout
LC	locus coeruleus
LTD	long-term depression
LTP	long-term potentiation
mAChR	muscarinic acetylcholine receptor
MAO	monoamine oxidase
Mg^{2+}	magnesium
mLTD	muscarinic long-term depression
Na^{+}	sodium

NE	norepinephrine
NE LTD	norepinephrine LTD
NET	norepinephrine transporter
NMDAR	*N*-methyl-D-aspartate receptor
PD	Parkinson's disease
PFA	paraformaldehyde
Phe	phenylephrine
PFC	prefrontal cortex
s. radiatum	stratum radiatum
TH	tyrosine hydroxylase

CHAPTER 1

INTRODUCTION

The Hippocampus and Learning and Memory

The hippocampus, located in the medial temporal lobe, is a brain structure essential for normal learning and memory processes (Scoville and Milner, 1957). As reported by Scoville and Milner in 1957, a patient know as H.M. suffered from a severely debilitating form of epilepsy and underwent a bilateral resection of the medial temporal lobe which resulted in ablation of epileptic events. However, H.M. subsequently experienced anterograde amnesia for all events following surgery while memories prior to the procedure remained intact (Scoville and Milner, 2000). The resection included removal of the hippocampal formation, which encompasses the hippocampus proper, dentate gyrus, subiculum and entorhinal cortex, whereas the parahippocampal gyrus received only slight damage (Corklin et al., 1997). In humans, the hippocampus is also susceptible to damage by ischemia as reported in the case of patient R.B., who experienced an episode of global ischemia that resulted in a bilateral lesion of the entire CA1 field of the hippocampus (Zola-Morgan et al., 1986). R.B.'s coincident memory impairment following the lesion was not as severe as that of H.M. (Squire and Zola-Morgan, 1991) but was indicative of the importance of specific hippocampal regions to memory function (Squire and Zola-Morgan, 1991).

Nonhuman primate and rodent lesion studies have since confirmed the importance of the hippocampus and associated cortices upon normal learning and memory (Squire and Zola-Morgan, 1991) while also highlighting discrepancies in hippocampal lesion specificity for memory loss between humans and animals. In humans, circumscribed hippocampal damage is sufficient to induce anterograde and retrograde amnesia with deficits lasting from weeks to years. In nonhuman primates, extensive lesions which include the hippocampus and associated entorhinal, perirhinal, and parahippocampal cortices are required to generate similar memory impairment as seen in humans with localized hippocampal damage (Squire and Zola-Morgan, 1991). Nevertheless, the importance of the hippocampus for normal learning and memory is clear and further understanding of the cellular mechanisms that correlate to these processes is necessary.

Hippocampal Synaptic Plasticity

Synaptic plasticity refers to the activity-dependent modification of synaptic strength in response to various forms of stimuli and is considered to be the cellular correlate to learning and memory (Malenka and Bear, 2004). Alterations in synaptic strength can persist for seconds, minutes, hours, days, or even weeks depending upon the mechanism of activation. Short-term plasticity, or synaptic changes that last for short periods of time are primarily dependent on presynaptic modification which usually involves a decrease in neurotransmitter release from readily releasable pools of synaptic vesicles (Zucker and Regehr, 2002; Dobrunz and Stevens, 1997). In contrast, long-term plasticity can last from hours to weeks and usually entails various postsynaptic

modifications mediated by kinases, phosphatases, and receptor trafficking (Malenka and Bear, 2004).

The two most extensively characterized forms of long-term synaptic plasticity are long-term potentiation (LTP) and long-term depression (LTD), which are mediated by the strengthening and weakening of synapses, respectively. It is believed that these forms of synaptic transmission work in concert with one another and serve as the cellular model for learning and memory. Various forms of LTP and LTD have been discovered that differ based upon brain region, the mode of induction (via electrical stimulation or chemical activation), the age of the animal, and molecular signaling cascades (Malenka and Bear, 2004).

It is well established that LTP and LTD exist throughout the brain and the vast majority of studies focus on their properties at the Schaffer collateral pathway at area CA3-CA1 in hippocampus. Here, LTP is typically induced by a brief train of high frequency stimulation [HFS-LTP, 100 Hz for 1 sec (100 pulses)] or a theta burst stimulation protocol (4 pulses at 100 Hz every 200 msec) (Bliss and Lomo, 1973; Larson et al., 1986; Hernandez et al., 2005), whereas the induction of LTD requires a prolonged low frequency stimulation [LFS-LTD, 0.5-5 Hz (900 pulses)] (Dudek and Bear, 1992).

In addition to the activity dependence required for the induction of LTP and LTD, postsynaptic Ca^{2+} flux through *N*-methyl-D-aspartate receptors (NMDARs) is also necessary. Although 2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl)propionic acid receptors (AMPARs) are primarily responsible for basal levels of current [i.e. excitatory postsynaptic potentials (EPSPs)] flow across the postsynaptic membrane, sufficient

depolarization allows for the removal of the voltage-dependent Mg^{2+} block of NMDARs, permitting Na^{+} and Ca^{2+} flux through these ligand-gated ion channels. Ca^{2+} entry into the postsynaptic spine of CA1 pyramidal cells is imperative for activation of various signaling cascades, including protein kinases for the induction of LTP and phosphatases for inducing LTD. Rapid increases in the intracellular Ca^{2+} concentration are responsible for the expression of LTP via an upregulation of AMPARs at the membrane surface. Conversely, prolonged increases in intracellular Ca^{2+} are responsible for the expression of LTD by activating endocytosis of AMPARs from the surface of the postsynaptic membrane.

NE and Cognitive Dysfunction

Alzheimer's disease and Parkinson's disease. In normal aged humans, the LC experiences a 25% decline is cell body number and the total brain concentration of NE decreases by 50% beginning at 40 years of age (Mann, 1983; Mann et al., 1983). Additionally, patients with Alzheimer's disease (AD) and Parkinson's disease (PD) also experience extreme degeneration of LC cell bodies of up to 67.9% and 83.2%, respectively (Marien et al., 2004). Importantly, the LC-NE system has been implicated as a potential compensatory and protective mechanism that acts to minimize the effects of other neurotransmitter system damage as it occurs in these disease states (Marien et al., 2004). Thus, a decrease in the NE neuronal population leads to gradual impairment of cognitive function and ultimately results in dementia via disturbance of the NE projections that aide in the regulation of brain homeostasis (Mann et al., 1983).

Posttraumatic stress disorder (PTSD). In addition to AD and PD, the LC has also been implicated in neuropsychiatric disorders including PTSD, anxiety, and panic (Rioja et al., 2006). Previous electrophysiological studies imply that LC neuronal activity is primarily activated by aversive stimuli (Redmond and Huang, 1979; Grant and Redmond, 1984), with substantial evidence showing stressor-elicited activities lead to increases in NE release (Anisman, 1978; Dunn, 1988; Dunn and Kramarcy, 1984; Finlay et al., 1995; Korf et al., 1973; Nisenbaum et al., 1991; Stone, 1973 and 1975; Thierry et al., 1968; Weiss et al., 1970, 1975, and 1980; Zigmond and Harvey, 1970). The hippocampus has been shown to be highly responsive to stressful environmental stimuli (Fuchs and Flugge, 1998; McEwen, 1999). Thus, exposure to acute stress could be associated with neurological and psychological disorders (Browne and Finkelhor, 1986; Kerr et al., 1991; Kulka et al., 2002). Further, it has been shown that excessive stimulation of ARs during stress leads to cognitive dysfunction in the prefrontal cortex (PFC) (Birnbaum et al., 1999), a brain region that also receives NE input from the LC (Arikuni and Ban, 1978; Gerfen and Clavier, 1979; Morrison et al., 1979; Porrino and Goldman-Rakic, 1982) and is important in working memory (Goldman-Rakic, 1995). Thus, it can be postulated that excessive release, in addition to depletion, of NE could also lead to deficits in hippocampal dependent learning and memory, and therefore, synaptic plasticity.

Attention-deficit hyperactivity disorder (ADHD). ADHD is a common behavioral disorder among children and adults that consists of age inappropriate behavior, increased motor activity, impulsivity, and inability to maintain one's attention during tasks (Biederman, 2005; Biederman and Faraone, 2002). Although initially thought to be mediated through disturbances in dopamine neurotransmission (Pliszka et al., 1996;

Castellanos et al., 1996; Zametkin and Rapoport, 1987), evidence has also implicated NE as a key factor in the pathophysiology of the disorder (Zametkin and Rapoport, 1987; Pliszka et al., 1996; Arnsten et al., 1996; Biederman and Spencer, 1999). While patients who suffer from ADHD do not exhibit decreases in the NE neuronal population, they do exhibit malfunctions in the neurotransmission of NE, where decreases in NE release can have virtually the same effects on learning and memory function as seen in patients who experience neurodegeneration of NE input (e.g. AD and PD).

LC-NE Innervation: Learning and Memory

The locus coeruleus (LC), located in the pontine nucleus of the brain stem, is the primary source of NE for the central nervous system (CNS) (Baloyannis et al., 2006; Aston-Jone et al., 1995 and 2000). NE was first proposed to be involved in memory in the early 1970s by S. Kety who believed that behaviorally mediated arousal would facilitate changes at synapses concurrently in a state of excitation (Kety, 1970 and 1972). The dense population of cell bodies located in the LC is characterized by divergent efferent projections throughout the CNS and NE release from these various projections is then modulated in a regionally specialized manner (Berridge and Waterhouse, 2003). NE innervation plays a role in the maintenance of homeostasis within the brain, in regulation of motivation, selective attention, alertness, orientation (Masson, 1980), defense reactions (Levine et al., 1990), and coordination of state-dependent cognitive function (Berridge and Waterhouse, 2003; Usher et al., 1999). The role NE plays in memory has been examined extensively at the level of memory acquisition and shows that NE interacts

with other neurotransmitter systems and stress hormones to establish long-term memory formation (McGaugh and Roozendaal, 2008).

Further evidence for the importance of NE in learning and memory comes from transgenic mouse studies where mice with a double knockout (KO) of dopamine β-hydroxylase (DβH), the enzyme responsible for NE synthesis, show cognitive deficits. When tested in the Morris water maze, DβH KO mice exhibited deficits in memory consolidation and subsequent behavioral studies have shown that these mice also experience deficits in active-avoidance learning (Thomas and Palmiter, 1997).

In addition to the role of NE synthesis to learning and memory, reports also show that AR activation is also important. Rats receiving intracerebral injections of β-AR antagonists 2 hours after learning a task show memory loss when re-tested 48 hours later (Sara et al., 1999; Tronel et al., 2004). α1-ARs have also been shown to be necessary for spatial memory learning tasks, as α1-AR agonists and antagonists block the formation of memory (Pussinen, et al., 1997; Puumala et al., 1998; Riekkinen et al., 1997). Furthermore, $\alpha 1_D$-AR KO mice exhibit impaired working memory when tested using a Y-maze, as these receptors are globally expressed throughout hippocampus (Mishma et al., 2004).

The role the LC-NE system plays in modulating synaptic plasticity at glutamatergic synapses has been well documented and may provide an avenue through which NE participates in learning and memory (Bramham et al., 1997; Brocher et al., 1992; Hopkins and Johnston, 1984; Huang and Kandel, 1996; Izumi and Zorumski, 1999; Katsuki et al., 1997; Pelletier et al., 1994; Thomas et al., 1996). Furthermore, reports

have shown that activation of α1- and β-ARs by NE can facilitate tetanus-induced LTP (Hopkins and Johnston, 1984; Huang and Kandel, 1996; Lin et al., 2003; Thomas et al., 1996) and induce de novo LTD during basal transmission (Scheiderer et al., 2003), respectively. Therefore, NE innervation from the LC may be a general mechanism by which synaptic efficacy can be modulated throughout the brain.

Hippocampal NE Innervation and Synaptic Plasticity

The hippocampus receives it sole NE input from the LC (Berridge and Waterhouse, 2003), therefore, it can hypothesized that alterations in the structure and/or function of the LC can lead to the deficits in synaptic plasticity localized to the hippocampus. As noted earlier, changes in long-term synaptic plasticity are believed to be the cellular correlate of learning and memory. *In vitro* studies have shown that NE depletion reduces or eliminates LTP in hippocampal slices (Harley, 1991), and application of NE increases LTP (Brocher et al., 1992; Izumi and Zorumski, 1999; Katsuki et al., 1997; Hopkins and Johnston, 1988) and LTD (Scheiderer et al., 2004). Collectively, these studies show that hippocampal NE innervation is important for the activity-dependent processes that underlie synaptic modifications that contribute to hippocampal-dependent memory acquisition and storage (Kirkwood et al., 1999; Stanton and Sarvey, 1985). Previously, Scheiderer et al. has shown that when NE or a selective α1 agonist pharmacologically activates α1-ARs, LTD at CA3-CA1 synapses is induced and is dependent on postsynaptic NMDAR activation (Scheiderer et al., 2003). Both methods of α1-AR activation, which induce LTD, remain unaffected in the presence of α2- and β-AR antagonists. This plasticity also persists in the presence of $GABA_A$

receptor antagonism via bicuculline, which indicates that NE LTD is not a result of NE induced changes in synaptic inhibition (Scheiderer, 2003). NE LTD has also been shown to be dependent upon presynaptic activity, where in a dually stimulated brain slice preparation, LTD is only expressed in the pathway turned on during α1-AR agonist application (Scheiderer, 2003). In addition to the dependence of this plasticity on presynaptic activation, coincident postsynaptic NMDAR activation is also required as NE LTD can be blocked in the presence of an NMDAR antagonist (Scheiderer, 2003). Therefore, NE innervation from the LC to the hippocampus may be a requirement for normal learning and memory processing and thus synaptic efficacy.

Conclusions

It is clear that NE is an important catecholamine necessary for normal neurotransmission throughout the brain, and specifically the hippocampus. Many neurodegenerative and neuropsychiatric disorders are characterized by malfunctions in NE neurotransmission usually resulting in learning and memory deficits. In addition to NE-mediated deficits, other neurotransmitters such as acetylcholine, dopamine, and serotonin are also affected. Each of these neurotransmitters functions in close association with one another as they collectively exhibit deficits in neurotransmission in disease states. However, NE has been shown to play a distinct compensatory role when other neurotransmistter systems are damaged (Marien et al., 2004). Thus, it is imperative to determine the function of NE innervation in normal and disease states. The data presented in this study will attempt to elucidate the effects of NE degeneration on hippocampal synaptic plasticity related to learning and memory.

CHAPTER 2

METHODS

LC Lesion

All experiments were conducted with an approved protocol from the University of Alabama at Birmingham Institutional Animal Care and Use Committee in compliance with the National Institutes of Health guidelines. Hippocampal NA degeneration was performed using the NE specific neurotoxin *N*-(2-Chloroethyl)-*N*-ethyl-2-bromobenzylamine hydrochloride (DSP-4) (Tocris, Ellisville, MO). Six-week old male Sprague Dawley rats (Charles River) were lightly anesthetized with isofluorane and injected intraperitoneally with DSP-4 (50mg/kg) in saline or saline alone (immediately prior to injection) at 48-hour intervals for a total of 3 injections. Electrophysiological recordings and immunohistochemical analysis were performed 7-21 days following the initial DSP-4 injection.

Slice Preparation and Electrophysiological Recordings

Coronal slices from dorsal hippocampus were cut at 400μm-thickness from animals 7-21 days following the first injection of DSP-4. Animals were decapitated following deep isofluorane inhalation and the brain was rapidly removed and placed in

ice-cold "high-sucrose" artificial cerebrospinal fluid (aCSF) containing (mM): 85 NaCl, 2.5 KCl, 4 $MgSO_4$, 0.5 $CaCl_2$, 1.25 NaH_2PO4, 25 $NaHCO_3$, 25 glucose, 75 sucrose, 2 kynurenic acid, and 0.5 ascorbate. This type of aCSF was used to increase neuronal survival during the slicing procedure, as provided by the high concentration of sucrose and low Na^+ and Ca^{2+}. A vibratome (Vibratome Co., St. Louis, MO) was used to cut coronal slices from dorsal hippocampus, which were then incubated for 30 min post-slicing in high-sucrose aCSF, and then transferred to a standard aCSF containing (mM): 119 NaCl, 2.5 KCl, 2.5 $CaCl_2$, 1.3 $MgSO_4$, 1 NaH_2PO_4, 26 $NaHCO_3$, 10 glucose, and 2 kynurenic acid, saturated with 95% O_2-5% CO_2 (pH 7.4) for an additional 30 min. For recordings, slices were transferred to a submersion recording chamber and were perfused continuously at 3-4 ml/min with aCSF, without kynurenic acid, warmed to 26-29°C.

Extracellular dendritic field excitatory postsynaptic potential (fEPSP) recordings were recorded (Axoclamp 2B, Axon Instruments, CA) from stratum (s.) radiatum in area CA1 of hippocampus. A stainless steel bipolar stimulating electrode (FHC, Bowdoinham, ME) was used to stimulate the Schaffer collateral pathway in s. radiatum, and a glass microelectrode filled with standard aCSF without kynurenic acid was placed in CA1 of s. radiatum to record extracellular dendritic fEPSPs. A 0.1 Hz (100μs in duration) stimulus frequency was used with the intensity adjusted to elicit fEPSPs of 0.7-1.0 mV in amplitude.

All drugs (Sigma, St. Louis, MO) were prepared as stock solutions and diluted to the appropriate working concentration at the time of recording. Phenylephrine (Phe, α1-AR agonist; in deionized water), propranolol (β-AR antagonist; in DMSO) and prazosin (α1-AR antagonist; in DMSO) were prepared fresh daily and atomoxetine (Atmx, NET

inhibitor; in deionized water) and clorgyline (Clor, MAO inhibitor; in deionized water) were frozen in 300μL aliquots until used for recordings.

α1-AR LTD was induced using 100μM Phe that was bath applied for 10 min following a stable baseline of fEPSPs of ≥20 min as previously reported in Scheiderer et al. (2004). Experiments to test the functionality of NE fibers remaining following DSP-4 treatment were conducted using 500nM Atmx plus 10μM Clor bath applied for 10 min following stable baseline transmission. Atmx and Clor were used to induce accumulation of endogenous NE in the synapse; however, coupled with the equal affinity NE shows for α- and β-ARs and the competing inhibitory and excitatory effects of receptor activation, it was unclear whether LTD was successfully being induced. To eliminate potential issues in the evaluation of results (i.e. synaptic potentiation due to β-AR activation), the remainder of these experiments were performed in 10μM propranolol, a selective β-AR antagonist. To ensure the α1-AR specificity of the LTD induced by endogenous NE in these recordings, interleaved experiments were conducted in the presence of 10μM prazosin (in addition to propranolol, Atmx, and Clor).

Data Analysis

All data were stored on a computer using Labview data acquisition software (a gift from Richard Mooney, Duke University) after being filtered at 3kHz and digitized at 10kHz. The fEPSP slope was measured and evaluated as a series of 5 averaged raw data points plotted versus time. Unpaired student's *t* tests or one-way ANOVAs were used to evaluate statistical significance between groups and paired student's *t* tests were used for statistical analysis within groups. The significance level was set at $p<0.05$ and the data

are presented as the mean ± s.e.m, and the n number refers to single animals. Within experimental groups, the percentage of LTD was evaluated 10 min into stable baseline transmission versus 20 min into the drug washout period.

Immunohistochemistry

Following electrophysiological recordings, 400μm-thick hippocampal slices were stored in 4% paraformaldehyde at ~5°C until the time of staining. Twenty-four hours prior to staining, slices were rinsed in phosphate buffered saline (PBS) and then transferred to a 30% sucrose/PBS solution. Tissue was resectioned to 50μm using a freezing microtome. Sections were washed 3 x 10 min in PBS at room temperature and then blocked in 10% normal donkey serum (NDS) in 0.3% PBS Triton/PBS for 1-2 hours. Primary antibodies were diluted in 10% NDS in 0.3% Triton/PBS [rabbit anti-tyrosine hydroxylase (TH, 1:200) and mouse anti-dopamine β-hydroxylase (DβH, 1:300); Chemicon, Temecula, CA], and applied to free-floating sections for overnight incubation at ~4°C. Slices were washed 3 x 10 min with PBS and were labeled with fluorescence-activated secondary antibodies diluted in 10% NDS in 0.3% Triton/PBS [donkey anti-rabbit Alexa 594 (1:200) and donkey anti-mouse Alexa 488 (1:200); Invitrogen, Eugene, OR] for 1-2 hours at room temperature. Slices were washed 3 x 30 min and incubated with Hoescht nuclear stain [1μl stock/10ml PBS] for 15 min at room temperature. Slices were mounted on slides using Permafluor (Immunon, Waltman, MA) and viewed on a Leica (Exton, PA) DM IRBE laser scanning confocal microscope. Sequential scans of blue, green, and red channels were obtained and ~20μm stacks of images were collected in a z-axis of 1.0-1.5μm step size, averaging 2 scans per image. Maximum projections

were generated and used for NE fiber quantification. DβH-positive fibers were measured and counted, with the criterion that only fibers with 4 or more consecutive boutons be considered a fragment of axon. The entire CA1 region of s. radiatum was analyzed using ImageJ software.

CHAPTER 3

RESULTS

α1-AR Activation Induces LTD at CA3-CA1 Synapses in Rat Hippocampus

Our laboratory has previously reported that *in vitro* application of NE (40μM) or a selective α1-AR agonist is sufficient to induce a NMDAR dependent long-lasting depression of extracellular fEPSPs at CA3-CA1 glutamate synapses in hippocampus (Scheiderer et al., 2003). Here, I show that application of Phe (100μM) is also reliably able to induce α1-AR LTD of the same magnitude [Fig. 1, CON: 84 ± 4% of baseline fEPSP slope (n=6)]; p=0.001]; To test whether this α1-AR mediated LTD can also be induced via accumulation of endogenous NE in hippocampus, I applied the selective NET inhibitor Atmx (500nM) in addition to an inhibitor for MAO, Clor (10μM). Previous reports have well documented the ability of NET inhibition to block reuptake of NE into the presynaptic membrane and induce increases in extracellular NE (Youdim and Riederer, 1993). Additionally, selective inhibition of MAO, the enzyme responsible for NE degradation, has also been shown to cause accumulation of NE extrasynaptically (Youdim and Riederer, 1993). These inhibitors were used collectively in order to stimulate maximal accumulation of extracellular NE. Accumulation of endogenous NE following NET and MAO inhibition appears to facilitate the expression of varying forms of synaptic plasticity (Fig. 2), where potentiation as well as depression are able to be

induced following washout. When these experiments were pooled, Atmx and Clor did not show a significant level of depression with respect to baseline, [Fig. 3, CON: 94 ± 5% (n=6); p=0.144]. This variable response can be attributed to coincident global activation of α1, α2, and β-ARs, as NE shows equal binding affinity for each receptor type. To that end, Winder and colleagues have reported that bath application of the β-AR agoinst isoproterenol is able to induce LTP and the α1-AR agonist methoxamine is responsible for LTD in the bed nucleus of stria terminalis (BNST) (Egli et al., 2005; McElligott and Winder, 2008). Thus, the activation of β-ARs by endogenous NE could be masking any α1-AR LTD expression induced by Atmx and Clor application. To determine whether blockade of β-AR activation would unmask LTD, propranolol (10μM) was applied for the duration of the recording period during the Atmx and Clor experiments, and this resulted in a significant magnitude of LTD [Fig. 4, CON: 83 ± 6% (n=4); p=0.020]. The magnitude of LTD induced by the selective α1-AR agonist Phe (Phe group) was compared to the LTD induced via NET, MAO, and β-AR inhibition (CPA group) and was not found to be significantly different, lending to the idea that these forms of synaptic plasticity are the same (data not shown; CON: CPA vs. Phe p=0.801). The NE-induced LTD reported previously by Scheiderer et al. was shown to be mediated by specific activation of α1-ARs because coincident application of NE and the α1-AR antagonist was able to block LTD (Scheiderer et al., 2003). To confirm that the LTD I see following accumulation of endogenous NE is also mediated by α1-AR activation, interleaved experiments were performed with prazosin (10μM) in the presence of propranolol, Atmx, and Clor, and this resulted in a block of the LTD (Fig. 5, CON: CPA plus prazosin, 96.5 ± 4%, p=0.398)

DSP-4 Causes a Significant Decrease in NE Innervation in CA1 of Hippocampus

In order to determine whether depletion of NE input to hippocampus is sufficient to cause deficits in α1-AR LTD, the NE specific neurotoxin DSP-4 (50mg/kg, in saline), which is known to cause a decrease in NE input from the LC to the hippocampus (Fritschy et al., 1989 and 1990; Jonsson et al., 1981) by targeting the NE uptake system and inducing alkylation of vital neuronal structures (Ross, 1976), was administered intraperitoneally at 48-hour intervals for a total of 3 injections (control animals received injections of saline only). This robust treatment protocol was used because a recent study has shown that mice treated with one dose of DSP-4 had an increased probability of hippocampal NE regeneration compared to mice treated 3 times with the toxin (Puolivali, 2000). Levels of NE innervation in s. radiatum of CA1 following DSP-4 treatment were evaluated using anti-DβH immunohistochemical staining of NE fibers, which were then imaged via confocal microscopy. DSP-4 was able to induce a significant decrease in NE fiber number and length in s. radiatum of CA1 in animals sacrificed 7-21 following the first injection [Figs. 6 and 7; Total NE fiber number, DSP-4 (n=25) vs. CON (n=7) $p<<0.001$; Total NE fiber length, DSP-4 vs. CON $p<<0.001$)].

α1-AR LTD Remains Intact Following NE Degeneration.

Systemic treatment of DSP-4 does not significantly alter α1-AR LTD induced via direct activation of α1-ARs by the selective α1-AR agonist Phe (Fig. 8A, DSP-4: 85 ± 3% (n=7); $p<<0.001$) and the magnitude of depression was not different from control (Fig. 8B, CON v. DSP-4, $p=0.852$). Despite an 85% decrease in NE innervation in s. radiatum of area CA1, α1-ARs remain coupled to downstream signaling cascades necessary for the induction of LTD. However, it is unclear whether α1-AR LTD can be

induced by endogenously released NE from the remaining 15% of NE fibers following DSP-4 treatment.

To determine whether the NE fibers surviving neurotoxic damage are able to functionally release NE, NET, MAO, and β-ARs antagonist were again bath applied and were able to induce α1-AR LTD the was not different from control (Fig. 9A, DSP-4: 83 ± 3% (n=11); $p<<0.001$; Fig. 9B, CON v. DSP-4, $p=0.972$). Furthermore, the magnitude of LTD induced via indirect activation of α1-ARs was not significantly different from the magnitude of LTD mediated through direct α1-AR activation in DSP-4 treated animals (data not shown, DSP-4, CPA vs. Phe $p=0.575$), which provides solid evidence supporting the functionality of the NE fibers remaining after DSP-4 treatment.

Figure 1. **α1-AR LTD is induced by the selective α1-AR agonist Phe. 100μM Phe is able to induce α1-AR LTD in control animals treated with saline only (CON 84 ± 4%, n = 6). (scale bar: 0.5mV, 10ms)**

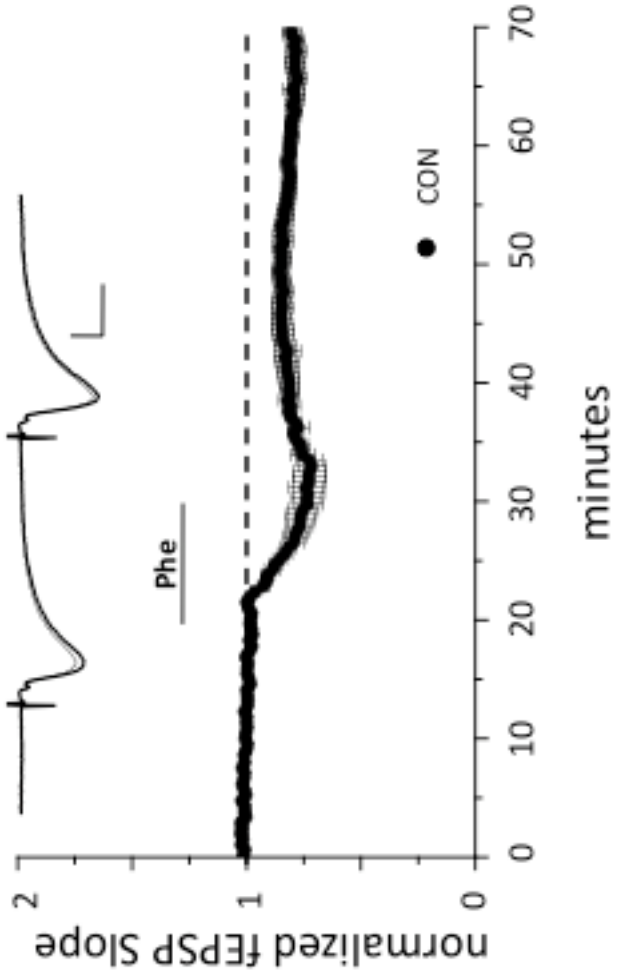

normalized fEPSP Slope
2
1
0
Phe
CON
0
10
20
30
40
50
60
70
minutes

Figure 2. **Collective activation of α1-, α2-, and β-ARs by endogenous NE results in variable forms of synaptic plasticity. (A) Representative example of LTD induced via NET and MAO inhibition by Atmx (500nM) and Clor (10μM), respectively. (B) Single example of LTP induced via endogenous NE accumulation.**

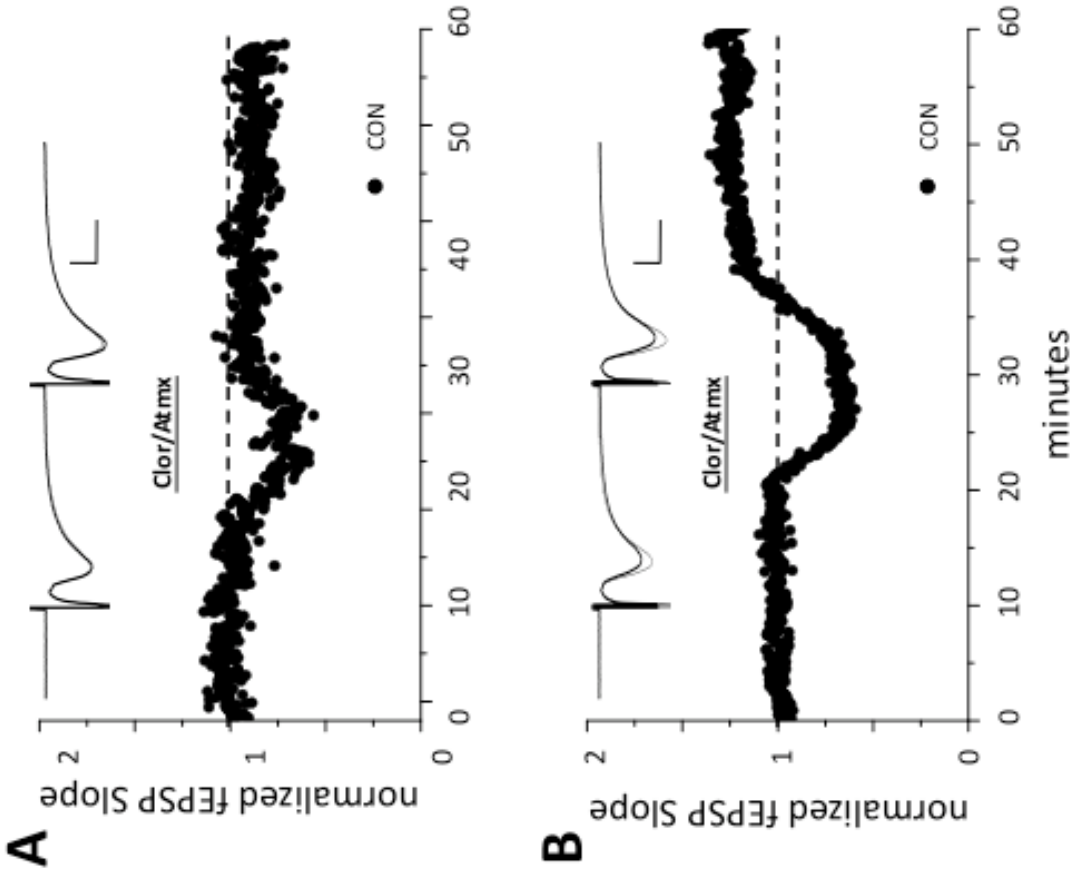

A
B
normalized fEPSP Slope
Clor/Atmx
CON
minutes
0
10
20
30
40
50
60
1
2

Figure 3. **Averaged excitatory and inhibitory synaptic plasticity induced by Atmx and Clor results in no significant change in baseline transmission (CON 94 ± 5%, n–6).**

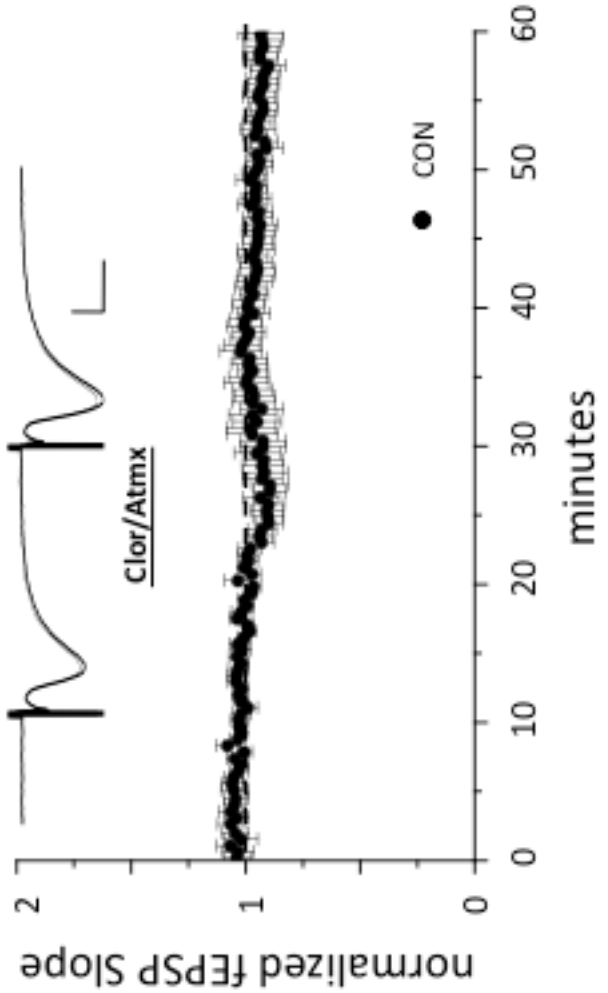

normalized fEPSP Slope
2
1
0
0
10
20
30
40
50
60
minutes
CON
Clor/Atmx

Figure 4. **The β-AR antagonist propranolol is able to unmask α1-AR LTD when used in addition to Atmx and Clor. (1μM) Propranolol inhibits excitatory transmission mediated by β-AR activation and endogenous NE is able to specifically induce α1-AR LTD in control (CON 83 ± 6%, n=4).**

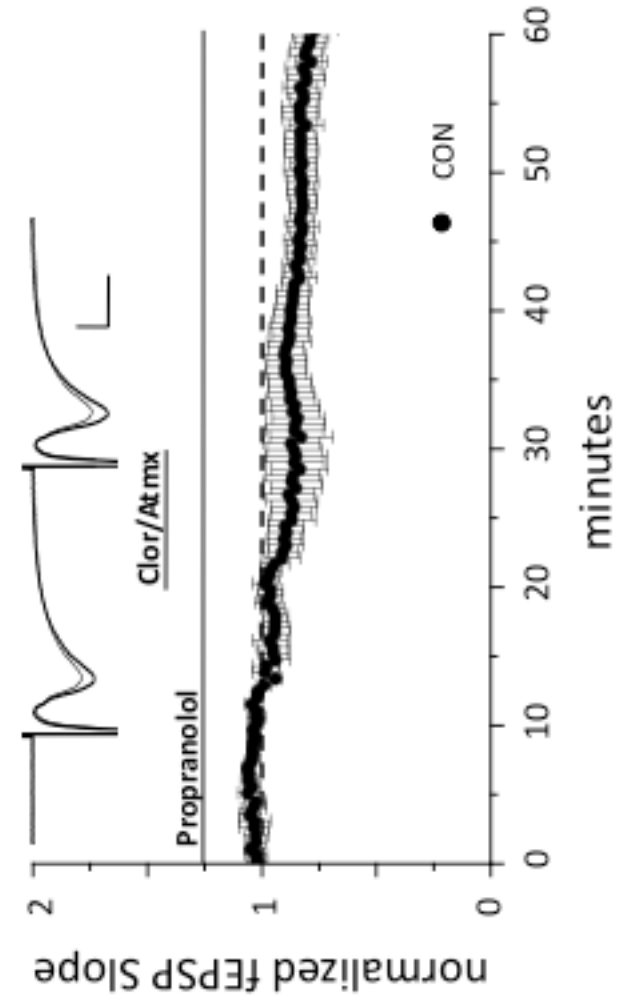

normalized fEPSP Slope
2
1
0
Propranolol
Clor/Atmx
CON
0
10
20
30
40
50
60
minutes

Figure 5. **α1-AR LTD is occluded by application of the α1-AR antagonist prazosin (10μM) in the presence of propranolol, Atmx, and Clor ($p > 0.05$).**

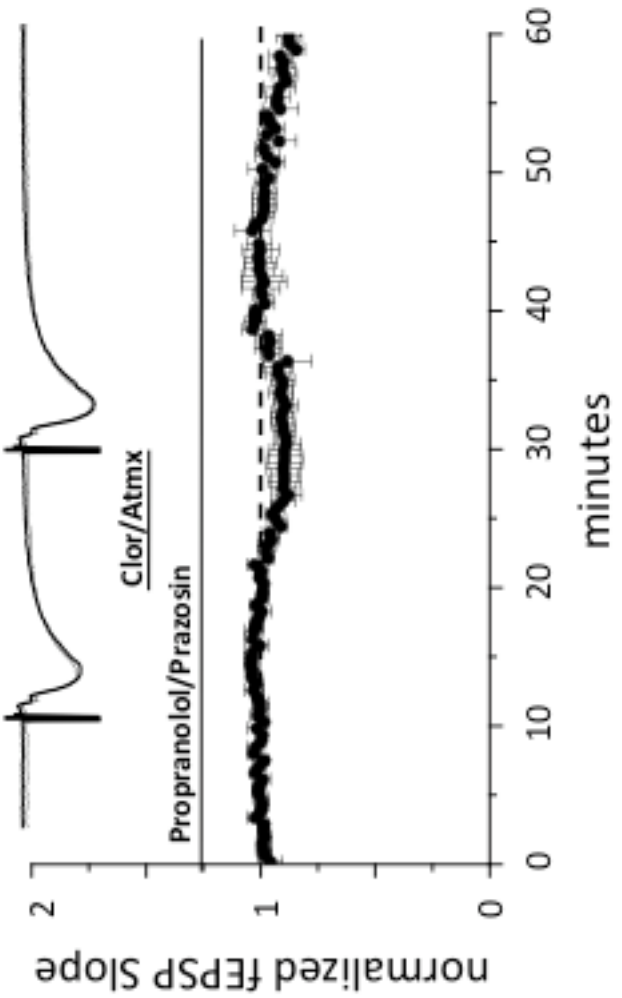

normalized fEPSP Slope
2
1
0
Clor/Atmx
Propranolol/Prazosin
0
10
20
30
40
50
60
minutes

Figure 6. DSP-4 treatment causes significant NE degeneration in CA1 of hippocampus. *Left*, anti-DβH staining of NE fibers from a saline treated (control) animal. *Right*, representative hippocampal section following DSP-4 treatment (scale bar: 40μm).

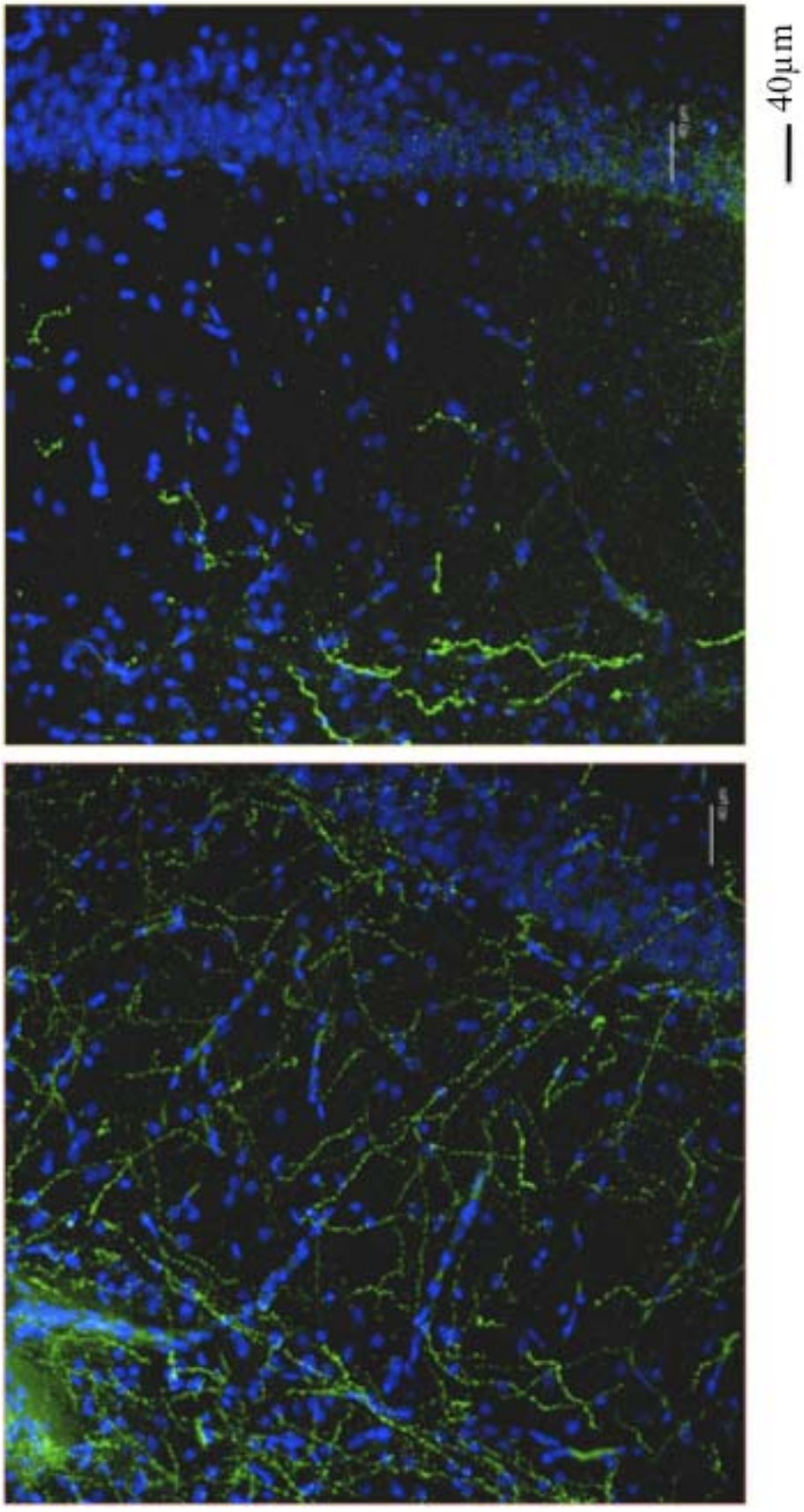

40µm

Figure 7. Total NE fiber length and number is significantly decreased following DSP-4 treatment. (*A*) Significant degeneration of NE fiber length results after DSP-4 treatment. (*B*) Total NE fiber number is significantly decreased by DSP-4 treatment by ~ 85% (CON, n=7; DSP-4, n=25).

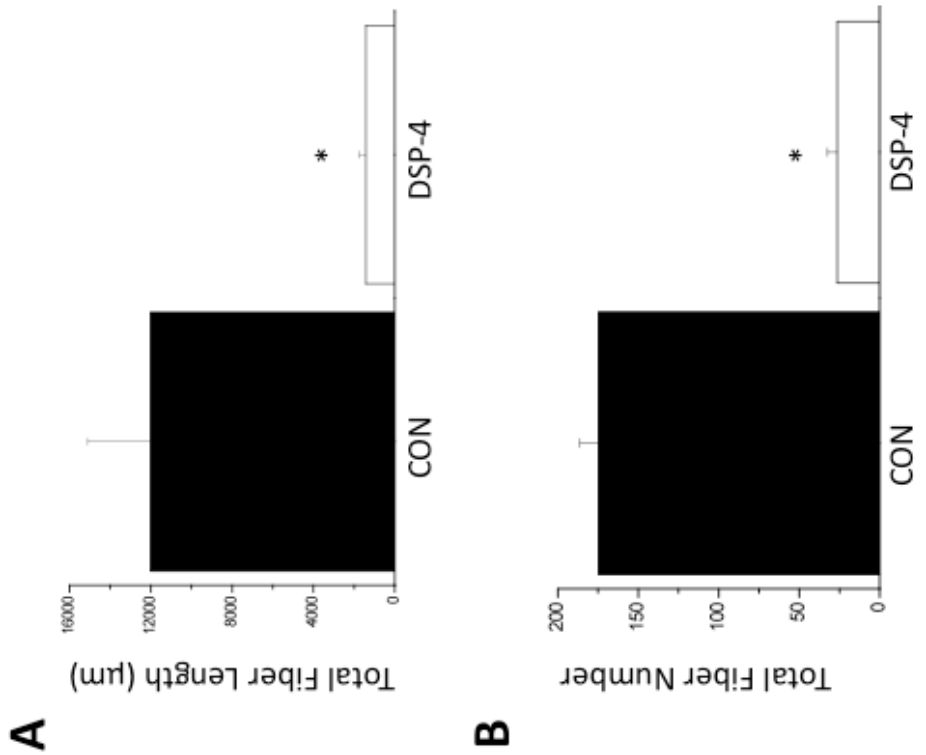

A
Total Fiber Length (μm)
16000
12000
8000
4000
0
CON
DSP-4
*
B
Total Fiber Number
200
150
100
50
0
CON
DSP-4
*

Figure 8. α1-AR LTD remains intact following NE degeneration (A, DSP-4 85 ± 3%, n=7). The magnitude of α1-AR LTD induced by Phe is not significantly different between control and DSP-4 treatment groups (CON v. DSP-4, $p > 0.05$).

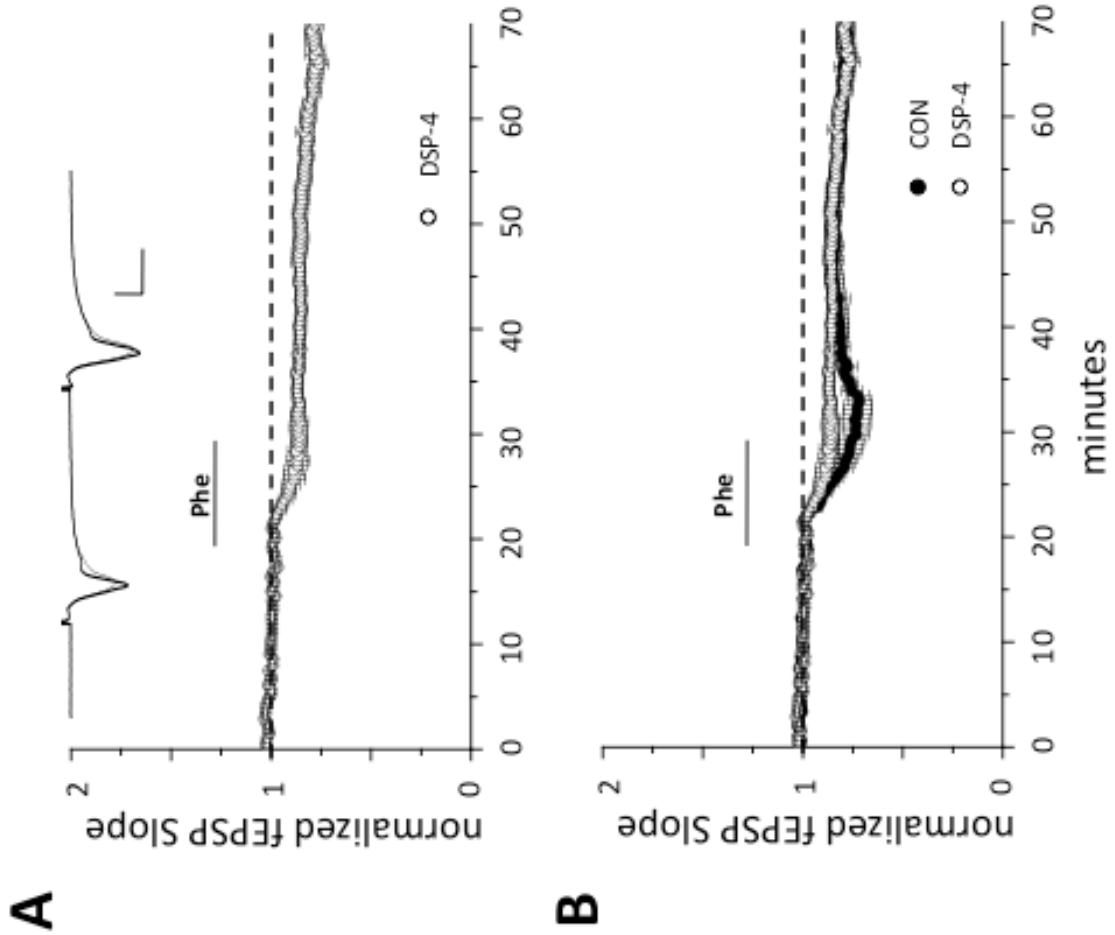
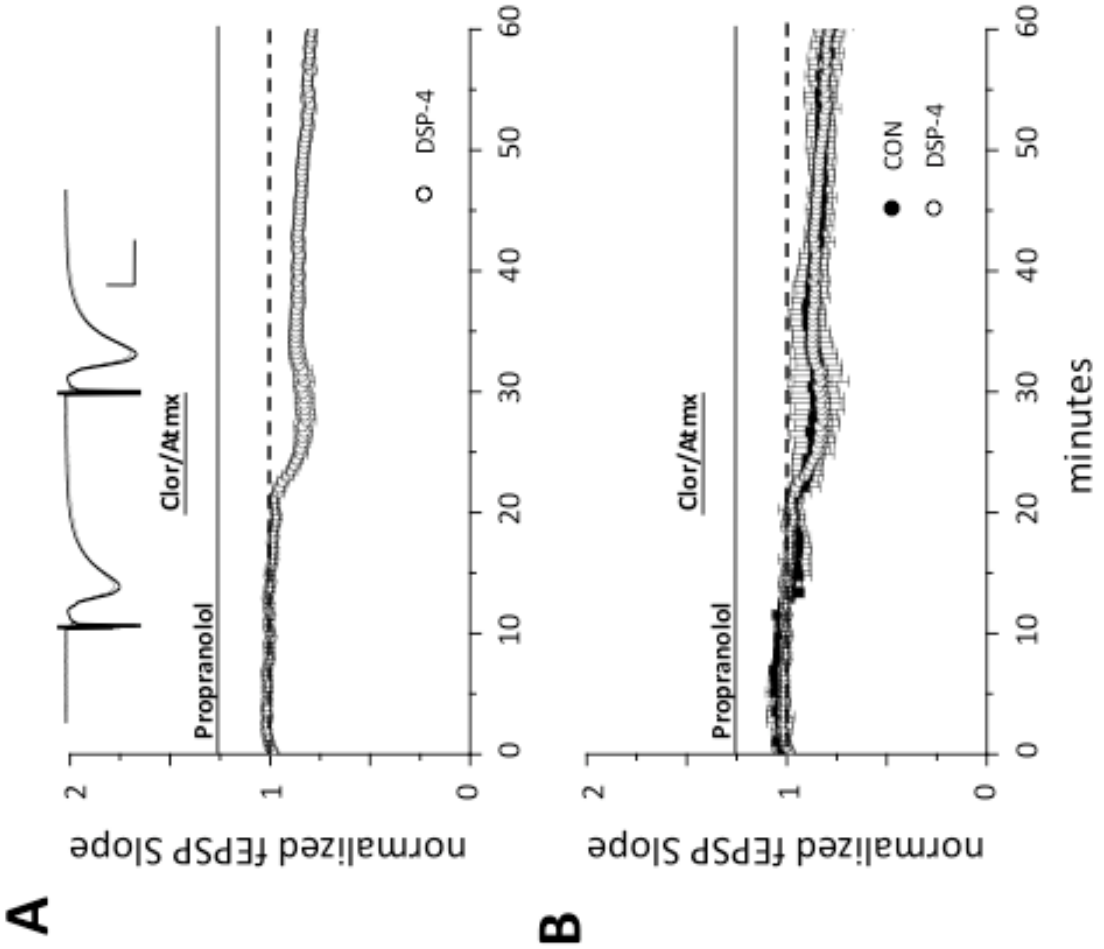

A
normalized fEPSP Slope
2
1
0
Phe
DSP-4
0
10
20
30
40
50
60
70
B
normalized fEPSP Slope
2
1
0
Phe
CON
DSP-4
0
10
20
30
40
50
60
70
minutes

Figure 9. Endogenous NE is able to induce LTD that is dependent on α1-AR activation. (***A***) α1-AR LTD is induced by endogenous NE and is unaffected by NE degeneration via DSP-4 treatment (DSP-4 83 ± 3%, n=11). (***B***) The magnitude of α1-AR LTD induced by accumulation of endogenous NE is not significantly different between control and DSP-4 treatment groups (CON v. DSP-4, $p > 0.05$).


A
normalized fEPSP Slope
2
1
0
Clor/Atmx
Propranolol
DSP-4
0
10
20
30
40
50
60
B
normalized fEPSP Slope
2
1
0
Clor/Atmx
Propranolol
CON
DSP-4
0
10
20
30
40
50
60
minutes

CHAPTER 4

DISCUSSION

Here I have established that α1-AR mediated LTD at CA3-CA1 synapses in hippocampus, as previously described by Scheiderer and colleagues (Scheiderer et al., 2003), remains intact following significant degeneration of NE input from the LC. This NMDAR dependent form of synaptic plasticity can be induced via direct activation of α1-ARs by specific α1 agonists, in addition to accumulation of endogenous NE by selective blockade of NET and MAO. Lesion of NE input to hippocampus from the LC did not prevent the α1-AR mediated synaptic plasticity, as the NE fibers that remained following DSP-4 treatment did not exhibit deficits in the expression of LTD. Therefore, the data suggest that in light of severe NE degeneration, α1-ARs remain coupled to their signaling cascade and are able to be activated by pharmacological agonists and endogenously released NE from surviving NE fibers to induce LTD.

DSP-4 Induced Lesion of Hippocampal NE Innervation

It has been well documented that DSP-4 will cause marked reductions in NE innervation in brain regions that receive projections from the LC (Fritschy and Grzanna,

1989; Jonsson et al., 1981). In addition to NE degeneration, a profound loss in DβH, the enzyme responsible for NE synthesis, occurs 4-5 days following a single dose of the toxin (Fritschy et al., 1990; Ross, 1976). It has also been shown that the NE lesions induced by DSP-4 have the ability to regenerate after a variable period of time with hyperinnervation of NE fibers occurring several months after treatment (Fritschy and Grzanna, 1992). The DSP-4 treatment used here is far more robust than those used previously (Fritschy and Grzanna, 1989 and 1992; Fritschy et al., 1990). While other studies have used single doses of DSP-4 that were able to induce "near complete" NE lesions (Fritschy and Grzanna, 1989 and 1992; Fritschy et al., 1990), my data show that even after 3 treatments NE innervation cannot be reduced by more than ~85% (Fig. 6 and 7). Additional injections or increases in DSP-4 concentration were not used due to possible increases in animal mortality. The NE lesion used here is a variable model of NE cell loss as DSP-4 only provides a temporary decrease in NE innervation, whereas neurodegeneration is permanent. Several studies have shown that DSP-4 induced NE degeneration is not permanent and can be reversed several months following treatment (Fritschy and Grzanna, 1989 and 1992; Fritschy et al., 1990). The LC-NE system is also known to be capable of initiating extreme compensatory mechanisms (as described above) in response to damage, which includes an increase in NE turnover (Jonsson et al., 1979) and release (Abercrombie and Zigmond, 1989) in surviving cells, as well as receptor supersensitivity (Berridge and Dunn, 1990; Starke, 2001). Furthermore, the lesion protocol used here may cause increased activation of the NE compensatory mechanisms thought to be responsible for lesion reversal. Thus, the 85% loss in NE

innervation observed here might not have been long enough, where α1-ARs have time to uncouple in the absence of endogenous NE.

α1-AR LTD

In the absence of normal NE innervation, it is considered likely that α1-AR LTD is altered due to AR G-protein uncoupling caused by a decrease or complete absence of endogenous NE. Data presented here demonstrate that the magnitude of α1-AR LTD is maintained despite 85% degeneration of NE input (Fig. 8). Recently, our lab has shown that M1-acetylcholine receptor (AChR) LTD is lost following hippocampal cholinergic degeneration from the medial septum but is rescued by sympathetic sprouting and cholinergic reinnervation that is 15% of control levels (Scheiderer et al., 2006). Interestingly, both M1 and α1 receptors couple to the same Gαq signaling pathway (Porter et al., 2002; Hague et al., 2003); therefore, a NE lesion has the potential to induce similar effects on LTD via α1-AR activation. Thus, the expression of α1-AR LTD at synapses in the remaining 15% of NE fibers in the data presented here is consistent with the rescued mLTD following cholinergic reinnervation as it occurs after medial septal lesion.

NE Fiber Functionality and LTD

It is clear that pharmacological activation of α1-AR by α1 specific agonists, such as phenylephrine, is sufficient to induce LTD in the presence of significant NE degeneration. In addition, the data presented here also indicate that the remaining NE

fibers are functional and α1-ARs are able to be activated by endogenously released NE and thus induce LTD with the same magnitude as that of direct α1-AR activation.

Drugs used to stimulate accumulation of endogenous NE by inhibition of the NET and MAO (Atomoxetine and Clorglyine, respectively) are widely used as therapeutic targets in disorders such as ADHD and depression, where imbalances in catecholamine neurotransmission, specifically NE, are known to occur (Castellanos et al., 1996; Zametkin and Rapoport, 1987). Interestingly, PD patients participating in clinical trials for major depression were found to exhibit improvements in vigilance, psychomotor speed, and long-term memory when administered the MAO inhibitor moclobemide (Kerr et al., 1992; Allain et al., 1992; Fairweather et al., 1993). These data suggest that in neurodegenerative diseases and psychological disorders where NE depletion or misregulation occurs, early detection and pharmacological intervention targeting α1-ARs may be able to reduce or prolong the period of time prior to the manifestation of cognitive deficits.

Future Directions

Mechanism of NE Degeneration. DSP-4 induced lesions of the LC-NE system are widely documented throughout the literature with respect to elucidating the mechanisms underlying learning, memory, and behavior in animal models of human disease where NE transmission is disrupted. Unfortunately, this neurotoxin does not cause permanent damage to the NE cell bodies located in the LC, as highlighted by studies showing regeneration and hyperinnervation of NE input several months following DSP-4 treatment (Fritschy and Grzanna, 1992). Performing electrolytic lesions of the LC

would be more effective and permanent with regard to NE degeneration as it occurs in AD and PD. The caveat to this proposed tool, and why it was not initially used in this study, is that the LC is an extremely small nucleus located deep within the brain stem. Due to the location and size of the LC, NE degeneration induced by electrolytic lesion would be extremely difficult and animal mortality rates would increase dramatically. However, these obstacles must be overcome before a complete model of LC-NE degeneration can be developed that directly mimics the effects of NE cell body loss and its relation to the pathophysiology of AD and PD.

Excessive NE Release. This study has highlighted the effects of NE depletion on neurotransmission, but in order to have a more thorough understanding of the role of NE in learning and memory the effects of excessive NE release/concentration must also be elucidated. The data shown here demonstrate that despite a decrease in NE input from the LC, surviving fibers are able to compensate for the reduction in innervation. However, it is unclear whether excessive stimulation of α1-ARs will cause a change in this form of synaptic plasticity. In addition to the hippocampus, the prefrontal cortex (PFC) also receives NE input from the LC (Arikuni et al., 1978; Gerfen and Clavier, 1979; Morrison et al., 1979; Porrino and Goldman-Rakic, 1982) and early studies suggest that the activity of the LC is mediated by aversive stimuli (Remond and Huang, 1979; Grant and Redmond Jr., 1984) and excessive release of NE results (Anisman, 1978; Dunn, 1984; Finlay et al., 1995; Kerf et al., 1973; Nisenbaum et al., 1991; Stone, 1973 and 1975; Thierry et al., 1968; Weiss et al., 1970 and 1975; Zigmond and Harvey, 1970). Marzo et al. have shown that α1-AR mediated LTD in PFC is lost when over stimulation occurs following acute restraint stress (Marzo et al., 2008).

In addition to the LC-NE system's role in hippocampal dependent learning and memory processes, it may also play a role in the learning and memory deficits seen following stress, anxiety, and panic. Cecchi and colleagues have recently shown that blocking α1-ARs in the bed nucleus of stria terminalis (BNST), a brain region implicated in stress-induced relapse behavior in addiction (Wang et al., 2001), is able to reduce anxiety in conjunction with decreased hypothalaminc-pituitary-adrenal axis activation (Cecchi et al., 2002). These results imply that α1-ARs could be a viable target for therapeutic intervention in anxiety disorders where NE neurotransmission is disrupted (McElligott and Winder, 2008). Furthermore, several studies have also shown that patients exhibiting symptoms related to PTSD show improvements with administration of α1-AR antagonists (Raskind, 2000 and 2002; Taylor and Raskind, 2002; Peskind et al., 2003; Taylor et al., 2006). Although the BNST exhibits NMDAR-independent α1-AR LTD, whereas in hippocampus it is NMDAR-dependent, the use of pharmacological tools targeting α1-ARs could improve deficits in learning attributed to PTSD and similar stress-induced disorders.

The results from studies implicating hyperfunction of NE neurotransmission in brain regions such as PFC and BNST highlight the role α1-ARs play in synaptic plasticity. Therefore, similar experiments should be conducted to elucidate whether the same deficits and improvements in synaptic plasticity also occur in hippocampus.

Behavioral Studies Evaluating NE Transmission. The data I have presented in this study show that despite significant NE degeneration, the mechanisms (i.e. α1-ARs) necessary for normal synaptic plasticity remain intact. Although changes in synaptic plasticity are widely considered to be the cellular correlate to learning and memory, it is

important to remember that experiments testing the efficacy of such plasticity are performed *in vitro;* therefore, it remains unclear how NE depletion affects synaptic plasticity *in vivo.*

Previous studies show conflicting results when rodents were tested in various learning and memory required tasks following DSP-4 treatment (Ohno et al., 1993 and 1997; Decker and McGaugh, 1989; Prado de Carvalho and Zornetzer, 1981). Reports indicate that DSP-4 confers minimal impairment in the acquisition of inhibitory avoidance as well as spatial tasks (Ohno et al., 1993 and 1997), whereas others show that long-term retention of learned tasks are damaged (Decker and McGaugh, 1989; Prado de Carvalho and Zornetzer, 1981). The reversibility of DSP-4 treatment, as well as variable treatment paradigms could lead to the confounding behavioral results following NE degeneration. In light of the robust treatment protocol used in this study and the resulting 85% decrease in NE innervation, behavioral studies examining hippocampal-dependent learning tasks should be performed in order to gain further insight into the role of NE degeneration on synaptic plasticity *in vivo.*

Coupled with the effects of NE depletion on behavioral tasks, it is also important to examine the impact excessive NE release may have on similar tasks. Microdialysis studies have shown that NE release is increased following acute and chronic stress in rodents (Nowakowska et al., 2001). Furthermore, Marzo et al. reports that rats undergoing acute restraint stress immediately prior to *in vitro* electrophysiological recordings exhibited a loss in α1-AR LTD in PFC (Marzo et al., 2008). In order to have a more complete understanding of the mechanisms underlying learning and memory as it is

modulated by extremes in NE signaling (i.e. release and depletion) behavioral studies must be performed.

LTD and ERK Phosphorylation. Scheiderer and colleagues recently reported that ERK (extracellular signal related kinase) phosphorylation is required for the induction of mLTD and NE-LTD (e.g. α1-AR LTD) in CA1 of rat hippocampal slices, where *in vitro* inihibition of ERK phosphorylation was sufficient for blockade of both forms of LTD (Scheiderer et al., 2008). Given the ability of selective α1-AR agonists (e.g. phenylephrine) and endogenous NE (via NET and MAO inhibition) to induce LTD via α1-AR activation in animals with intact and significantly depleted NA input to hippocampus, it is important to further establish the role of ERK signaling in this form of synaptic plasticity. Therefore, α1-AR activation and subsequent of ERK phosphorylation should be evaluated following *in vitro* application of phenlyephrine and clorgyline plus atomoxetine to confirm the dependence of this form of LTD on α1-AR activation. In addition to confirming the role of α1-AR activation, these experiments have the potential to reveal varying levels of ERK phosphorylation in response to NE degeneration when compared to controls, which would imply that decreases or increases in ERK activation are sufficient to maintain LTD.

The experiments performed in this project demonstrate that α1-AR mediated LTD is a stable form of synaptic plasticity that remains intact despite significant decreases in NE input to the hippocampal CA3-CA1 region. Additionally, the NE fibers remaining following neurotoxic damage continue to release NE sufficient to induce LTD in the absence of exogenous ligand. This study has provided additional characterization to what has previously been described as NE LTD (Scheiderer, 2003); however, further

investigation is required to fully understand the role of NE modulation in hippocampal function.

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APPENDIX

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL FORM

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

Notice of Approval for Protocol Modification

DATE: June 18, 2009

TO: Lori McMahon, Ph.D.
MCLM-701 0005
FAX: 975-9028

FROM: Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee

SUBJECT: Title: Muscarinic Receptor Inducted LTD in Rat Hippocampus
Sponsor: NIH
Animal Project Number: 081006879

On June 18, 2009, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the modification as described: Personnel- Katie Dyer. The sponsor for this project may require notification of modification(s) approved by the IACUC but not included in the original grant proposal/experimental plan; please inform the sponsor if necessary. The following species and numbers of animals reflect this modification.

Species	Use Category	Number in Category
Mice	B	Zero - Procedural modification only
Rats	B	Zero - Procedural modification only

Animal use is scheduled for review one year from October 2008. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files.

Refer to Animal Protocol Number (APN) 081006879 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at 934-7692.

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