LOCUS COERULEUS-DEPENDENT DOPAMINE RELEASE IN THE DORSAL HIPPOCAMPUS: MECHANISMS AND MODULATION OF SYNAPTIC PLASTICITY

APPROVED BY SUPERVISORY COMMITTEE

Robert W. Greene, M.D., Ph.D., Mentor

Julian Meeks, Ph.D., Chair

Kimberly Huber, Ph.D.

Saïd Kourrich, Ph.D.

Todd Roberts, Ph.D.

DEDICATION

I consider this work to be a long-term collaboration between mentors (*Robby, Julian, Kim, Saïd, & Todd*), teams (*stay hard, Dartos)*, family (*August, Claire, Dad, Franklin, Mom, Pat, Steve, Sue, and above all, Marissa*), and friends who helped me over the past 6 years (Adan, Ayako, Brad, Florian, Francisco, Karine, Lijing, Stephanie, Theresa, To Thai, Vamsi). A special dedication goes out to my grandfather, whose last spoken words to me were “I hope I live long enough to see you get your Ph.D.” Thank you all.

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ALEX JAY SONNEBORN

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ALEX JAY SONNEBORN, Ph.D.

The University of Texas Southwestern Medical Center at Dallas, 2019

ROBERT W. GREENE, M.D., Ph.D.

ABSTRACT

Locus coeruleus (LC) neurons coordinate the overwhelming majority of norepinephrine (NE) signaling throughout the mammalian neocortex and hippocampus. Recent discoveries indicate that dopamine (DA), the biosynthetic precursor of NE, is also released from LC axons. These axons innervate most brain regions, and are especially prevalent in the rodent dorsal hippocampus, including area CA1. It was previously thought that the only supply of CA1 dopamine was the ventral tegmental area, but several recent studies have identified LC fibers as the main source of DA in

this region. However, both the mechanism by which LC-DA is released, and whether or not it is released in sufficient quantities to influence DA-dependent processes in the hippocampus, remain unclear. These questions have major implications for theories concerning the molecular basis of learning, since the consolidation of episodic memories in CA1 requires activation of dopamine D1-like receptors. Therefore, the focus of this dissertation is to determine if LC-originating DA can modulate synaptic plasticity, and therefore learning and memory, in CA1 of the mouse hippocampus. We also sought to uncover the molecular mechanism of this LC-DA release.

The following experiments study the effects of LC-dopamine on CA1 function using optogenetic, electrophysiological, pharmacological, and behavioral approaches. We show that optogenetically evoked LC-DA release is sufficient to activate D1/D5 receptors (D1/5R) on CA1 pyramidal neurons and modulate synaptic potentiation at Schaffer collateral synapses, a necessary step for the consolidation of learning. In accordance with this, we find that LC-specific knockdown of DA synthesis can block learning at the behavioral level (**Chapter 2**). We also demonstrate that one possible LC-DA release mechanism is reverse transport through the norepinephrine transporter (NET), and advance the idea that presynaptic NMDA receptors on LC terminals may play a role in this release. Furthermore, as DA and NE should be co-released in dorsal CA1, we show that they act together to enhance synaptic strength (**Chapter 3**). Since LC activity is known to be involved in attention and memory, our results contribute new insight into how the LC can link attentional processes to memory formation at the molecular, circuit, and behavioral levels (**Chapter 4**).

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CHAPTER ONE: Background and Introduction

Some Definitions: In this dissertation, I will frequently refer to the following items using their abbreviations (in parenthesis): locus coeruleus (LC), dopamine (DA), norepinephrine (NE), dorsal hippocampus (DH), dopamine receptors (D#Rs, where # is a number 1-5), adrenergic receptors (ARs), and long-term potentiation (LTP). In every experiment, LTP is measured as an increase in the initial slope of extracellular field potentials (fEPSPs) recorded from stratum radiatum dendrites of dorsal hippocampal CA1 after high-frequency electrical stimulation of CA3 axons (see **Fig. 2.1B**). This slope approximates the average strength of fast glutamatergic synaptic transmission between CA3 axons and CA1 dendrites, which should be largely mediated by AMPA receptors. A significant long-lasting increase (~1 hour) in fEPSP slope after high-frequency stimulation is an indicator of successful LTP induction. Several factors are known to contribute to this long-lasting slope increase, including: AMPA or NMDA receptor phosphorylation leading to greater channel conductance, AMPA receptor insertion into the postsynaptic density, or the *de novo* synthesis of proteins including AMPA receptors and postsynaptic scaffolding proteins (Reymann and Frey, 2007).

Preface

Scientific study of the locus coeruleus (LC) dates back to the late 1700's, when it was first described by French anatomist Félix Vicq-d'Azyr as a small brain region appearing darker and more blue than the surrounding postmortem human brain tissue (Tubbs et al., 2011). It took until 1964 for Dahlström and Fuxe to discover that the LC releases large amounts of norepinephrine (NE) from its axons (Dahlstroem and Fuxe,

1964a, b; Dahlstroem et al., 1964). Since then, the LC-noradrenergic system has consistently been a popular area of research, and firing activity of its neurons has been positively correlated with numerous important cognitive processes including: general arousal, novelty detection, salience signaling, attention, working memory, and memory formation, consolidation, and retrieval. However, even with the abundance of studies on LC function over the last few decades, we are only beginning to uncover the effects of LC activity at the molecular, circuit, and behavioral levels. The following sections will provide a summary of the current state of the field, with a focus on the LC's role in dopamine release and hippocampus-dependent memory formation. The function and anatomy of hippocampal circuits in the context of their modulation by the LC, dopamine and norepinephrine will also be discussed.

Anatomy of the locus coeruleus-noradrenergic system

The LC is a small pontine nucleus containing less than 1,000 neurons in mice. Despite its size, it sends diffuse axonal projections throughout the entire central nervous system (with the exception of the striatum) (Berridge and Waterhouse, 2003), and provides the only source of norepinephrine (NE) to the cerebellum, cerebral cortex, and hippocampus (Aston-Jones and Cohen, 2005; Sara, 2009; Schwarz and Luo, 2015). LC axons are thin and unmyelinated, and often do not form conventional synapses with neurons in their target regions. Instead, they contain numerous release sites along their length called varicosities (Chiti and Teschemacher, 2007; O'Donnell et al., 2012). These varicosities can be found throughout all subregions of the dorsal hippocampus, where

they are thought to primarily release neurotransmitter via *en passant* volume transmission (Agnati et al., 1995; Cimarusti et al., 1979; Pickel et al., 1996).

Due to the widespread nature of their termination, noradrenergic neurons residing within the LC nucleus were historically thought to all become active simultaneously and exert global control over general arousal (Sara and Bouret, 2012). However, fully synchronous LC firing only occurs early in postnatal development, and recedes as its neurons mature (Christie et al., 1989), before nearly disappearing in older rodents (Totah et al., 2018). Rather, recent data has uncovered an enormous amount of heterogeneity between individual neurons within the LC (Chandler, 2016; Schwarz et al., 2015; Totah et al., 2019; Uematsu et al., 2015). Notably, single noradrenergic cells are now known to exhibit specificity in their afferent and efferent connections (Schwarz and Luo, 2015), and can display different morphological and electrophysiological properties depending on the region they connect with (Chandler et al., 2014). In relation to the content of this dissertation, it is important to note that hippocampus-projecting LC somas are located within the dorsal, anterior potion of the nucleus in mice, and are usually large, fusiform-shaped cells with broad action potential widths (Schwarz et al., 2015). In spite of the clear projection preference of individual LC neurons based on their location within the nucleus, many LC neurons send highly branched axons that usually terminate in more than one brain region at once (Kebschull et al., 2016). So, it seems that LC neurons would be better labeled as regionally specific, but not usually globally or individually as some studies have suggested.

Afferent projections onto LC neurons arrive less universally, typically from just a handful of sources. These include the amygdala, hypothalamus, neocortex, and various midbrain/brainstem structures such as the periaqeductal grey, ventral tegmental area, and Raphe nucleus (RN). Intriguingly, the densest reciprocal connections between the LC and cortex occur in the prefrontal area, paralleling well known LC contributions to problem solving, decision making, and sustained attention (Samuels and Szabadi, 2008a). Most of these inputs, with the exception of the VTA (dopamine) and RN (serotonin), are glutamatergic, and LC neurons have been shown to express mRNA for all major AMPA and NMDA receptor subtypes in their cell bodies (Chandley et al., 2014). Overall, the LC is emerging as a more intricate and anatomically segregated system than originally thought, and is likely involved in many different cognitive processes to variable extents (Uematsu et al., 2017).

Release of norepinephrine from LC terminals

Before going into detail about the behavioral consequences of LC activity, it will be useful to briefly mention the basic mechanisms involved in the synthesis and release of norepinephrine. LC-derived norepinephrine begins as the amino acid tyrosine. More specifically, tyrosine is converted into L-DOPA by the rate-limiting enzyme tyrosine hydroxylase (TH). L-DOPA is then decarboxylated into dopamine by a second enzyme, L-amino acid decarboxylase. Both of these steps occur in the cytoplasm of LC terminal boutons (Benarroch, 2009; Szabadi, 2013). Dopamine is next transported into synaptic vesicles via a subtype of vesicular monoamine transporter, VMAT2, where it is converted into norepinephrine by the membrane-bound enzyme, dopamine β-

hydroxylase, within the lumen of the vesicle (Rush and Geffen, 1980; Saxena and Fleming, 1983). Norepinephrine is then released when vesicles fuse with the membrane after sufficient calcium entry into the axon (diagram of this process shown in **Fig. 3.9**).

The levels of extracellular NE can drastically vary over a broad range of LC patterns corresponding to different behavioral states (Waterhouse and Navarra, 2019). These include a slower, tonic LC firing pattern (~1-2 Hz) seen during periods of quiet waking (e.g. grooming), a faster tonic firing pattern (~4-5 Hz), seen during periods of increased general arousal (e.g. exploratory behavior), and a phasic, bursting pattern achieved when an animal experience something novel, salient, rewarding, surprising, or threatening (Grilli et al., 2009; Waterhouse and Navarra, 2019). More detail covering the role of these firing patterns in behavior and physiology will be given in the section titled 'General functions of the locus coeruleus-noradrenergic system' below.

Adrenergic receptor activation by norepinephrine

Once norepinephrine is in the extracellular space, it binds to and activates three general classes of adrenergic receptors (ARs). The highest affinity ARs are the α2-ARs, which are primarily expressed presynaptically on LC terminals to act as inhibitory autoreceptors. As such, α2-ARs are coupled to $G_{i/o}$-type G-proteins and decrease the production of cyclic AMP when activated, which dampens excitatory intracellular neuronal signaling pathways. Turning on α2-ARs can also open G-protein coupled inwardly rectifying (GIRK) potassium channels (Torrecilla et al., 2013) and inhibit multiple types of calcium channels (McBurney-Lin et al., 2019), thereby reducing excitability and neurotransmitter release in LC terminals (Arnsten, 2000; Marzo et al.,

2009). Indeed, pharmacological activation of α2-ARs by clonidine hyperpolarizes noradrenergic neuron membrane potential (Aghajanian and VanderMaelen, 1982), thereby lowering excitability (Svensson et al., 1975). In contrast, blockade of α2-ARs with an antagonist, idazoxan, increases LC firing rate (Simson and Weiss, 1987), and NE release in terminals regions (Fernandez-Pastor and Meana, 2002).

A second type of AR present in the dorsal hippocampus with a slightly lower affinity for NE is the α1-AR. These receptors are mainly localized to interneurons in CA1 (Hillman et al., 2007; Hillman et al., 2005b) and are coupled to G_q G-proteins. This coupling means that α1-ARs enhance phospholipase C signaling when an agonist is bound, leading to IP3 production and increased Ca^{2+} release from intracellular stores. There is also evidence that turning on these receptors lowers the conductance through potassium leak channels (Bergles et al., 1996). In line with this, activation of α1-ARs has been shown to increase both excitability of CA1 interneurons and spontaneous GABA release onto neighboring pyramidal cells via a presynaptic mechanism (Hillman et al., 2009). This increased probability of GABA release effectively creates weaker CA3-CA1 glutamatergic connections (Mynlieff and Dunwiddie, 1988), which in turn curtails excitability of CA1 pyramidal neurons (Mueller et al., 1981).

The last type of AR found in dorsal CA1, the β-adrenergic receptor, is found exclusively on pyramidal cells (Hillman et al., 2005b), which express both the β1 and β2 subtypes (Guo and Li, 2007). Like the previously mentioned α-ARs, β-ARs are also GPCRs, but they differ due to their downstream coupling to G_s effector proteins and their relatively low affinity for NE (Arnsten, 2000; Marzo et al., 2009). When activated,

they increase the production of cyclic AMP in CA1 pyramidal cells, which can have major positive impacts on fast glutamatergic synaptic transmission in the region (Hillman et al., 2005a; Mueller et al., 1982). β-ARs have also been implicated in multiple mechanisms of CA1 synaptic plasticity (O'Dell et al., 2015). These include upregulation of surface AMPA receptors and increased inward current mediated by AMPA receptor phosphorylation (Hu et al., 2007). They are capable of increasing the excitability of CA1 pyramidal cells as well (Liu et al., 2017), often by shortening the afterhyperpolarization and preventing accommodation of action potential firing (Haas and Konnerth, 1983; Madison and Nicoll, 1982).

Taken as a whole, there exists a complex interplay between the levels of extracellular NE, which are controlled by a range of LC firing patterns, and differential activation of adrenergic receptors on multiple cell types in dorsal CA1. It is not yet clear what this complexity accomplishes at the hippocampal circuit level, but it has been hypothesized that α1 and β receptors are involved in gain control of incoming sensory information (Aston-Jones and Cohen, 2005), which may direct the efficient routing of environmental representations for more accurate hippocampal memory formation and reduced overgeneralization.

Clearance of norepinephrine from the synapse

After NE is released, a small portion is broken down into metabolites by the enzymes catechol-O-methyltransferase (COMT) and monoamine oxidase (MAO). The remainder is reuptaken back into the presynapse and recycled for later use. This process occurs through the norepinephrine transporter (NET), which is located on or

near NE release sites. As a Na^+/Cl^--dependent transporter, the NET heavily relies on extracellular Na^+ and Cl^- gradients to facilitate the flux of synaptic NE back into cells (Mandela and Ordway, 2006). In the context of this dissertation it is important to note that the NET also has a high affinity for DA (Carboni and Silvagni, 2004; Weinshenker and Schroeder, 2007), and is the main contributor to DA reuptake in dorsal hippocampus (Borgkvist et al., 2012).

Critically, the NET has also been shown to efflux both DA and NE from the cytosolic compartment under certain conditions. The most widely studied is the presence of the drug amphetamine (AMPH), which causes the reverse transport of both DA and NE from the NET (Sulzer et al., 2005). This reversal relies on intracellular signaling of multiple effectors including Ca^{2+}, PKC, and CAMK2 (Robertson et al., 2009). Phosphorylation of these transporters is thought to drive a conformational change to an outward facing configuration that allows for their reversal (Foster and Vaughan, 2017). So, it is possible that the NET transport can be bidirectionally modified in physiological environments based on the state of a circuit. More information about this topic can be found in **Chapter 3**.

General functions of the locus coeruleus-noradrenergic system

As detailed above, the LC sends projections to nearly all regions of the central nervous system, and activation of a single LC neuron is capable of initiating NE release in multiple brain regions at once. Because of this, LC activity can be causally linked to numerous cognitive and behavioral processes ranging from general arousal during the sleep-wake cycle (Berridge et al., 2012), to complex problem solving and decision

making (Sara, 2009). The LC was originally studied in the context of the sleep-wake cycle, as single unit LC activity is highly correlated to an animal's current level of arousal (Samuels and Szabadi, 2008b). Throughout the transition from active waking to sleep, LC neurons gradually reduce their tonic output, and firing rate drops to zero during the deeper stages of sleep (Takahashi et al., 2010). In accordance with this, optogenetically suppressing or enhancing the tonic firing of LC neurons will cause mice to rapidly fall into a sleeplike state or awaken from sleep and increase their locomotor behavior, respectively (Carter et al., 2010).

During more active waking periods of increased environmental awareness, short, higher-frequency bursts of LC activity emerge on top of the underlying tonic discharge. Interplay between these two firing modes is thought to control a range of behaviors over varying complexity (Atzori et al., 2016). For instance, if an animal is awake but doing something simple like grooming or consuming nutrients, LC neurons fire at a slower tonic rate of about 1-2 Hz. During more active and vigilant environmental exploration, this tonic rate increases to ~5 Hz, and can even exceed this value during periods of intense stress and anxiety (Devilbiss, 2019). If an animal then encounters a meaningful, novel, alerting, or otherwise salient stimulus in its environment, the LC will transiently switch to a phasic mode of activity and fire action potentials at rates of up to 20 Hz within individual bursts (Waterhouse and Navarra, 2019). The levels of extracellular NE measured from LC terminal regions rise linearly from 0.5-5 Hz tonic LC activity (Berridge and Abercrombie, 1999; Devilbiss et al., 2006), but an equal number of LC

stimulations given in bursts of 3-4 action potentials at speeds of ≥10 Hz produces a non-linear surge in the amount of extracellular NE (Florin-Lechner et al., 1996).

Not surprisingly, these bursts of LC activity can bring about global shifts in network connectivity (Grella et al., 2019; Zerbi et al., 2019), and brainwide synchronization changes that are large enough to be measured by EEG (Berridge and Foote, 1991; Vazey et al., 2018). Compared to tonic activity, phasic firing greatly enhances the signal-to-noise ratio of incoming sensory information, and therefore helps an animal more effectively attend to specific stimuli in rapidly changing environments (Devilbiss, 2019; Devilbiss and Waterhouse, 2011). Furthermore, LC bursting is thought to encode the salinece value of a stimulus (Corbetta et al., 2008; Vazey et al., 2018), and permit greater cognitive flexibility in the cortex when enhanced attention and problem solving is required (Bouret and Sara, 2005; Sara and Bouret, 2012).

Locus coeruleus-dependent memory formation and dopamine-dependent synaptic plasticity in the hippocampus

In addition, and most relevant to this dissertation, phasic LC firing (bursts of 3-4 action potentials at speeds of ≥10 Hz) has been associated with successful long-term memory consolidation in rodents (Hansen, 2017). Much of this data has been gathered from studies in the amygdala, a brain region crucial for emotional learning, especially the association of pain with a particular sensory cue. Disrupting NE signaling within the amygdala using AR antagonists (especially the β-AR antagonist propranolol) yields large deficits in remembering emotional events (Giustino and Maren, 2018). More specifically, blocking β-ARs in the amygdala as a mouse learns to associate an auditory

tone with a painful shock nearly abolishes recall of the association during playback of the tone (Bush et al., 2010). Likewise, silencing LC input to the amygdala greatly reduces aversive learning and anxiety about an impending shock (Llorca-Torralba et al., 2019).

In the hippocampus, the dentate gyrus (DG) receives the densest LC projections of all subregions (Hagena et al., 2016). Within the DG, norepinephrine binding to ARs is essential for episodic memory formation (Harley, 2007), and blocking β-ARs is known to reduce potentiation of population spike amplitude elicited by entorhinal cortex stimulation (Hagena et al., 2016). The opposite manipulation, phasic LC stimulation with simultaneous entorhinal cortex activation, can enhance dentate gyrus LTP in a β-AR-dependent manor (Hansen and Manahan-Vaughan, 2015; Straube and Frey, 2003). Since the dentate gyrus is more involved in pattern separation of incoming sensory information than memory storage/distribution like CA1 (Aimone et al., 2011; Kesner, 2007; Kesner et al., 2004), a common hypothesis proposes that LC-noradrenergic signaling in DG is effective at regulating the acquisition phase of associative memory than the CA1-dependent consolidation phase.

In support of this, β-AR-dependence for LTP expression is not present in the CA1 region, which relies much more heavily on DA signaling (Swanson-Park et al., 1999). Instead, NE seems to play a more subtle facilitative role of only low-frequency glutamatergic stimulation and prevention of long-term synaptic depression (Katsuki et al., 1997). However, LTP's dependence on DA over NE in CA1 is odd considering the lack of canonical dopamine-releasing VTA fibers innervating this region (Gasbarri et al.,

1997; Kwon et al., 2008; Nomura et al., 2014; Smith and Greene, 2012; Swanson, 1982b). This anatomical mismatch has lead to a multitude of recent work (including data from this dissertation) showing that CA1 dopaminergic signaling is actually mediated by locus coeruleus inputs (Kempadoo et al., 2016; Smith and Greene, 2012; Takeuchi et al., 2016; Wagatsuma et al., 2018). Scatton et al. (1981) pioneered the chemical detection of dopamine release in hippocampus after electrical LC stimulation. Since then, many groups have found similar results with more specific LC stimulation, and have connected this dopamine release to hippocampal circuit and behavioral level phenomenon.

The first study to suggest a lone contribution of the LC to hippocampal DA release instead of the VTA was Smith and Greene Smith and Greene (2012). They found that knocking out catecholamine signaling in the VTA had no effect on DA-dependent potentiation of CA1 synapses caused by amphetamine application. Instead, this potentiation was successfully eliminated by catecholamine knockdown in the LC. Several years later, Kempadoo et al. (2016) chemically detected an increase in extracellular NE and DA after targeted LC optogenetic stimulation in dorsal CA1. In the same paper, they showed that mice performed better on tests for novel object recognition and contextual memory when the LC was stimulated during the learning phase of these tests. The observed cognitive improvements were dependent on D1-like receptors in dorsal CA1, but not β-ARs.

Around the same time, Takeuchi et al. (2016) demonstrated an analogous finding, as VTA stimulation in dorsal CA1 was ineffective at promoting learning of an

everyday memory paradigm, but LC stimulation or novelty presentation had an appreciable effect. Again, this LC-dependent memory augmentation seemed to only rely on D1-like receptors in DH and not β-ARs. A third study was focused on the LC's ability to promote place-cell formation, and found that archaerhodopsin (ArchT) inhibition of LC fibers in the dorsal CA3 region, but not CA1, impairs spatial memory formation of novel contexts. They further reported deficits in the stability of place cell representations in CA3, which presumably lead to inadequate neuronal ensemble formation downstream in CA1 (Wagatsuma et al., 2018).

Using a complementary approach to the behavioral and chemical findings in the above papers, the following chapters will look into LC-DA's probable role in CA1 synaptic potentiation by means of slice electrophysiology. The strengthening of dorsal CA1 synapses, measured as an increase in the extracellular voltage response to CA3 stimulation, robustly correlates with memory formation in live animals (Lisman and McIntyre, 2001) and is thought to be the molecular substrate of episodic learning (Lynch et al., 2007; Lynch, 2004). Crucially, this process is contingent on the activation of dopamine D1-like receptors, especially when measuring long-lasting memory formation over days to years (Frey et al., 1991; Lisman et al., 2011) (See **Chapter 2** for more detail). Hence, the main question of this dissertation revolves around the ability of LC-DA to initiate and maintain the process of synaptic potentiation.

Taking the above summary into account, the LC-noradrenergic system is a prime candidate to co-release dopamine alongside norepinephrine and aid modulate dorsal hippocampal plasticity using two separate catecholamines. This concept is a paradigm

shift in the field of associative memory, but fits well with studies indicating an interaction of DA and NE systems is required for successful synaptic plasticity in dorsal CA1 (Darracq et al., 1998; Jenson et al., 2015; Moncada, 2017). Below we present the first direct evidence that LC-DA is sufficient to modulate synaptic strength in dorsal CA1, and also put forth a hypothesis for the mechanism and physiological purpose of its co-release alongside norepinephrine with respect to the formation and consolidation of memories.

CHAPTER TWO: Dopamine released from the locus coeruleus modulates contextual learning and synaptic plasticity in dorsal hippocampal CA1

Data from the following chapter has been published as:

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***Equal authorship contribution**

Contributions as co-first author:

- Designed, performed and analyzed slice electrophysiology experiments to determine if locus coeruleus-dopamine release had an effect on synaptic plasticity in CA1.
- My data went into creating Figure 5 and Extended Data Figure 7 of the article, and writing some of the paper.

Introduction

The relationship between dopamine (DA) and hippocampus-dependent synaptic plasticity and episodic learning was established over 30 years ago, and has since drawn an enormous amount of attention in the literature. Specifically, both the release of DA and activation of dopamine D1 receptors (D1Rs) are required for the encoding and persistence of episodic memories in dorsal hippocampus (Bethus et al., 2010; O'Carroll et al., 2006). For example, blocking (Li et al., 2003) or knocking out (Granado et al., 2008) D1Rs in this region causes considerable LTP deficits, and consequently spatial memory impairment. At the cellular level, activation of D1Rs in hippocampus is required

for the expression and maintenance of long-term potentiation (LTP) (Matthies et al., 1997; Navakkode et al., 2007), the cellular substrate of learning (Malinow et al., 2000). At least part of this LTP process occurs via a PKA-dependent increase in the synaptic expression of AMPA receptors (AMPARs) (Gao et al., 2006; Navakkode et al., 2007). Accordingly, activation of D1Rs in DH is sufficient to increase not only AMPAR transmission (Gonzalez-Islas and Hablitz, 2003; Yang, 2000), but also transmission mediated by NMDA receptors (NMDARs) (Varela et al., 2009). Strong enough enhancement of both of these processes leads to a separate but equally essential process in LTP maintenance involving protein synthesis (Reymann and Frey, 2007; Sajikumar and Frey, 2004).

It is assumed that dopaminergic fibers originating in the ventral tegmental area (VTA) contribute the released DA in dorsal hippocampus (Bethus et al., 2010; Gasbarri et al., 1996; Gasbarri et al., 1997; Lisman and Grace, 2005). Remarkably, however, little evidence exists to support this assumption. The primary input from VTA is actually to the ventral subregion of hippocampus, with minimal input to the dorsal hippocampus (DH) (Gasbarri et al., 1994; Gasbarri et al., 1997; Nomura et al., 2014; Swanson, 1982a). In fact, dopaminergic innervation is barely observed in DH, and what little is observed is primarily in the stratum oriens region of dorsal CA1 (Gasbarri et al., 1994). This is quite puzzling given that activation of D1Rs is required for dorsal CA1-dependent learning and memory.

In an attempt to resolve this discrepancy, our attention turned to the noradrenergic system. The locus coeruleus (LC) is the only other known source of DA in

the vicinity of DH-D1Rs, and provides a dense innervation to the dorsal CA1 region (Loughlin et al., 1986; Zhang et al., 2019). This dopamine is present in the cytosolic compartment of LC axons in high concentrations before it gets transported into synaptic vesicles by VMAT2 (Yamasaki and Takeuchi, 2017). As a consequence of this, electrical and chemical stimulation of the LC increases norepinephrine (NE) and DA release in terminating cortical regions (Devoto and Flore, 2006; Devoto et al., 2005a, b; Kawahara et al., 2001). Likewise, reversal of the norepinephrine transporter, but not dopamine transporter, by amphetamine causes a DA-dependent potentiation of glutamatergic signaling in CA1 (Smith and Greene, 2012). These studies hint at activation of LC fibers being able to increase both NE and DA in areas of termination.

Mechanistically, LC-DA may be preferentially released in situations of higher-frequency noradrenergic fiber stimulation. This would hinder the ability of the enzyme dopamine β-hydroxylase (DBH) to convert DA to NE inside of synaptic vesicles (Ahn and Klinman, 1989), as vesicles would be recycled and re-released too rapidly for DBH to be 100% effective. Alternatively, there may be a separate pool of vesicles that do not contain the DBH enzyme and therefore only release dopamine. Furthermore, the norepinephrine transporter (NET) has a higher affinity for DA than for NE (Devoto and Flore, 2006; Horn, 1973), thus permitting preferential transport of DA into presynaptic terminals for subsequent release, and possible reverse transport of cytosolic DA from LC terminals before it is carried into vesicles by VMAT2 and converted to NE.

Regardless of the mechanism, direct evidence for the release of DA from LC terminals has never been shown. Thus, given the disparity between DA released in DH

and the lack of dopaminergic innervation, as well as supporting evidence that the noradrenergic system readily releases DA in terminating cortical regions, we pursued the possibility that a significant amount of DA arises from noradrenergic afferents, and that this DA is sufficient to modulate synaptic plasticity in dorsal CA1 and long-term memory in awake, behaving mice.

Results

Optogenetic stimulation of LC terminals in CA1 increases extracellular dopamine

To probe whether locus coeruleus dopamine is a legitimate candidate for modulating DA-dependent synaptic processes in the dorsal hippocampus, we first needed to determine if DA is even released from LC terminals under physiological conditions. Answering this question required selective and precise control of locus coeruleus inputs to the dorsal hippocampus. This was achieved using genetically engineered mice designed to express the enzyme Cre recombinase under the promotor for tyrosine hydroxylase (TH), an enzyme expressed by all catecholamine-releasing neurons. These mice were then microinjected with an AAV2 virus containing DIO-hChR2-eYFP, a Cre-inducible channelrhodopsin 2 (hChR2) conjugated to enhanced yellow fluorescent protein (eYFP), into the LC (**Fig. 2.1A**). The outcome of this process is expression of ChR2 only in LC cells, and importantly for this study, not in VTA neurons. This expression can be visualized in **Figures 2.1C** through **H**. The images show strong TH expression in red and eYFP localization in green, measured at least 3 weeks after LC viral injection. Notice how in the bottom images, which depict LC axons

in dorsal CA1, every single TH-positive fiber also contains eYFP and is therefore sensitive to optogenetic stimulation and coming from LC neurons.

After at least three weeks, injected Cre(+) animals were sacrificed, and their brains were removed to make hippocampal slices for optogenetic experimentation (see methods). To gather chemical evidence that DA is released from these LC terminals, we illuminated dorsal CA1 slices with 470 nm light pulses (**Fig. 2.1B**) and collected extracellular fluid for subsequent DA measurement with HPLC and electrochemical detection (Opto group, see Chapter 2 methods for more detail). Light pulses were designed to mimic phasic firing activity of LC neurons; a condition under which we hypothesize DA is released (diagram for phasic optogenetic firing in **Fig 2.4B**). Slices from Cre(-) animals (Control group) also underwent the same optogenetic treatment.

Results from this experiment are quantified in **Figure 2.2**, and representative chromatogram elution-time plots are shown. Five minutes after phasic stimulation, we found a trend toward increased DA in the Cre(+) group (white bars) compared with the Cre(-) control group (black bars). Although these results are not statistically significant, other labs doing similar experiments have managed to gather statistically significant data to support LC-DA release. These experiments include: similar sample collection in hippocampal slices (Kempadoo et al., 2016), *in vivo* microdialysis with HPLC and electrochemical detection in the paraventricular thalamus (Beas et al., 2018), and *ex vivo* hippocampal tissue collection and detection in hippocampus after chemogenetic activation of LC (Zerbi et al., 2019). So, it seems likely that the LC is capable of physiological DA release not only in DH, but possibly throughout its entire projection.

Dopamine is both necessary and sufficient for potentiation of CA3-CA1 synapses

Before testing if LC-DA is sufficient to modulate DA-dependent synaptic potentiation in dorsal hippocampus, we needed an experimental readout that could reliably indicate the presence of dopamine signaling and approximate dopamine's role in plasticity. Therefore, we developed two separate long-term potentiation protocols to demonstrate that dopamine is both necessary and sufficient for the enhancement of synaptic strength in the DH. To do this, we recorded the slope of field potentials from stratum radiatum dendrites in hippocampal CA1 before and after weak or strong high-frequency electrical stimulation of CA3 axons (Schaffer collaterals) in acute slice preparations (**Fig. 2.1 B**). This way, we could either block strong LTP with dopamine D1R-like receptor antagonists to show necessity, or enhance a weaker LTP with D1R-like receptor agonists to show that DA is sufficient to enhance synaptic strength.

Data from these experiments can be seen in **Figure 2.3**. In panel **A**, baseline slope recordings were measured for 15 minutes prior to a strong 100Hz LTP stimulation (black arrow). In the no-drug control group (black circles) this 100Hz stimulation produced a large increase in LTP lasting at least 45 minutes. In the drug group, SCH23390, a dopamine D1R-like receptor antagonist, was applied to the bath during recording. This caused a significant reduction in LTP magnitude after 45 minutes, which means that DA receptor activation was necessary to achieve long-lasting potentiation in response to this particular strong high-frequency stimulation. In contrast, application of a D1-like receptor agonist, SKF81297 to the bath during the 15 minute baseline recording

was sufficient to convert a weak theta-burst LTP (wLTP, see **Fig. 3.1**) into a strong and seemingly longer-lasting LTP (**Fig. 2.3B**).

Bursting optogenetic stimulation of LC fibers in dorsal CA1 enhances the magnitude of a weak LTP stimulation in a dopamine-dependent manor

The next step was to figure out if the dopamine released from LC varicosities under phasic firing conditions was similarly sufficient to enhance a weaker form of LTP. Therefore, TH-Cre(+) mice were once again injected with AAV2-DIO-hChR2-eYFP and given 3 weeks to recover from surgery. Mice were then sacrificed and dorsal hippocampal slices were cut so that simultaneous field potential recordings and phasic optogenetic stimulation could be performed (**Fig. 2.1B**). LTP experiments began with a 15 minute baseline recording comparable to that mentioned above, followed by an extra 10 minute baseline during which optogenetic stimulation was given. This stimulation is represented as a blue bar under the baseline data points in **Figure 2.4A**, and the optogenetic protocol is shown in **Figure 2.4B**. Phasic optogenetic stimulation for 10 minutes before electrical wLTP stimulation (black arrow and **Fig. 2.4B,** bottom) was able to significantly augment wLTP (**Fig. 2.4A**, blue circles). Interestingly, the augmentation was blocked after putting SCH23390, a D1R-like antagonist, in the bath throughout the recording (**Fig. 2.5A**, red circles). One interpretation is that phasic LC firing is releasing enough DA to activate D1-like receptors and facilitate LTP maintenance.

A noticeable consequence of phasic optogenetic stimulation is an upward trend in baseline slope even before high-frequency LTP stimulation (**Fig. 2.4A**, blue circles, minutes 16-25). Therefore, an important control experiment in **Figure 2.4A** is a group

that received ONLY phasic optogenetic stimulation but not electrical wLTP stimulation. Field potential slopes from this group (open circles) increased passively initially, but leveled off at about 106% of baseline for the rest of the recording. This indicates that phasic optogenetic stimulation alone is not enough to account for the large increase in wLTP, and is somehow interacting with weak high-frequency glutamate activity to produce the observed LTP augmentation.

A second important control group is shown in **Figure 2.6A**. Here, in grey, the same optogenetic and wLTP stimulations were given, but this time in the presence of both SCH23390 and propranolol (a β-adrenergic receptor antagonist) to make sure that norepinephrine release was not also contributing to the LC-dependent wLTP augmentation. We observed no further block of LTP enhancement at 45 minutes post-stimulation with the addition of propranolol; however, there was a trend toward decreased early LTP compared to the SCH23390 only group (**Fig. 2.6A**, 26-30 minutes, comparison shown as hashtag). Moreover, when compared to the no-drug + wLTP + phasic optogenetic stimulation group (**Figs. 2.4A, 2.5A & 2.7A**, blue circles), post-tetanic LTP at 26-30 minutes was significantly lower in the SCH23390 + propranolol group (**Fig. 2.6A**, comparison shown as asterisk). These results hint at the LC possibly controlling post-tetanic LTP (e.g. rapid changes in synaptic connectivity and cellular excitability after high-frequency stimulation) using norepinephrine via β-AR activation, while simultaneously controlling late/long-lasting LTP (long-term changes in synaptic strength after high-frequency stimulation) with dopamine via D1-like receptors.

Slow, tonic, optogenetic LC stimulation does not enhance weak LTP

The LC can perform a range of different cognitive functions by transitioning through multiple states of firing activity. These states include a phasic bursting mode seen during active cognition (up to 16 Hz firing frequency within bursts), and a slower tonic firing mode seen during rest or tiredness (1-2Hz steady firing). Since phasic optogenetic LC stimulation was able to enhance wLTP, we wanted to test whether a tonic optogenetic protocol would have any effect on field potential strength. If DA is only released under burst firing conditions as we hypothesized above, then we should not see any effect of tonic optogenetic stimulation on LTP magnitude at 45 minutes after wLTP stimulation. This conjecture was confirmed in **Figure 2.7A** (green circles). A 2 Hz tonic optogenetic LC stimulation (in place of the phasic stimulation) was given to hippocampal slices prepared similarly to previously mentioned slices. Indeed, this 2Hz light stimulation had no effect on long-lasting LTP magnitude at 45 minutes (green circles, **X** represents non-significance. Intriguingly, it seems as though post-tetanic LTP is slightly enhanced in tonic optogenetic conditions (**Fig. 2.7A**, green circles, 26-30 minutes) which substantiates our prior findings that β-AR activation is necessary for post-tetanic LTP over 5 minutes post high-frequency stimulation.

Knocking down catecholamine production in LC neurons attenuates both dopamine- and norepinephrine-dependent influences on learning and memory

The next step in deciphering the LC's role in dopamine signaling was to check if blunting LC neurotransmission would impact DA-dependent memory at the behavioral level. Our strategy was to effectively block catecholamine synthesis in LC neurons by knocking down the enzyme tyrosine hydroxylase (TH). Normally, TH is the rate-limiting enzyme in the production of dopamine, and lowering its levels in LC will hinder the

production of both DA and NE. Thus, we injected a short hairpin RNA that targets TH mRNA into the LC of wild type mice (**Fig. 2.8A**). This TH-shRNA is stably expressed and is processed into a TH-siRNA that binds to TH mRNA, which leads to its degradation. An incubation and recovery period of three weeks after injections was given so that the shRNA had enough time to knock down TH enzyme expression in the LC. An example of TH protein knockdown in the LC is shown as a lack of red immunostaining in **Figure 2.8B** compared to a non-injected control (shown three weeks after shRNA injection).

After the three week recovery period, wild type and injected mice were trained in a “trace” fear conditioning paradigm to associate a specific tone with a foot shock in time (see methods for greater detail). This 2-day paradigm is thought to behaviorally isolate the dorsal hippocampus (Quinn et al., 2005), making it useful for comparison with our LTP data from dorsal hippocampal slices. Unlike the non-injected controls (black bars), TH-shRNA injected animals (white bars) showed deficits in acquisition learning of the tone/shock pairing on Day 1 (**Fig. 2.9A, right side**) and also the Day 2 long-term memory test (**Fig. 2.9B, right side**).

We then compared the above results to another set of experiments using the same behavioral test, but instead of a targeting the LC with a TH-shRNA viral injection, we performed IP injections of D1-like or β-adrenergic receptor antagonists into wild type mice. Interestingly, β-adrenergic receptor antagonist (propranolol, blue bars) administration, 30 minutes before training, significantly reduced acquisition of the tone/shock association over the last 3 tone/shock pairings (**Fig. 2.9A, left side**).

Acquisition was not blocked by SCH23390. Furthermore, D1-like receptor antagonist (SCH23390, red bars) administration, 30 minutes before training, strongly trended toward reduced long-term tone/shock association memory 24 hours after training (**Fig. 2.9B, left side**). Memory retention at 24 hours was not blocked by propranolol, indicating that β-adrenergic receptors are not involved in long term fear memory consolidation.

If one compares our findings from the IP pharmacology behavioral experiment (**Fig. 2.9, left side**) with those from the TH-shRNA injection experiment (**Fig. 2.9, right side**), a notable relationship is observed. It seems as though knocking down tyrosine hydroxylase has an effect on both acquisition and consolidation of emotional memories of painful associations. On the other hand, acquisition is only pharmacologically blocked by β-adrenergic receptor antagonists and not D1-like receptor antagonists. The reverse is true for memory consolidation, as SCH23390, but not propranolol, blocks fearful memory of the tone 24-hours after training. This implies that knocking down tyrosine hydroxylase in LC neurons depletes both DA and NE signaling in terminals regions, and indicates that LC-DA has a behaviorally relevant function.

It is possible that impairments in acquisition learning and long-term memory retention seen in the TH-shRNA injected mice were caused by reduced anxiety, which usually follows decreased LC-norepinephrine release (Llorca-Torralba et al., 2019; McCall et al., 2017). We therefore subject the mice to an elevated plus maze test of anxiety, where more time spent in the open arms of a plus-shaped maze means lower levels of anxiety (see methods for greater detail). In this experiment, propranolol

injected mice experienced significantly lower levels of anxiety (**Fig. 2.10, blue bar**) than vehicle injected controls. Reports of similar effects are shown in the above cited studies. Surprisingly, TH-shRNA injected mice did not exhibit a reduction in anxiety as measured by time spent in the open arm (**Fig. 2.10, cyan bar**). This result was unexpected, but there are at least two plausible explanations for this outcome. One is that, since neurons of the LC are so heterogeneous in their projections (Chandler et al., 2014), it is possible that a few of the injected mice did not have TH knocked down in cells projecting to the amygdala, which is the main area where the LC mediates emotional learning (Uematsu et al., 2017). Second, it is possible that the intraperitoneal injection of propranolol produced a stronger anxiolytic effect by not only acting centrally, but also peripherally in the sympathetic nervous system (for example by lowering blood pressure and heart rate) (Pohjavaara et al., 2003; Steenen et al., 2016). Tyrosine hydroxylase knockdown specifically in LC would have had minimal peripheral impact, leading to a weaker suppression of anxiety.

Discussion

A few main points arise from the findings in this chapter. The first is that dopamine seems to be released from LC terminals in dorsal CA1 (**Fig. 2.2**). We are not the only lab to demonstrate this, and several other groups have reported a significant amount of DA in the extracellular space after selective activation of LC terminals (Beas et al., 2018; Devoto et al., 2005a; Kempadoo et al., 2016; Zerbi et al., 2019). Second, this dopamine is sufficient to enhance a weak form of LTP, and is likely only released during a phasic LC firing (compare **Figs. 2.4A** and **2.7A**). And third, LC-DA is important

for the consolidation of aversive/emotional memories in live animals. These points will all be expanded upon below.

The earliest researchers to propose that the LC could co-release dopamine were Scatton, Dennis, and Curet (Scatton et al., 1984). However, their data to support this observation was collected using very non-physiological and non-selective methods. For example, they stimulated LC cell bodies at 50 Hz for 10 minutes with an electrode implanted into the LC of live mice. This technique has at least 2 issues. The first is that LC neurons are known to fire in bursts of 2-6 action potentials at a max of 20 Hz, and these bursts only occur, at most, at a rate of once per second (Waterhouse and Navarra, 2019). Secondly, the VTA sends divergent projections to the LC (El Mansari et al., 2010), and electrically stimulating the whole area of the nucleus may initiate antidromic action potentials in VTA neurons and cause unwanted DA release in other regions that the VTA projects to. Our targeted, phasic optogenetic stimulation is a much better paradigm than that used in the above study.

Similarly, Devoto et al. (2005b) found evidence for DA and NE co-release, but only looked in brain regions that simultaneously receive axonal projections from the ventral tegmental area (VTA), the canonical DA-releasing nucleus. The dorsal hippocampus is a much better system for approaching this problem, as VTA fibers are rarely observed here. The few that are present in CA1 are located in the stratum oriens layer, which is anatomically distinct from the stratum radiatum, the subregion where our recordings took place. Here, we can be sure that we are selectively stimulating LC fibers with our targeted viral + optogenetic strategy.

Using a sensitive chemical detection method, we were able to detect a strong trend toward increased extracellular DA in dorsal CA1 after phasic optogenetic LC stimulation. Consequently, the same phasic LC-stimulation pattern was also able to convert a weak LTP into a stronger one in a DA-dependent manor. Since LTP is thought to be a molecular correlate of memory, this result hints at the ability of the LC to modulate memory consolidation using its own DA. In contrast, giving 2Hz tonic optogenetic stimulation (while keeping all other parameters the same) did not produce any enhancement of LTP (**Fig. 2.7**). From these observations, we concluded that DA excretion from LC neurons is only achieved during higher frequency bursts of LC firing. During periods of tonic activity, it appears as though only NE is released.

These interpretations fit well with current theories regarding LC function. Phasic LC firing is commonly observed when an animal is actively engaged in a task, paying close attention to a salient stimulus, or exploring a novel environment (Vankov et al., 1995). When an animal is at rest but still awake, only slow, tonic LC activity can be detected (Sara, 2009). If DA is exclusively released during phasic firing that occurs during high arousal states, this may allow the LC to select which stimuli are committed to memory based on what an animal decides is most important. This is especially true for the long-term memory consolidation of novel experiences, which are known to recruit DA signaling in dorsal hippocampus and potentiate CA3-CA1 synapses (Clos et al., 2019; Duszkiewicz et al., 2019; Hansen and Manahan-Vaughan, 2014; Ihalainen et al., 1999; Lemon and Manahan-Vaughan, 2006; Moncada and Viola, 2007). Accordingly, LC neurons markedly raise their firing rates, and fire a greater proportion of action

potentials in bursts, when exposed to a novel stimulus (Takeuchi et al., 2016). This promotes long-term memory consolidation *in vivo* that can be blocked by the injection of D1-like receptor antagonists into dorsal CA1, but not β-adrenergic receptor antagonists.

We were able to emulate this phenomenon using slice physiology (**Fig. 2.3A**), but we did not run a phasic optogenetic group using ONLY β-adrenergic receptor antagonists to show that norepinephrine signaling is not necessary for LTP enhancement. I regard this as a considerable drawback to our methods, as multiple groups have reported that norepinephrine can promote certain types of CA1 LTP (Hu et al., 2007; Katsuki et al., 1997; Liu et al., 2017; Maity et al., 2015). Another weakness was the fact that we did not attempt to show chemical evidence (HPLC with electrochemical detection) for a lack of more DA release with tonic optogenetic stimulation. Moreover, the reason that we did not publish the behavioral data from this chapter is because we did not have a proper control group for the LC injections. Instead of comparing the LC-TH-shRNA injected animals to wild type mice that did not receive intracranial injections, we should have injected another group of mice with an AAV virus containing a scrambled oligonucleotide sequence into the LC. Alternatively, we could have also could have used the selective LC neurotoxin, DSP4, versus a sham lesion to more reliably eliminate all LC terminals. This approach would have likely reduced variability and taken less time and resources (Ross and Stenfors, 2015).

Overall, our results point to the locus coeruleus releasing enough DA to modulate plasticity and learning/memory in CA1. It is tempting to argue that LC-DA accounts for the majority of dopamine signaling in this region, since DA efflux from VTA terminals (by

amphetamine application) does not seem capable of driving synaptic potentiation (Smith and Greene, 2012). Although, this interpretation is contradictory to many previous results revealing VTA-mediated dopaminergic modulation of plasticity in the same brain area (Broussard et al., 2016; Lisman and Grace, 2005; McNamara et al., 2014; Rosen et al., 2015). One explanation for this discord is that the VTA and LC represent two different systems of DA, with each overseeing distinct classes of memory (Duszkiewicz et al., 2019; Hauser et al., 2019; McNamara and Dupret, 2017).

In either case, our data has helped establish that the LC is capable of independently controlling CA1 DA signaling. However, one possibility that our data have not ruled out is the fact that the excess NE may be activating DA receptors. The chemical structures of NE and DA only differ by one hydroxyl group, and several papers have revealed that NE can act as an agonist at D2-like receptors (Lei, 2014; Root et al., 2015; Sanchez-Soto et al., 2016). However, Smith and Greene (2012) did not find an effect of NE on DA receptors in dorsal hippocampus when all adrenergic receptors were blocked. In the context of our findings, D1-like receptors have a much lower affinity for catecholamines than D2, and therefore DA receptor activation by NE seems unlikely. Results from several experiments in the next chapter will also help to support this assertion. **Chapter 3** will take the conclusions from the above data and attempt to discover a mechanism for LC-DA release, leading us to a theory for the biological rationale for DA and NE co-release.

Materials and Methods

Animal Approval

All animal procedures performed were approved by the animal care and use committee (IACUC) at the University of Texas Southwestern Medical Center and comply with federal regulations set forth by the National Institutes of Health.

Tyrosine hydroxylase-Cre mice were purchased from The Jackson Laboratory (Bar Harbor, ME) (B6.Cg-Tg (TH-Cre)1Tmd/J; #008601)

Stereotactic injection of AAV2

Transgenic mice (aged 6-8 weeks) expressing Cre recombinase under the tyrosine hydroxylase promotor (TH; TH:Cre) (Lindeberg et al., 2004) on a mixed C57BL/6 and CD1 background were anesthetized with 1.5-2% isoflurane. A borosilicate glass electrode (10-15 MΩ) was pulled using a horizontal pipette puller (Sutter P-97) and filled with AAV2-EF1a-DIO-hChR2(H134R)-eYFP (UNC Vector Core Services). Injections were performed bilaterally by positioning the glass electrode in the LC (stereotactic coordinates; A/P, −5.45; M/L, ± 0.9; D/V, −3.0). Viral delivery was controlled by a picospritzer (Parker) under the guidance of a master-8 pulse stimulator (AMPI) and was set so that each delivery (~1 µl) occurred over a 10-15 min period. After each viral injection, the pipette was kept in place for 5 minutes to ensure proper diffusion of the virus. Animals were allowed to recover on a heating pad till normal behavior resumed. During the injection, 0.1 mg/kg buprenorphine was given for pain management. Animals were allowed to recover for at least 21 days prior to experimentation.

For tyrosine hydroxylase knock-down experiments, a similar procedure to the one described above was employed except that instead of AAV2-EF1a-DIO-hChR2(H134R)-eYFP injection into the LC, an AAV2 containing multiple short hairpin RNAs against tyrosine hydroxylase mRNA (TH-shRNA) was injected. Details of the hairpins can be found in Hommel et al. (2003), and were a generous gift from the lab of Dr. Ralph DiLeone.

Ex vivo slice preparation

Coronal slices (300 µm thick) were cut from injected transgenic mice or non-injected wild type control littermates (9-12 weeks) in low light conditions to prevent unwanted CHR2 activation or photooxidation of catecholamines. Animals were anesthetized under 1.5-2% isoflurane and brains were removed and blocked following rapid decapitation. Hippocampal slices were prepared using a Leica VT 1000S vibratome in ice cold NMDG ringer solution (in mM): 5 NaCl, 57 NMDG (N-Methyl-d-Glucosamine), 37.5 Na-Pyruvate, 12.5 Na-Lactate, 5 Na-Ascorbate, 2.5 KCl, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 25 Glucose, 10 $MgSO_4.7H_20$, 0.5 $CaCl_2.2H_20$, the pH was set between 7.3 and 7.4 using 12N HCl, the osmolarity was adjusted as needed to 310-315 mOsm using glucose and the solution was bubbled with 95% O_2 and 5% CO_2 gas. Slices were maintained in NMDG ringer at room temperature for no longer than 20 minutes and were transferred to artificial cerebral spinal fluid (aCSF; in mM): 125 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 1 $MgCl_2$, 2 $CaCl_2$, 25 $NaHCO_3$, and 25 dextrose continuously bubbled with 95% O_2 and 5% CO_2 gas, where they were kept up to 6 hours for experimentation while protected from light.

Field recordings

Slices were transferred to a submersion recording chamber and perfused with aCSF at a rate of 2 ml/min at 29-31ºC. Field recordings from the stratum radiatum of dorsal CA1 were acquired using a borosilicate glass electrode (~2 MΩ) filled with normal aCSF. A bipolar stimulating electrode (FHC) was also placed in the stratum radiatum of CA1 within 200-300µm of the recording electrode and stimulation (one stimulus every 30 seconds) was set to elicit a field EPSP slope that was ~50% of the maximum value. A stable 15 minute baseline was obtained, followed by 10 more minutes of baseline stimulation with or without simultaneous optogenetic stimulation. After the 25 minute total baseline, a weak theta-burst tetanus was applied consisting of 4 bursts (given at 10 Hz), with each burst containing 3 spikes at 50 Hz (12 total spikes). Baseline stimulation then resumed as described above for 45 minutes. Data was acquired using P-Clamp 10 (Molecular Devices).

For **Figure 2.3A**, a stronger electrical stimulation was applied which consisted of a 1-second long, 100 Hz train of Schaffer collateral electrical stimulation.

Optogenetic stimulation

For field recordings, optogenetic stimulation was applied simultaneously during the last 10 minutes of Schaffer collateral baseline stimulation as described above. This is represented as a blue line in all corresponding figures. Specifically, for the burst firing Opto protocol, every 30 seconds for 10 minutes an optogenetic stimulus was applied through the 10x objective (directly before the Schaffer collateral stimulus). Every 30 seconds saw four 200 ms bursts of 4 light pulses (10 ms light pulse duration, 16 Hz

intra-burst frequency), with each burst separated by 800 ms (16 total pulses every 30 seconds, 320 stimuli total). This same protocol was applied to the slices during sample collection for HPLC experiments as well. For the slow-tonic Opto protocol, light stimulation was given continuously for 10 minutes at 2 Hz, leading to a total of 1200 stimuli. Relevant data can be found in **Figures 2.1**-**2.2** and **Figures 2.4**-**2.7**.

Drugs

Where indicated, the following drugs were used: SKF-81297 (SKF; D1R agonist; 10 µM), prazosin (α adrenergic antagonist; 30 µM), propranolol (β adrenergic inhibitor; 30 µM), and SCH23390 (D1R antagonist; 1 µM).

In vitro HPLC

Hippocampal slices were mounted on glass slides, submerged in 20 µL of aCSF and held for 10 minutes to acquire a baseline for dopamine (DA) release. Effluent was removed, flash-frozen on dry ice and replaced. Slices were immediately exposed to the optogenetic light stimulation described above and held for an additional 5 minutes before effluent was removed and flash frozen on dry ice. Effluent was replaced a final time and held for an additional 10 minutes without optogenetic stimulation before being removed and flash frozen. All samples were stored at -80ºC until HPLC analysis. The fluid extracted from slices was mixed with an equal volume of 0.1 M perchloric acid containing 0.2 mM sodium metabisulfite. Twenty microliters were injected into a C18 HPLC column (ESA MD-150, 3 x 150 mm) on a Thermo Dionex HPLC system and separated by isocratic elution at a flow rate of 0.6 ml/min with MD-TM mobile phase (ESA). Neurotransmitter monoamines and metabolites were detected using an ESA

Coulochem III electrochemical detector with a model 5014B cell set to a potential of +220 mV. Peak areas were compared to a standard curve of external standards to calculate quantities of monoamines and metabolites.

Immunohistochemistry and confocal microscopy

The caudal block containing the LC that was removed prior to slice preparation of hippocampal slices (including slices used for electrophysiology experiments) was stored in 4% PFA in 1X PBS overnight, was transferred to a 30% sucrose + 1X PBS solution for cryoprotection, and was balanced and cut at 30 µm on a cryostat. Free floating sections were washed 3x with 1X PBS and then were treated with a H_2O_2 solution (PBS + 10% methanol + 1.05% H_2O_2) for at least 1 h. Sections were washed, blocked for 2 h in 10% normal donkey serum + 1X PBS + 1% Triton X-100 (blocking solution) and then were treated overnight at 4ºC with primary antibody diluted in blocking solution (rabbit anti-tyrosine hydroxylase (TH), 1:200, Millipore; chicken anti-GFP, 1:5000, Aves Laboratories). Slices were washed and incubated for 2h at RT covered with secondary antibody diluted in blocking solution (donkey anti-rabbit Alexa Fluor 594, 1:500, goat anti-chicken Alexa Fluor 488, 1:200, Invitrogen). Sections were washed, mounted on gelatin covered slides and coverslipped using PermaFluor (Thermo Scientific). A negative control was collected to account for background staining from the secondary antibody.

Hippocampal slices were prepared from slices used in electrophysiology experiments. After an Opto-LTP experiment slices were gently transferred to a well

containing 4% PFA, and were then processed as described above to confirm ChR2 expression. Hippocampal images were acquired in the z-axis at 20X using a Zeiss LSM 510 Meta confocal laser scanning microscope in steps equaling 2 µm each at a resolution of 1024 x 1024. LC images presented in the paper were acquired at 10X using an epifluorescent scope.

Trace fear conditioning

Procedural diagram found in Figure 2.8. Wild type or TH-shRNA injected mice (described above), age 10-12 weeks, were subject to the following 3-day experimental regimen: Acclimation to context and injection on Day 0, trace conditioning training on Day 1, and trace memory testing on Day 2. More specifically, on Day 0, mice were removed from their home cages and put into the context that the fear conditioning training would take place in (Context A). Each mouse was allowed 5 minutes to acclimate to the novel context. Then, the mice were returned to their home cages until the same time the next day. Importantly, mice that we planned to inject with drug on Day 1 also received an acclimating intraperitoneal 0.9% saline injection on Day 0 to prevent excess stress from the injection process on the first training day that might interfere with catecholamine signaling.

Twenty-four hours later on Day 1, mice in all drug groups (not the mice in the TH-shRNA groups) were either injected with vehicle or drug 30 minutes prior (amounts can be found in **Fig. 2.9**) to being put back into the same context that they were acclimated to the day before (Context A). Mice were then subject to a delayed tone/shock pairing protocol, presented a total of 6 times during the trial. An important feature of this

tone/shock protocol is that, unlike normal contextual fear conditioning (**Fig. 3.6**), the trace paradigm gives an 18 second delay between the presentation of a tone and a 40 milliamp shock. This delay, or “trace”, is thought to behaviorally isolate the dorsal hippocampus (Quinn et al., 2005), differing from normal fear conditioning which is thought to me more of a brain-wide phenomenon. During the 6 tone/shock pairings, behavioral freezing data was measured within a 1 minute epoch after each tone presentation. Data is shown as a percentage equal to the time a mouse spends “frozen” in fear divided by the total epoch time (1 min). A learning curve can then be generated and the average of freezing behavior over the last 3 tone/shock pairings was averaged and presented as the acquisition of the tone/shock association seen in **Figure 2.9A**. All data were generated automatically using the EthoVision program that tracks an animal’s movement over time and quantifies freezing behavior as a percent of total measurement time in a given epoch (Noldus, Leesburg, VA).

Finally, on Day 2 (24 hours later), the same mice were put into a different context (Context B) and the same tone was played again to test for long-term trace memory retention. Freezing behavior was then analyzed as the total percent freezing after the first tone presentation in the new context. This data is displayed in **Figure 2.9B**.

Elevated Plus Maze

Twenty-four hours after trace fear conditioning experiments, mice were tested on an elevated plus maze to assess their anxiety-like behavior. Specifically, the same mice were placed in an elevated plus maze under constant video monitoring using the EthoVision program to track an animal’s movement over time (Noldus, Leesburg, VA).

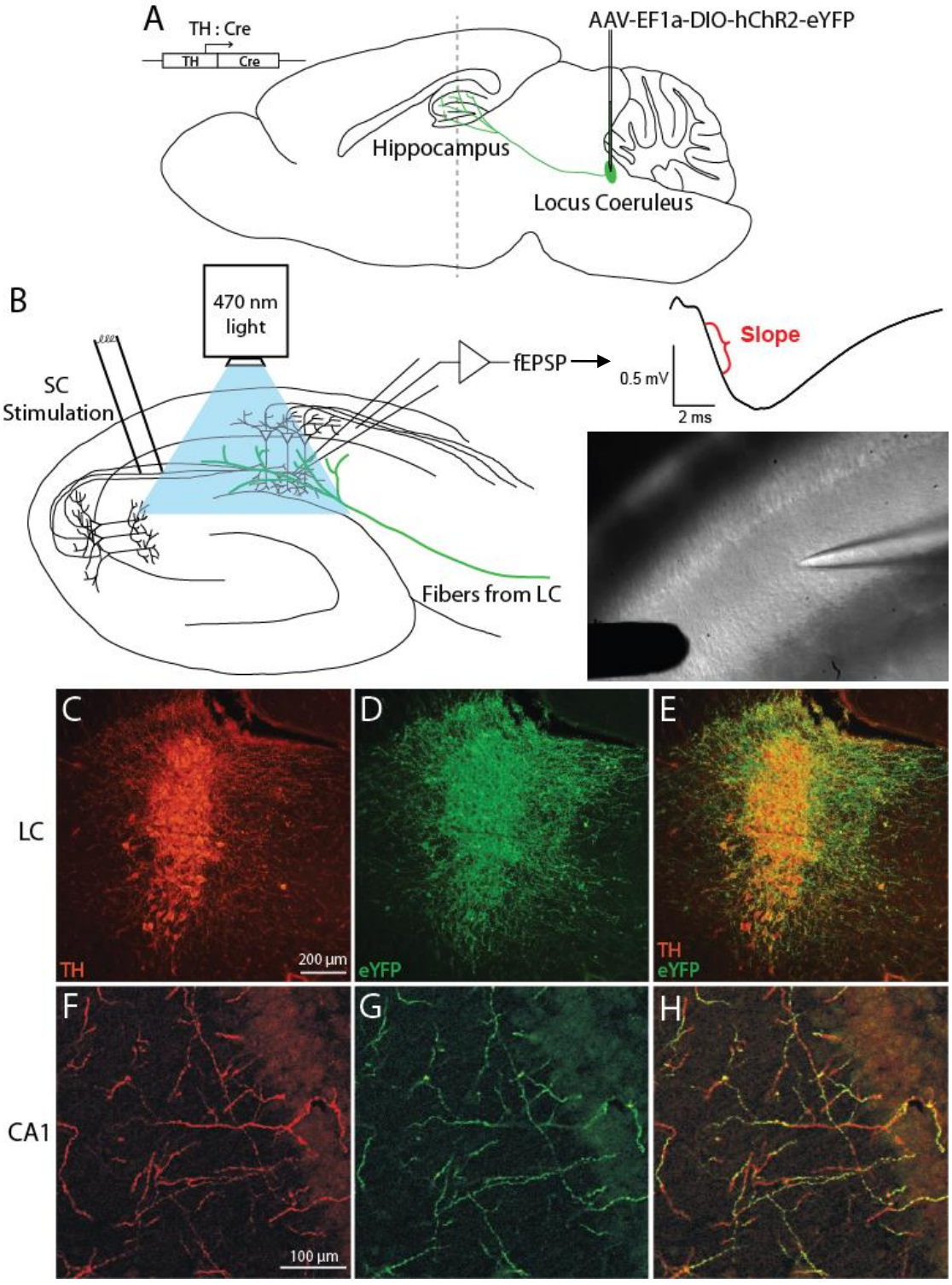
The maze consisted of four quadrants: two “closed” arms, and two “open” arms, that were arranged alternating in the shape of a plus (+). To begin the trial, each mouse was placed in the closed arm of the maze. Each mouse was given 5 minutes to explore the maze, and time spent in each quadrant was quantified using the EthoVision system. The most relevant data was time spent in the open arm, which is quantified in **Figure 2.10** for each group. The SCH 23390 group was omitted from the final analysis because blockade of D1 receptors caused them to spend nearly 100% of the time in the same arm they started in. This was likely due to attenuation of motor initiation by disrupting striatal function.

Statistical analysis

All data from electrophysiological and behavioral experiments are represented as the mean ± SEM. Electrophysiology data points are presented as occurring every minute, but are an average of every two slope recordings taken every 30 seconds. Field recordings were analyzed using a two-way repeated-measures ANOVA with time as an independent variable over a range of times, as noted in the corresponding figure legends. Behavioral tests were analyzed using a one-way ANOVA, while HPLC data was analyzed using a normal two-way repeated-measures ANOVA. Sidak’s or Dunnett’s post-hoc tests were used for multiple comparisons when necessary and are indicated in figure legends. All analyses were performed using GraphPad Prism 7 software (San Diego, CA).

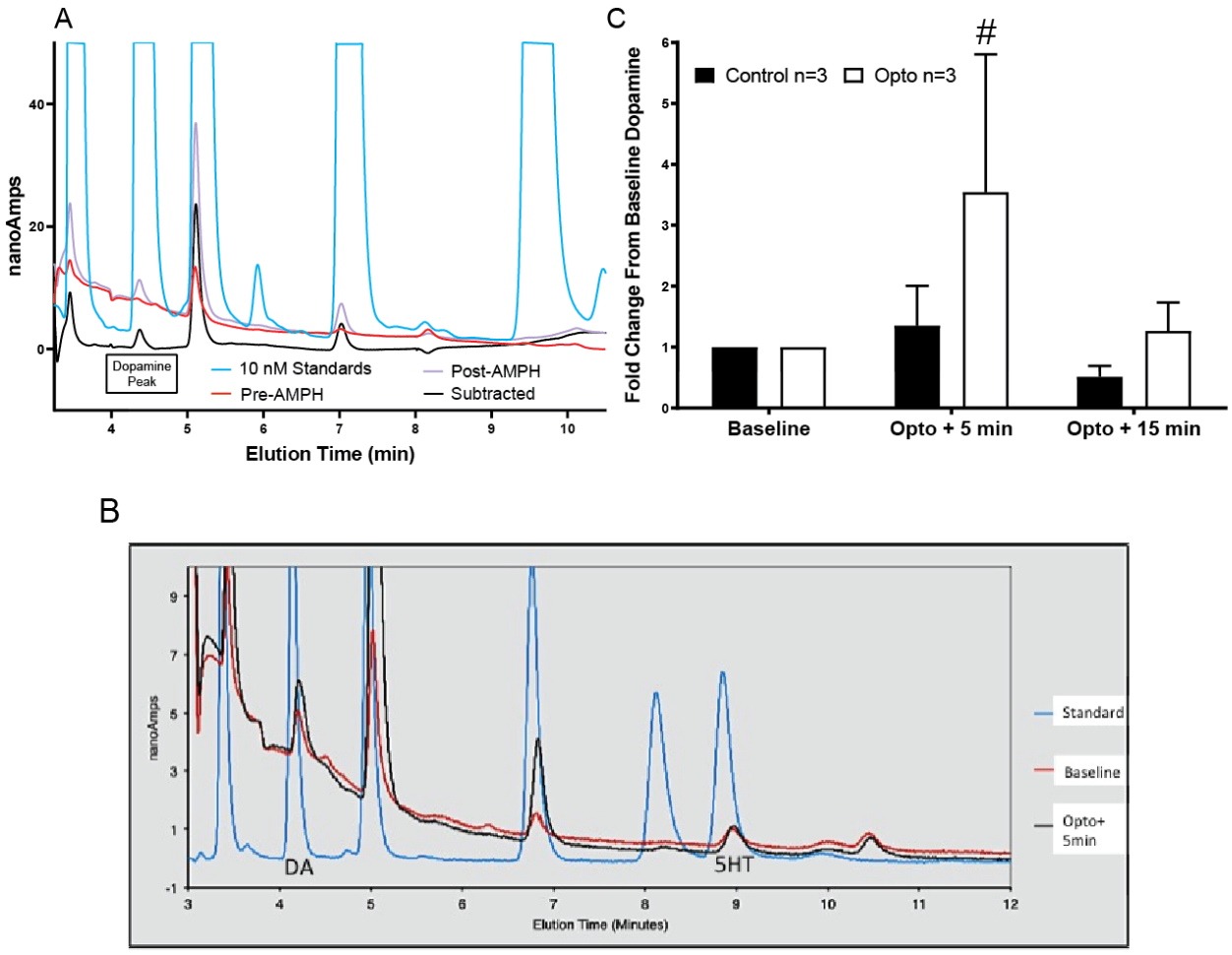
Figures

Figure 2.1 *Diagram: preparation of hippocampal slices and optogenetic stimulation of LC terminal fibers in CA1.*



A, Top, Schematic of AAV2-DIO-hChR2-eYFP viral injections into the LC of TH:Cre mice. Grey dotted line represents the approximate hippocampal slice plane. **B,** Left: schematic of hippocampal recordings. Schaffer collateral (SC) fibers were stimulated with a bipolar electrode while field EPSPs were recorded from the CA1 region. Optogenetic activation of LC fibers in CA1 was performed with a 470 nm LED applied through the 10x objective. Right (top): Example of electrical field excitatory postsynaptic potential recording with scale bars for time and voltage. All future data points will be shown as the slope of the voltage response shown here in red. Right (bottom): example picture of dorsal CA1 with electrodes in place taken during a recording. Stimulus electrode is on left and recording is on the right. **C,D,E,** Immunohistochemical staining of LC injection sites for tyrosine hydroxylase (TH, red), ChR2-eYFP (green), and a merged image (yellow) showing co-localization of TH and ChR2-eYFP staining in LC neurons. **F,G,H,** Immunohistochemical staining in hippocampal CA1 region for TH, ChR2-eYFP, and a merged image show co-localization of TH and ChR2-eYFP on LC terminals in CA1.

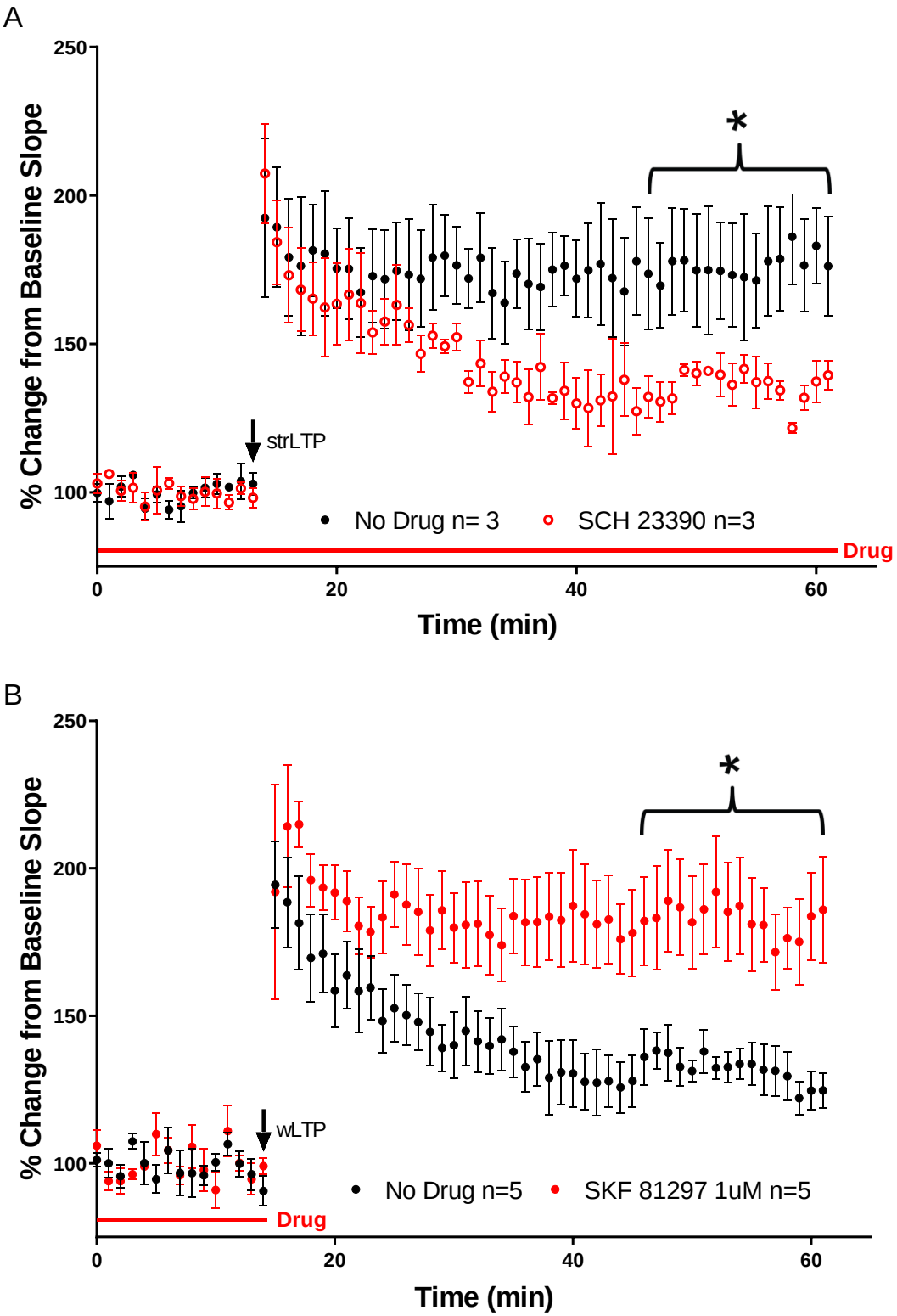
Figure 2.2 *Chemical measurement of dopamine from dorsal hippocampal slices after optogenetic LC stimulation.*



A, Example HPLC traces from an *ex vivo* slice superfusate, before and after exposure to AMPH (10uM for 10 minutes) and compared to a standard solution (blue line). The dopamine peak appears between four and five minutes. **B,** Example HPLC traces of superfusate from an *ex vivo* hippocampal slice, (expressing ChR2; Baseline, red line) and 5 minutes post optogenetic activation (Opto+5min, black line), together with trace

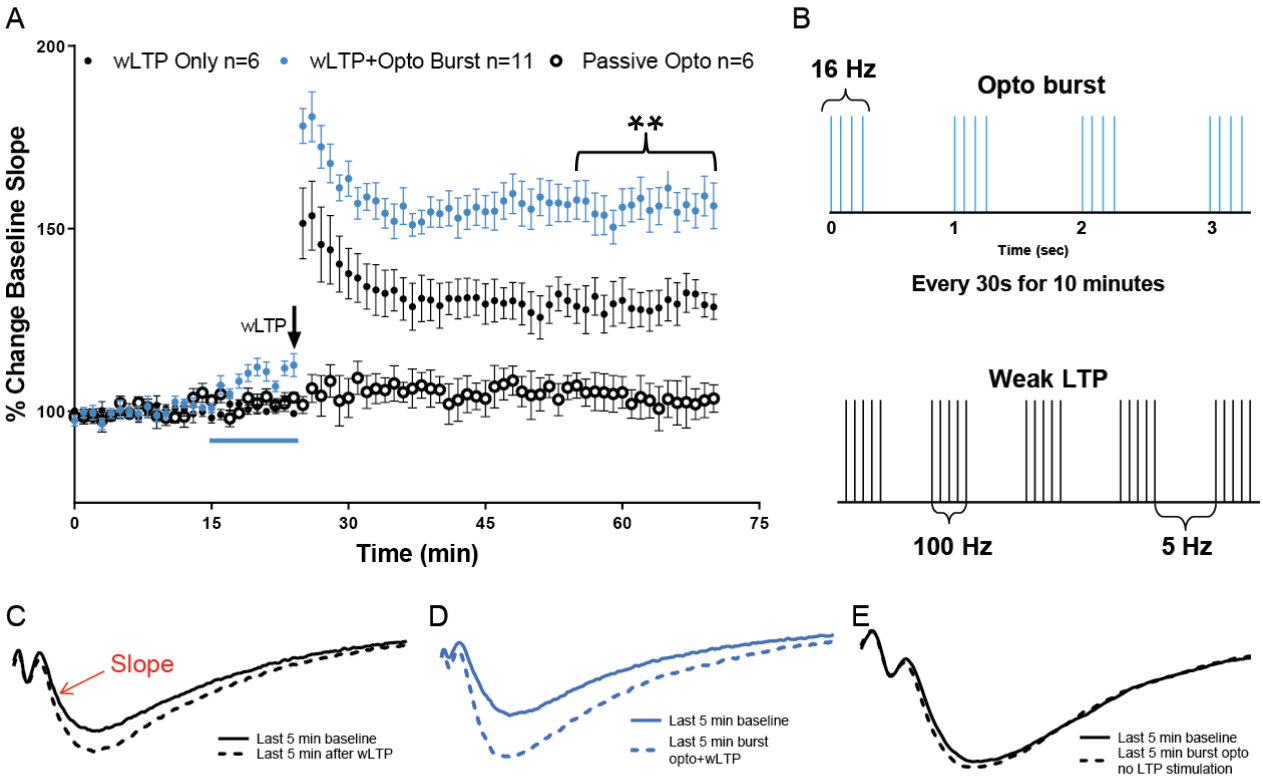
showing the elution time of standards (Standard, blue line). Slices were taken from a TH:Cre mouse, previously injected with AAV2-ChR2 into the LC bilaterally as described in the methods. **C,** Analysis of pooled HPLC data at three different time points for both groups. The control group (black bars) consisted of TH:Cre(-) mice and the Opto group (white bars) expressed ChR2. Both groups contain the averages of subtracted values after receiving the same phasic optogenetic stimulation. Baseline dopamine measurements were obtained prior to optogenetic activation of LC terminals for each animal. Then, phasic optogenetic stimulation (**Fig. 2.4B**) was applied and another sample was taken after a 5 minute period (Opto + 5 min). This was followed by another sample collection ten minutes after optogenetic stimulation (Opto + 15 min). Hashtag represents an adjusted p-value of 0.1113 after a two-way repeated-measures ANOVA with a Sidak's post-hoc multiple comparisons test.

Figure 2.3 *Dopamine D1/5R activation is necessary to maintain strong LTP and sufficient to enhance weak LTP in dorsal CA1.*



A, A strong, high-frequency, electrical stimulation was applied to Shaffer collateral fibers (a 1-second-long stimulation at 100 Hz, black arrow) in dorsal CA1 to induce a strong LTP (black circles) of synaptic responses at CA1 dendrites. This strong LTP was attenuated by the application of SCH 23390, a D1/D5 receptor antagonist, to the bath starting 15 minutes prior to LTP stimulation and lasting the duration of the recording (red bar/circles, $F(1, 4) = 9.439$, $p=0.0372$). **B,** A weak, high-frequency, theta-burst electrical stimulation was applied to Shaffer collateral fibers (see methods, black arrow) in dorsal CA1 to induce a weak LTP (black circles) of synaptic responses at CA1 dendrites. This wLTP was converted into a strong LTP by the application of SKF 81297, a D1/D5 receptor agonist, to the bath for 15 minutes prior to LTP stimulation (red bar/circles, $F(1, 8) = 10.47$, $p=0.0120$). All data points represent the field potential slope (as shown in **Fig. 2.1B**) mean ± SEM. Asterisks represent p-values < 0.05 measured by a two-way ANOVA using time as an independent variable over the last 15 minutes of recording.

Figure 2.4 *Optogenetic stimulation of LC terminals in CA1 is sufficient to strengthen weak LTP but does not produce an effect in the absence of high-frequency stimulation.*

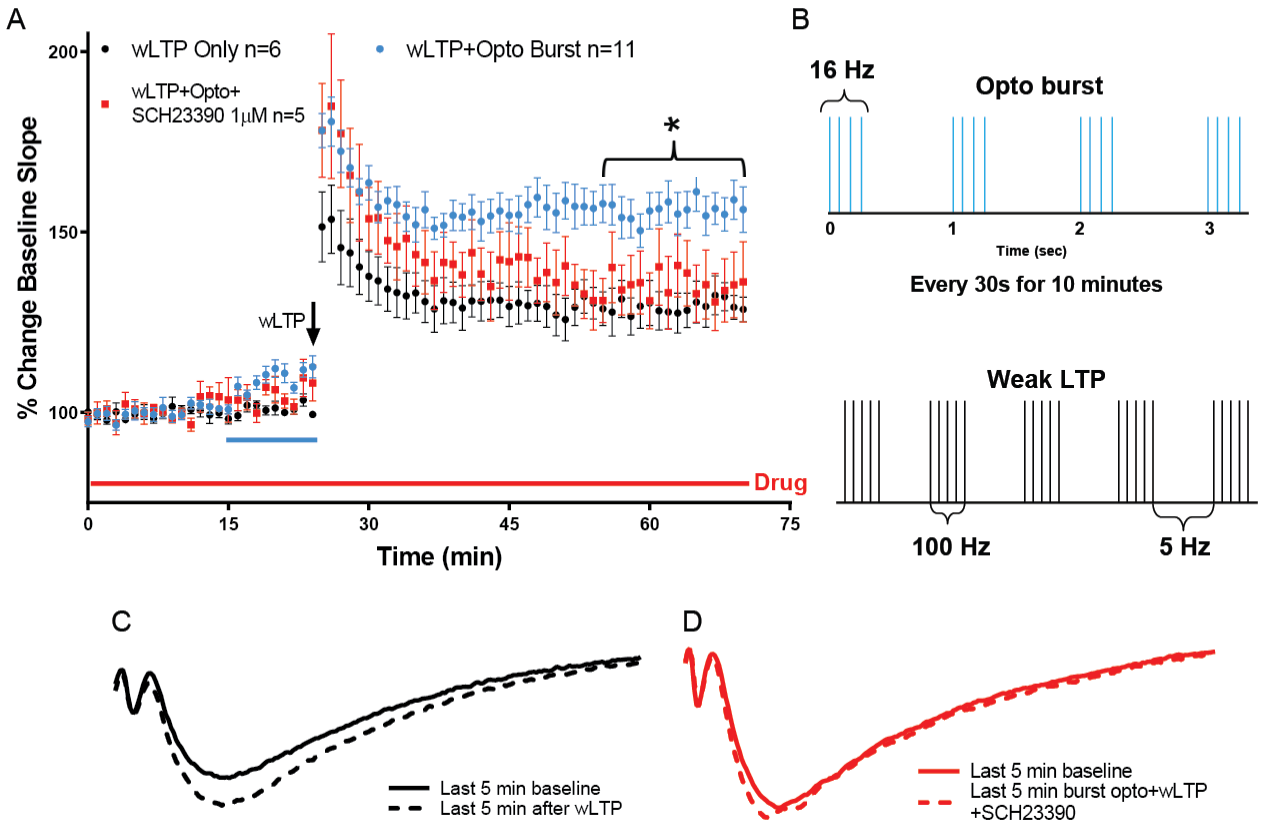


A, A weak, high-frequency, theta-burst electrical stimulation was applied to Shaffer collateral fibers (see methods, black arrow) in dorsal CA1 to induce a weak LTP (black circles) of synaptic responses at CA1 dendrites. This wLTP was converted into a strong LTP by the application of a bursting optogenetic protocol (shown in **B**) for 10 minutes prior to LTP stimulation (blue bar/circles, $F(1, 15) = 12.28$, $p=0.0032$). Opto stimulation alone, however, produced only a slight increase in baseline slope measurements, but did not induce a strong LTP when applied without high-frequency stimulation (open circles). **B,** Schematics of opto bursting and weak LTP theta-burst protocols (see

methods for more detail). **C,D,E,** Example averaged traces from the last 5 minutes of baseline before optogenetic or LTP stimulation (solid lines), and the last 5 minutes after wLTP stimulation (dashed lines).

All data points represent the field potential slope (as shown in **Fig. 2.1B**) mean ± SEM. Double asterisk represents p-value < 0.01 measured by a two-way ANOVA using time as an independent variable over the last 15 minutes of recording.

Figure 2.5 *Blocking dopamine D1-like receptors reduces the enhancement of LTP by optogenetic burst stimulation.*

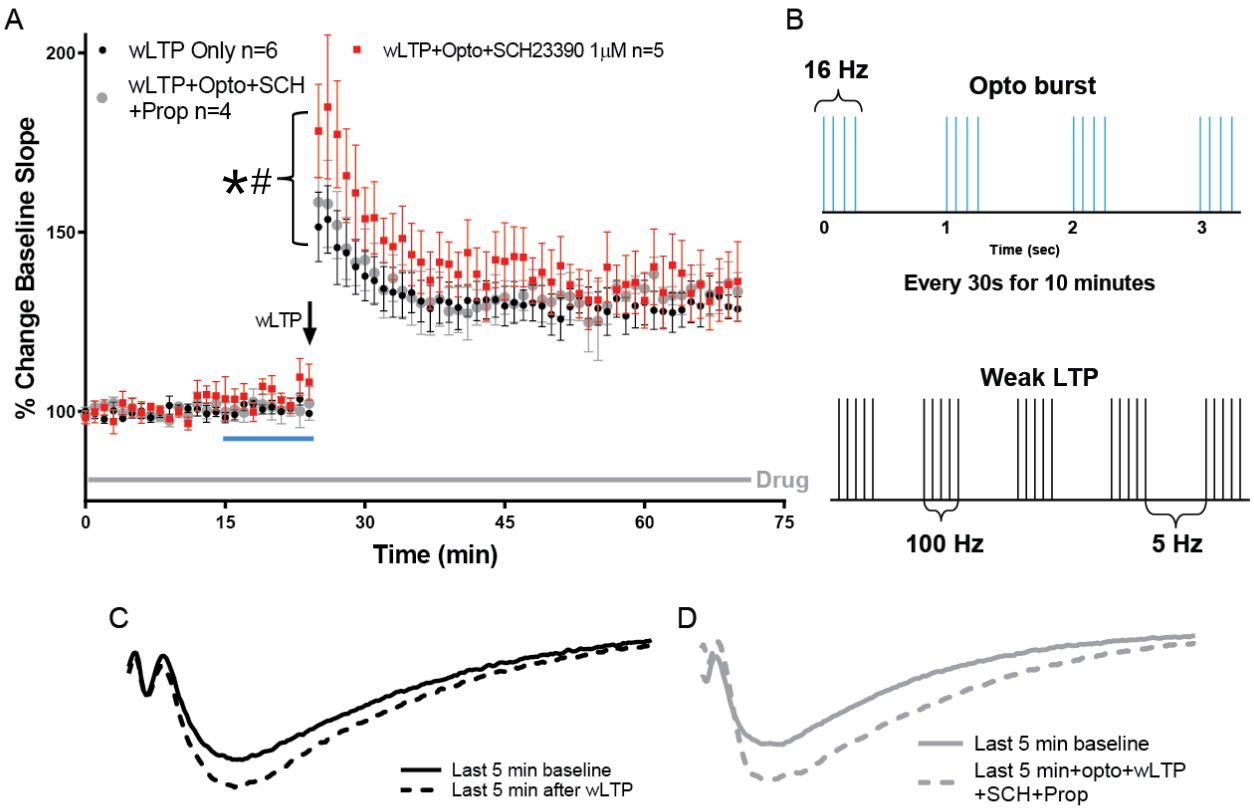


A, A weak, high-frequency, theta-burst electrical stimulation was applied to Shaffer collateral fibers (see methods, black arrow) in dorsal CA1 to induce a weak LTP (black circles) of synaptic responses at CA1 dendrites. This wLTP was converted into a strong LTP by the application of a bursting optogenetic protocol (shown in **B**) for 10 minutes prior to LTP stimulation (blue bar/circles). Opto-dependent LTP conversion was attenuated by the application of SCH23390, a D1/D5 receptor antagonist, to the bath starting 15 minutes prior to LTP stimulation and lasting the duration of the recording (red bar/circles, $F(1, 14) = 5.008$, $p=0.0420$). **B,** Schematics of Opto bursting and weak LTP

theta-burst protocols (see methods for more detail). **C,D,** Example averaged traces from the last 5 minutes of baseline before optogenetic or LTP stimulation (solid lines), and the last 5 minutes after wLTP stimulation (dashed lines).

All data points represent the field potential slope (as shown in **Fig. 2.1B**) mean ± SEM. Asterisk represents p-values < 0.05 measured by a two-way ANOVA using time as an independent variable over the last 15 minutes of recording.

Figure 2.6 *β-Adrenergic receptors may be involved in post-tetanic potentiation but have no effect on late LTP.*

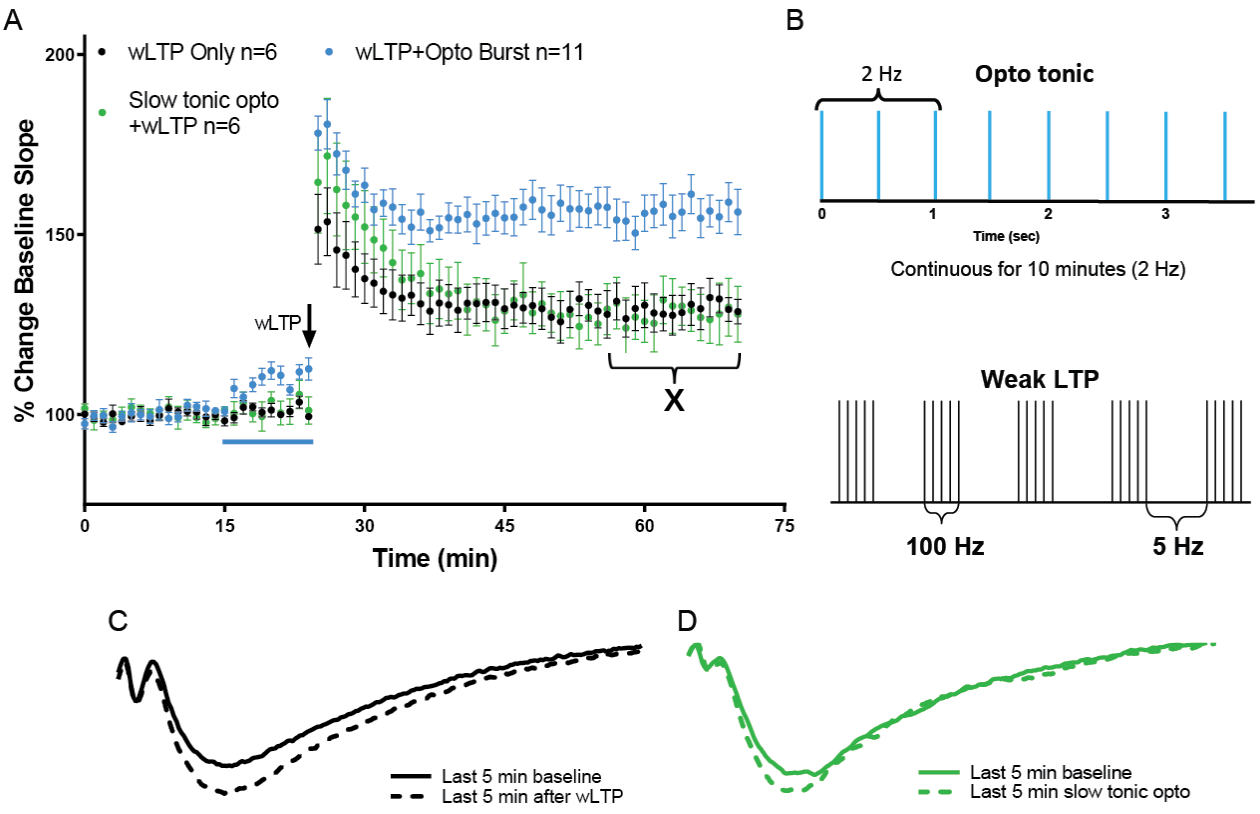


A, A weak, high-frequency, theta-burst electrical stimulation was applied to Shaffer collateral fibers (see methods, black arrow) in dorsal CA1 to induce a weak LTP (black circles) of synaptic responses at CA1 dendrites. Opto bursting LTP conversion (**Fig. 2.4A**) was attenuated by the application of SCH23390, a D1/D5 receptor antagonist, to the bath starting 15 minutes prior to LTP stimulation and lasting the duration of the recording (red circles). However, D1/D5 receptor blockade only seemed to affect the later part of LTP (50-70 minutes), and not the earlier part (26-30 minutes). The first 5

minutes of post-tetanic LTP trended toward attenuation by the addition of propranolol (a β-adrenergic receptor antagonist) in the bath with SCH23390 (grey bar/circles), but this was not significant ($F(1, 7) = 2.091$, $p=0.1914$, show as hashtag). However, when compared with the wLTP+Opto Burst group (**Figs. 2.4A, 2.5A & 2.7A**, blue circles), the SCH+Prop group was significantly lower over the first 5 minutes of post-tetanic LTP ($F(1, 13) = 5.004$, $p=0.0434$, show as asterisk). **B,** Schematics of Opto bursting and weak LTP theta-burst protocols (see methods for more detail). **C,D,** Example averaged traces from the last 5 minutes of baseline before optogenetic or LTP stimulation (solid lines), and the last 5 minutes after wLTP stimulation (dashed lines).

All data points represent the field potential slope (as shown in **Fig. 2.1B**) mean ± SEM. Hashtag represents a non-significant p-value measured with a two-way ANOVA using time as an independent variable over the first 5 minutes of recording after LTP stimulation.

Figure 2.7 *Slow tonic optogenetic stimulation does not enhance weak LTP.*

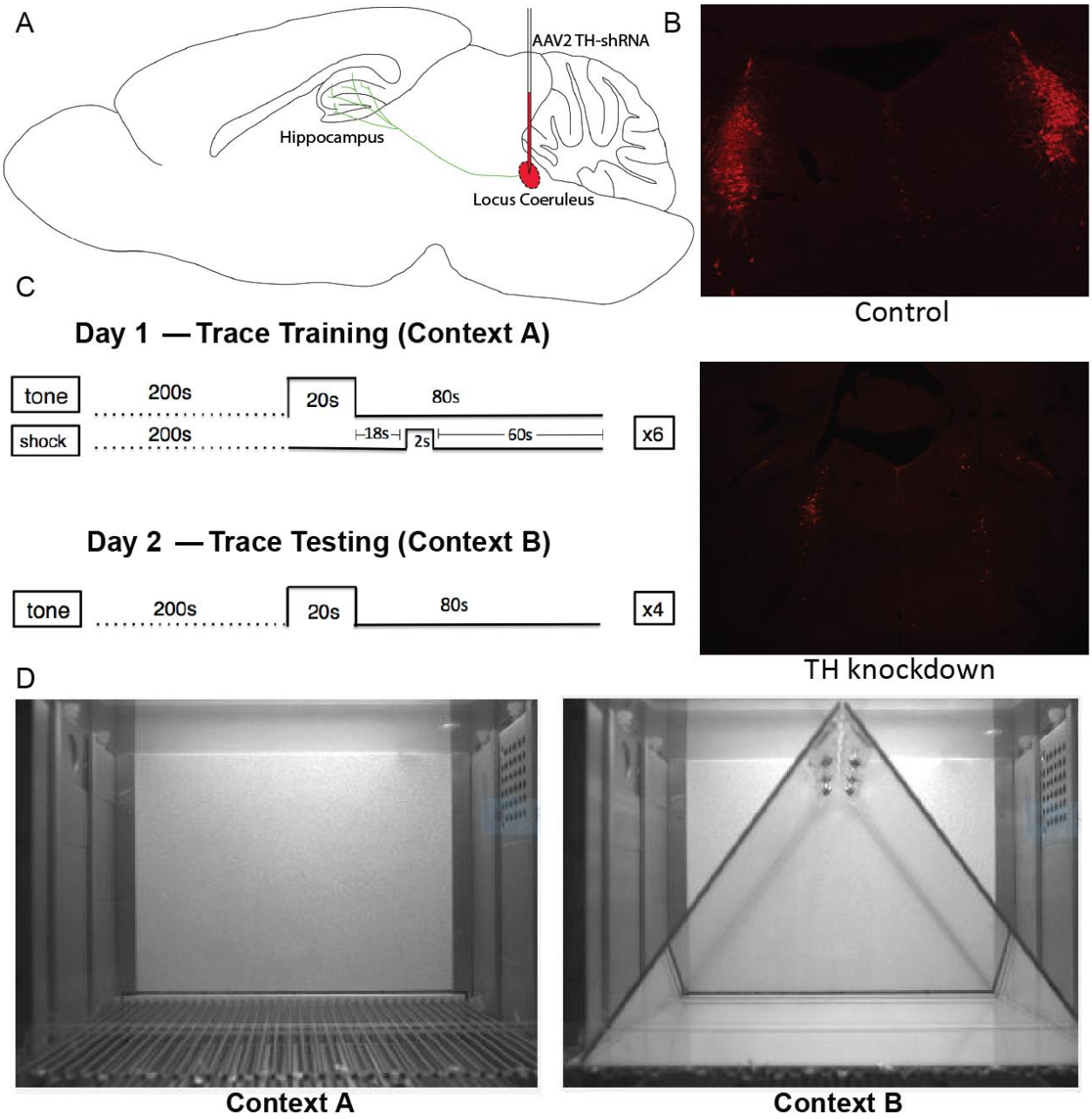


A, A weak, high-frequency, theta-burst electrical stimulation was applied to Shaffer collateral fibers (see methods, black arrow) in dorsal CA1 to induce a weak LTP (black circles) of synaptic responses at CA1 dendrites. This wLTP was converted into a strong LTP by the application of a bursting optogenetic protocol (shown in the **previous 3 figures**) for 10 minutes prior to LTP stimulation (blue bar/circles). In contrast, a slower, tonic Opto stimulation protocol was not shown to enhance late-wLTP (green circles compared to black, $F(1, 10) = 0.04697$, $p=0.8328$), although early wLTP magnitude seems to be marginally increased (26-33 minutes). **B,** Schematics of Opto tonic and

weak LTP theta-burst protocols (see methods for more detail). **C,D,** Example averaged traces from the last 5 minutes of baseline before optogenetic or LTP stimulation (solid lines), and the last 5 minutes after wLTP stimulation (dashed lines).

All data points represent the mean of the field potential slope (as shown in **Fig. 2.1B**) ± SEM. **X** represents highly non-significant p-value measured by a two-way ANOVA using time as an independent variable over the last 15 minutes of recording.

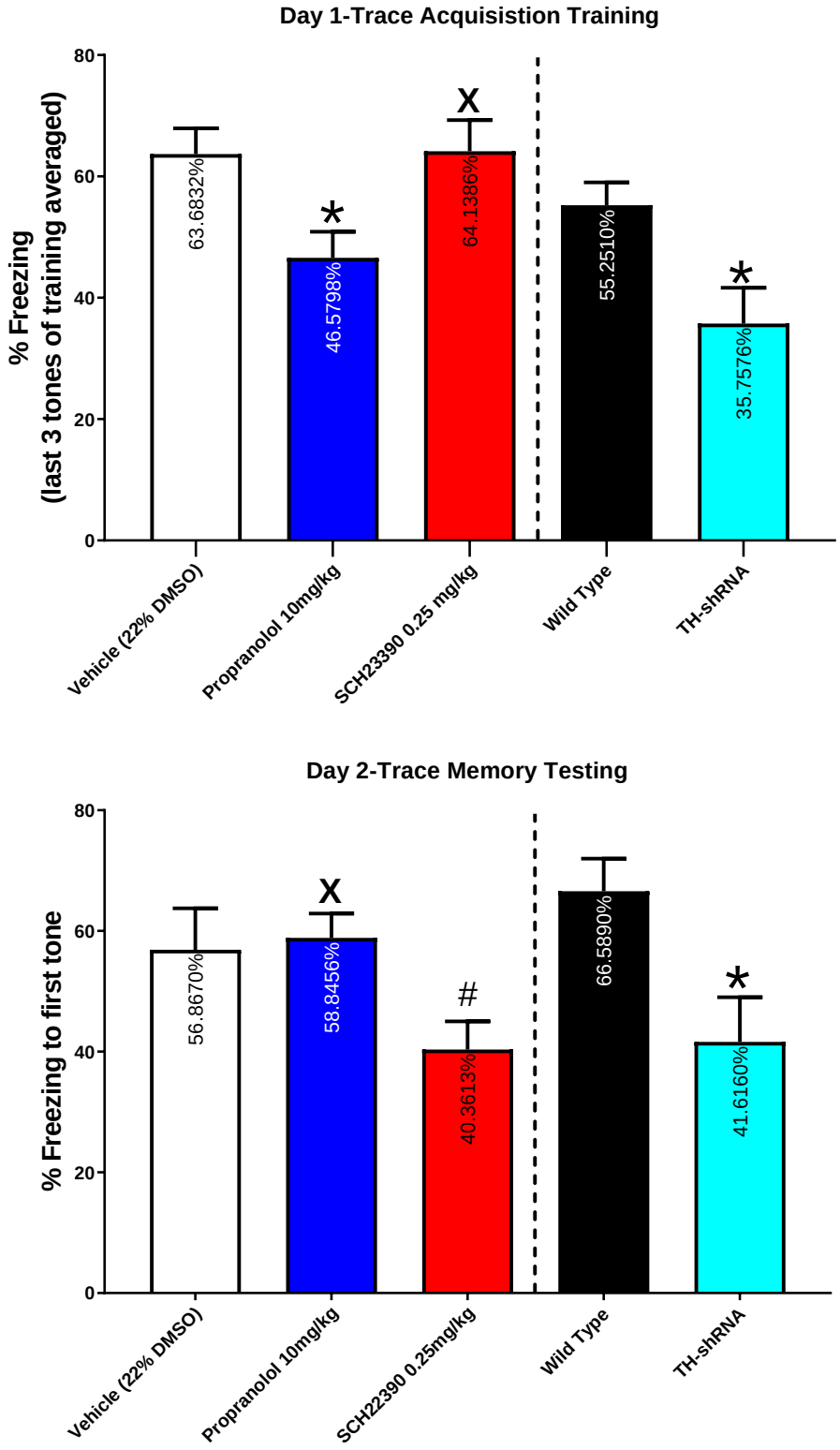
Figure 2.8 *Schematic of tyrosine hydroxylase knockdown in the LC and experimental procedure for trace fear conditioning.*



A, Schematic of AAV2-TH-shRNA viral injections into the locus coeruleus. **B,** Fluorescent images of the locus coeruleus showing tyrosine hydroxylase protein expression in red. Top image is from a control mouse and bottom is from an AAV2-TH-

shRNA injected mouse. **C,** Schematic of trace fear conditioning paradigm showing timing of tone/shock pairings on each day in each context. Dotted lines represent times when the animal was placed in the cage before tone/shock pairing started. **D,** Pictures of both contexts. In context B, a scent (vanilla) was also added to further increase the difference in the animal's sensory experience.

Figure 2.9 *Knocking down the enzyme tyrosine hydroxylase (TH) blocks both noradrenergic and dopaminergic aspects of contextual learning.*



Top (Day 1), Average freezing behavior over the last 3 tones of trace training was measured in drug and TH-knockdown subgroups (separated by dashed line). Left, mice that received an IP injection of the β-adrenergic receptor antagonist, propranolol, 30 minutes prior to training (blue bar) did not acquire as strong of a learned fear as mice injected with vehicle (white bar). SCH 23390 (D1/D5 receptor antagonist) did not seem to have an effect on memory acquisition compared to the vehicle control (red bar). After a one-way ANOVA and a Dunnett's multiple comparisons test, the asterisk represents an adjusted p-value of p=0.0196 and the **X** represents p=0.9965. Right, similarly, animals with tyrosine hydroxylase knocked down (cyan bar) seemed acquire the tone/shock association worse than their wild type controls (black bar). After a two-tailed t-test, the asterisk represents a p-value of p=0.0239 when. **Bottom (Day 2),** Twenty-four hours after the trace train trial, the trace memory test trial was completed. During this trial, four tones were played, and of those, the percent freezing during and the forty seconds after the first tone were averaged for each group. Left, blocking β-adrenergic receptors showed no significant impact on trace memory retention (blue bar), but blocking D1/D5 receptors (red bar) trended toward reduced memory retention compared to vehicle controls (white bar). After a one-way ANOVA and a Dunnett's multiple comparisons test, the hashtag for SCH 23390 represents an adjusted p-value of p=0.0857 and the **X** represents p=0.9534. Right, tyrosine hydroxylase knockdown (cyan bar) also reduced memory retention similarly to the SCH 23390 group. All groups contained 10 mice. After a two-tailed t-test, the asterisk represents a p-value of p=0.0139.

Figure 2.10 *Blockade of trace acquisition by TH-shRNA does not seem to be due to a decrease in anxiety-like behavior* in an elevated plus maze.

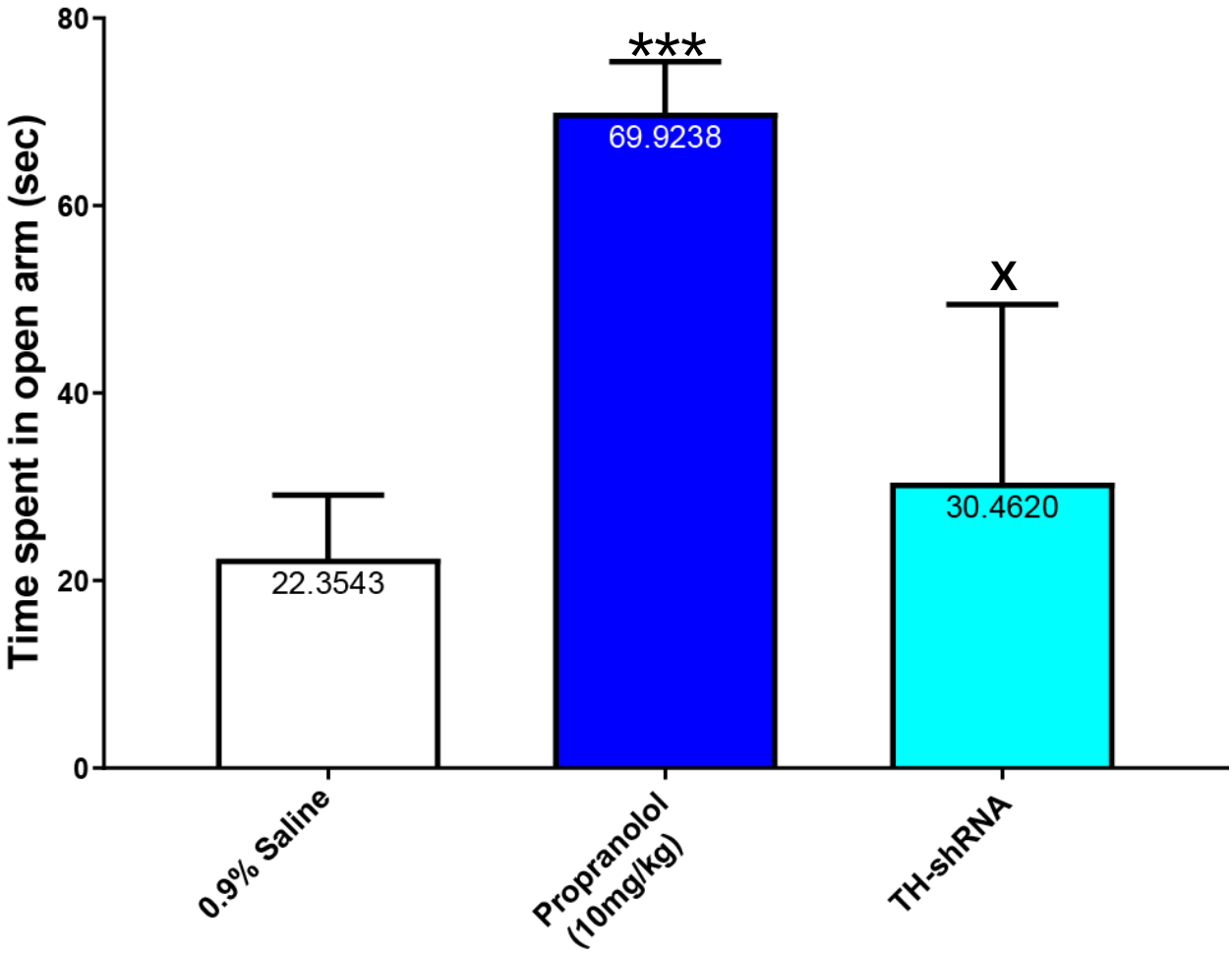


Figure 2.10, Intraperitoneal injection of propranolol increased the time mice spent in the open arm of an elevated plus maze test (blue bar, p=0.0001, n=10). In the same test, TH-shRNA injected mice did not display the same level of anxiety reduction as the propranolol group and did not spend more time than control mice in the open arm (cyan bar, p=0.6440, n=8). After a one-way ANOVA and a Dunnett's multiple comparisons test, the triple-asterisk represents an adjusted p-value of p=0.0001 and **X** represents a p-value of p=0.6440 when both were compared to controls (white bar, n=10).

CHAPTER THREE: A mechanism and practical hypothesis for locus coeruleus dopamine release

The following chapter is adapted from:

Sonneborn A, Greene RW. The norepinephrine transporter regulates dopamine-dependent synaptic plasticity in the mouse dorsal hippocampus. *Under review at Communications Biology*.

Contributions as lead author:

- Designed and performed all experiments to probe the mechanism of locus coeruleus dopamine release.
- Also analyzed all the data, made all the figures, and wrote the entire paper.

Abstract

The rodent dorsal hippocampus is essential for episodic memory consolidation, a process dependent on dopamine D1-like receptor activation. It was previously thought that the ventral tegmental area provided the only supply of dopamine to dorsal hippocampus, but several recent studies have established the locus coeruleus (LC) as a second major source. However, the mechanism for LC-dependent dopamine release has never been explored. Our data identify norepinephrine transporter reversal as one plausible mechanism by demonstrating that transporter blockade can reduce dopamine-dependent long-term potentiation in hippocampal slices. We also suggest that presynaptic NMDA receptors on LC terminals may initiate this norepinephrine transporter reversal. Furthermore, as dopamine and norepinephrine should be co-released from the LC, we show that they act together to enhance synaptic strength.

Since LC activity is highly correlated with attentional processes and memory, these experiments provide insight into how selective attention influences memory formation at the synaptic and circuit levels.

Introduction

Adrenergic signaling in the mammalian brain is largely controlled by a network of remarkably divergent axon projections arising from locus coeruleus (LC) neurons (Kebschull et al., 2016; Schwarz et al., 2015). These LC axons were once thought to exclusively release norepinephrine (NE) (Berridge and Waterhouse, 2003), but recent chemical evidence reveals that their specific activation can also increase extracellular dopamine (DA) (Beas et al., 2018; Devoto et al., 2005b; Kempadoo et al., 2016; Zerbi et al., 2019). In accordance with this, LC stimulation is sufficient to modulate DA-dependent changes in learning and synaptic physiology within the rodent dorsal hippocampus (Kempadoo et al., 2016; Lemon and Manahan-Vaughan, 2012; Takeuchi et al., 2016; Wagatsuma et al., 2018). Dopamine D1-like receptors are abundantly expressed in this region, where they play an essential role in promoting many forms of long-term synaptic potentiation (LTP), especially in area CA1 (Lisman et al., 2011). However, in CA1, projections from canonical DA-releasing nuclei such as the ventral tegmental area (VTA) are sparse compared to those of the LC (Nomura et al., 2014; Takeuchi et al., 2016), indicating that DA receptor activation in this area is mainly due to LC activity. Yet despite data supporting the LC as the main source of DA in dorsal hippocampus, the mechanism underlying its release has never been explored.

One hypothesis for the mechanism of LC DA release is by reverse transport through the norepinephrine transporter (NET). Under normal conditions, the NET is responsible for the reuptake of both NE and DA after they are released (Borgkvist et al., 2012; Moron et al., 2002). In contrast, the presence of amphetamines allows the NET to *efflux* catecholamines from LC varicosities (Robertson et al., 2009), and DA released in this way potentiates synaptic strength in dorsal CA1 (Smith and Greene, 2012). Furthermore, the closely related dopamine transporter (DAT) can reverse its flux under more physiological conditions than amphetamine application (for a review, see Leviel (2017)). These conditions include a rise in intracellular [Na^+] and [Ca^{2+}] following action potential firing (Gnegy et al., 2004; Khoshbouei et al., 2003), activation of NMDA receptors (Ihalainen et al., 1999; Olivier et al., 1995), and phosphorylation by CAMKII or PKC (Feenstra et al., 1999). Because the amino acid sequences of DAT and NET are almost 80% homologous (Andersen et al., 2015), we propose that the NET will also efflux cytosolic DA from LC axons under similar physiological conditions. Below we investigate this possibility in the dorsal hippocampus, where DAT expression is not detectable (Kwon et al., 2008; Smith and Greene, 2012), by designing a DA-dependent LTP that is significantly attenuated after the NET is blocked.

In support of a more detailed model for NET-mediated DA release, an existing theory posits that high-frequency glutamate activity may play a role. The authors speculate that elevated pyramidal cell firing in response to environmental stimuli can result in glutamate spillover (Frey et al., 1991), leading to activation of presynaptic NMDA receptors on LC terminals and enhanced vesicular NE release (Mather et al.,

2016). Taking this idea one step further, Olivier *et al.* discovered that an NMDA-dependent rise in striatal DA is nearly abolished by GBR12909, a selective DAT blocker (Olivier et al., 1995). This indicates that NMDA receptors can somehow interact with transporters and change the direction of catecholamine flux. Comparably, early studies in dorsal hippocampus reported increased extracellular NE and/or DA after NMDA receptor agonist application (Chaki et al., 1998; Grilli et al., 2009; Lalies et al., 1988; Malva et al., 1994; Mayer et al., 2009). To our knowledge, no studies have attempted to associate this DA transmission with NET reversal, or looked at its ability to regulate synaptic plasticity. With this in mind, we deleted NMDA receptors from catecholamine terminals and saw a decrease in DA-dependent LTP in dorsal hippocampus.

Lastly, given that both DA and NE can modulate synaptic plasticity in dorsal CA1 (Lawrence and Cobb, 2018), along with the indication of their co-release from the LC, we presume that they are working together to influence synaptic strength in this region (Jenson et al., 2015). Our final experiment shows that simultaneous application of DA and NE, but not either of them alone, can strengthen a weaker form of hippocampal LTP. The implications of these results are then discussed in the context of the LC's purported role in selective attention (Ramos and Arnsten, 2007), and how glutamate can interact with catecholamines to organize attention-driven memory formation at the synaptic level.

Results

The norepinephrine transporter (NET) contributes to dopamine-dependent potentiation in the dorsal hippocampus

If the NET is capable of controlling DA efflux in dorsal hippocampus, then blocking it should attenuate DA-dependent synaptic potentiation. To test this, we developed a strong theta-burst LTP protocol (strLTP) by stimulating CA3 axons and recording the slope of field excitatory postsynaptic potentials (fEPSPs) from stratum radiatum dendrites of CA1 (**Fig. 3.1A-D**). This protocol was based on previous methods used to generate catecholamine-dependent potentiation in hippocampus (Larson and Munkacsy, 2015; Nguyen and Kandel, 1997). Importantly, our strLTP was not blocked by co-application of β-adrenergic (propranolol) and α1-adrenergic (prazosin) receptor antagonists (**Fig. 3.1E**, comparison between strLTP with no-drug from **Fig. 3.1D** and strLTP with drug from **Fig. 3.4A**).

Even though propranolol and prazosin applied together were not able to reduce strLTP, they each seem to have effects on potentiation when applied separately. In **Figure 3.2**, propranolol alone produces a non-significant but noticeable decrease in overall LTP magnitude after a single 100Hz stimulation (blue circles). Conversely, prazosin alone produces a trend toward an increase of the same 100 Hz LTP (gold circles). This figure depicts a possible bidirectional control of LTP magnitude in the dorsal hippocampus by β- and α1-adrenergic receptors. Similar findings have been reported in the visual cortex (Salgado et al., 2012). Functionally, this dichotomy for LTP control may be necessary for determining the salience of a stimulus, and therefore the level of its memory consolidation in the hippocampus. This idea is expanded upon in the discussion section of this chapter.

Following the further addition of the D1-like receptor antagonist, SCH 23390 to the propranolol and prazosin group, a robust blockade of LTP occurred over the last 30 minutes of recording (**Fig. 3.4A**, red traces). This indicates that strLTP maintenance is dependent on DA receptors, but not adrenergic receptors. Similarly, combination of SCH23390 with propranolol alone also significantly reduced 100Hz LTP (**Fig. 3.3**, red circles). A corresponding experiment showed that protein synthesis inhibition by anisomycin blocks 100Hz LTP to the same extent (**Fig. 3.3**, cyan circles). This parallels the observations of multiple groups that new protein synthesis is required for D1/5 receptor-dependent LTP in dorsal hippocampus (Frey and Morris, 1998; Moncada and Viola, 2007; Smith et al., 2005). However, the fact that anisomycin application did not fully block the potentiation at 1 hour leads us to believe that a large part of our potentiation is not dependent on protein synthesis and may rely on AMPA receptor insertion and/or phosphorylation.

Next, we administered the same strLTP stimulation, but substituted nisoxetine, a NET blocker, for SCH 23390. Treatment with nisoxetine produced a similar reduction in LTP (**Fig. 3.4B**, green traces), suggesting that DA signaling in the dorsal hippocampus is mediated by the NET. Likewise, a genetic deletion of the NET from LC neurons also greatly reduced strLTP amplitude after 1 hour (**Fig. 3.5**). However, the latter experiment was performed in different experimental conditions than the former (100Hz LTP with no adrenergic antagonists in the bath), and non-physiological activation of adrenergic receptors was not controlled for.

To corroborate the results of our *ex vivo* LTP experiments, we wanted to test whether the NET could modulate dopamine-dependent behavioral processes. So, we subject mice to a contextual fear conditioning task known to be sensitive to dopamine D1/5-like receptor activation (Heath et al., 2015; Sarinana et al., 2014). In this two-day task, mice learned that a particular context (Context A, **Fig. 2.8**) was associated with a painful shock. Mice were trained to associate Context A with a shock on Day 1, and on Day 2 they were placed back into Context A to see how well they remembered getting shocked in that context. Their memory was measured by the percent of time during the trial on Day 2 that they spent behaviorally frozen, an indicator of fear, and therefore memory, of getting shocked in context A. This freezing behavior is quantified in **Figure 3.6B**. Interestingly, freezing behavior was significantly decreased when a dopamine D1/5 receptor antagonist, SCH23390, was injected into the mice before learning (red bar). However, injection of nisoxetine had no effect on their ability to remember the painful context, and mice in this group even trended toward enhanced memory of getting shocked in Context A (teal bar).

The above result was unexpected given the decrease observed after blocking the NET during the LTP experiments. However, it could be a consequence of a non-specific, intraperitoneal injection of nisoxetine, which would affect adrenergic signaling throughout the brain instead of locally affecting the dorsal hippocampus. The brain region likely responsible for producing the observed increase in freezing is the amygdala. Here, activation of β-adrenergic receptors is known to be essential for many types of fear-conditioned memory (Bush et al., 2010; LaLumiere et al., 2003). Therefore,

overactivation of these receptors, as would occur during NET blockade, could possibly lead to enhanced amygdala-dependent fear memory that is strong enough to override the dopamine-dependent hippocampal representation. If this was the case, then nisoxetine's effect of blocking dopamine release may not be noticeable.

Blocking α2-adrenergic receptors does not reduce the effect of NET antagonism

Because blocking the NET will flood synapses with NE, one possible confound is over-activation of inhibitory α2-adrenergic autoreceptors, leading to a decrease in overall LC excitability and neurotransmitter release (Abercrombie et al., 1988). This may cause a reduction in LTP based on an indirect decrease in total NE and/or DA levels. To control for this, we repeated the aforementioned experiments with the inclusion of RS79948, an α2-receptor antagonist, in the bath with propranolol and prazosin. Interestingly, adding RS79948 caused a significant increase in fEPSP slope over the first 30 minutes after strLTP stimulation, but not the last 30 minutes (**Fig. 3.4C**, blue traces). This effect could be due to greater vesicular NE release reaching concentrations high enough to displace propranolol and activate β-adrenergic receptors, a process known to enhance early LTP (Hagena et al., 2016). In line with our prior results, the further addition of nisoxetine was still able to diminish the magnitude of strLTP over the last 30 minutes (**Fig. 3.4D**, green traces), reinforcing the finding that NET may contribute to DA signaling in dorsal hippocampus.

NMDA receptor knock-out from catecholamine neurons reduces the magnitude of dopamine-dependent LTP

Activation of glutamate receptors, in particular NMDA, is capable of enhancing catecholamine release in hippocampus (Chaki et al., 1998; Malva et al., 1994; Raiteri et al., 1992). To expand on the mechanism of DA release from the NET, we asked if presynaptic NMDA receptors on LC terminals could functionally be coupled to NET reversal, and thus involved in our NET/DA-dependent LTP. To approach this question, we first confirmed that NMDA receptors co-localized with the norepinephrine transporter on LC axon terminals in the dorsal hippocampus (**Fig. 3.7B,C,D**). To our knowledge, this is the first time that co-localization of these proteins has been shown in LC terminals in any area of the brain.

We next wanted to check if this co-localization was important for the expression or maintenance of LTP in dorsal CA1. To do this, the NR1 subunit of NMDA receptors had to be genetically deleted from catecholamine neurons, since blocking NMDA receptors would prevent LTP. This was done by crossing a mouse expressing Cre recombinase under the control of the tyrosine hydroxylase promoter with a floxed NMDA-NR1 subunit mouse. Cre-negative controls for these mice showed normal strLTP (**Fig. 3.7**, filled circles), whereas the TH-NR1 knockouts exhibited decreased LTP throughout the full hour after LTP induction (**Fig. 3.7**, open circles). Since dorsal CA1 receives very little VTA input, we interpreted these effects as being predominantly due to NMDA deletion from LC neurons. However, the results do not rule out possible compensatory effects of NMDA receptor knockout resulting from Cre expression in TH-positive neurons during development (Matsushita et al., 2002). One approach for ruling out this possibility would be to test if the reduction in LTP in our TH-NR1 knockout mice

could be further blocked by the addition of the D1R antagonist SCH23390. If NMDA receptors are functionally linked to LC-DA release, then TH-NR1 knockout should occlude the effect of SCH23390.

Concurrent activation of DA and NE receptors is required for LTP enhancement in CA1

The regulation of synaptic plasticity by DA or NE is well documented in dorsal hippocampus (Harley, 2004; Palacios-Filardo and Mellor, 2019). For example, D1/D5 receptor antagonists in CA1 can inhibit synaptic potentiation (Huang and Kandel, 1995) and contextual learning (O'Carroll et al., 2006), while agonists of β-adrenergic receptors are sufficient to lower the threshold for LTP initiation (O'Dell et al., 2010). Yet in dorsal CA1, whether or not coincident activation by both catecholamines is needed to enhance LTP has never been examined. This question remains of great importance, as it has been well established that the LC can release DA and NE together. Accordingly, we developed a weak LTP (wLTP) stimulation paradigm (**Fig. 3.1C&D**) and tested if an interaction between DA and NE is necessary to strengthen it.

Bath application of NE alone had no effect on the magnitude of wLTP; although a decrease in baseline glutamatergic signaling before wLTP stimulation was apparent (**Fig. 3.8A**, 16-30 mins). The latter phenomenon has been recorded previously (Katsuki et al., 1997; Mynlieff and Dunwiddie, 1988), and is likely due to NE activating α1-receptors on interneurons to increase their feed-forward/lateral inhibitory drive (Bergles et al., 1996). Surprisingly, washing in DA alone had no effect on either wLTP magnitude or basal glutamate transmission (**Fig. 3.8B**). This seems counterintuitive considering

that activation of D1-like receptors by selective agonists (e.g. SKF-81297) can reliably evoke LTP in dorsal CA1 (Lisman et al., 2011). However, the result is consistent with multiple reports showing no change in CA1 excitatory transmission in response to bath applied DA (Ito and Schuman, 2007; Otmakhova and Lisman, 1999; Rosen et al., 2015). It is unclear why this occurs, but one explanation could be that over activation of inhibitory D2-like receptors negates the excitatory D1-like receptor activation in this region. Interestingly, even though neither of the catecholamines in isolation was able to produce stronger LTP, their simultaneous application resulted in a significant increase compared to the control wLTP (**Fig. 3.8C**). These findings pair well with the following conclusion that the LC utilizes both DA and NE to optimize memory storage, mainly during periods of enhanced attention to salient stimuli.

Discussion

Taken together, these results allude to the LC orchestrating coincident release of NE and DA in the dorsal hippocampus using two separate mechanisms. The first is the widely accepted vesicular release of NE (Chiti and Teschemacher, 2007), and the second is reverse transport of DA from the NET as shown in this study. A reason for separate release mechanisms is still unclear, but one plausible explanation is that they are used to facilitate a molecular link between attention and memory (Chun and Turk-Browne, 2007), especially since the LC is heavily involved in both cognitive processes at the behavioral level (Sara, 2009). Below we postulate that their co-release should only occur when an animal devotes a large amount of attention to a stimulus that is worthy of memory storage. If this happens, NE and DA can interact in CA1 to help

exclusively potentiate the most relevant synapses for future memory consolidation (**Fig. 3.8**).

When an animal is awake but not experiencing anything particularly interesting in its environment, the LC releases a low, background level of NE from vesicles using a slower, tonic firing pattern (~3 Hz). In the hippocampus, this leads to a general suppression of activity by activating higher affinity α1-adrenergic receptors on interneurons in the area (Bergles et al., 1996; Katsuki et al., 1997). During times of elevated arousal and selective attention to salient stimuli, higher frequency (~16 Hz) phasic activity (Devilbiss, 2019) transiently boosts extracellular levels of NE (Abercrombie et al., 1988; Berridge and Abercrombie, 1999). These quick increases in NE are theorized to recruit lower affinity β-adrenergic receptors on hippocampal pyramidal cells to amplify more active glutamatergic inputs, while the α1-receptors continue to reduce the noise generated by less active ones (Aston-Jones and Cohen, 2005). Therefore, NE helps the most immediately relevant and strongest signals prevail over those that are firing slower and likely carrying less important information. Elaborating on this, Mather et al. (2016) propose that very active glutamatergic synapses can in turn augment the release of NE after glutamate spillover activates presynaptic NMDA receptors on nearby LC axons. This would create a local positive feedback loop between the most rapidly firing glutamatergic input and phasically active LC terminals, with less active circuits remaining suppressed as they are unable to trigger this positive feedback. In the hippocampus, this system presumably optimizes circuit organization to reduce the overlap between stored memory traces.

Our data expand on these theories and suggest that presynaptic NMDA receptors can similarly initiate DA signaling from LC axons in dorsal hippocampus, since their deletion weakens DA-dependent LTP (**Fig. 3.7**). This effect makes sense within the framework of attention being a driving force for memory formation (Chun and Turk-Browne, 2007). For instance, when strong glutamatergic signaling in response to salient environmental cues couples with phasic LC firing in CA1, excess glutamate can overflow from the synapse and bind to NMDA receptors on LC terminals (Mather et al., 2016). At the same time, salience-guided, phasic action potential firing in LC terminals will influx Na^+ and Ca^{2+}, removing the Mg^{2+} block and allowing even more cation influx through NMDA receptors. Calcium entering the neuron via this process may then promote the function of CAMKII or PKC, kinases capable of interacting with (Fog et al., 2006) and phosphorylating transporters to reverse their direction (Darracq et al., 1998). Likewise, since monoamine transporters are known to move neurotransmitters using the energy stored in Na^+ gradients (Kesner, 2000), a switch to higher intracellular Na^+ during action potential bursting could supply the energy needed to flux DA out of the NET. In favor of this idea, it is known that the NET can reuptake DA nearly as well as NE in the hippocampus under normal conditions (Borgkvist et al., 2012), hinting that the reverse mechanism may be possible. **Figures 3.4 & 3.5** explore this idea and highlight that DA released in this way seems to be physiologically relevant, as blocking or deleting the NET is capable of reducing DA-dependent LTP.

Functionally, this non-canonical DA efflux likely arose as a form of coincidence detection in dorsal CA1. Here it will potentiate only the most prevalent glutamatergic

inputs that were selected by the preceding NE modulation of glutamatergic attentional resources. In other words, once a stimulus becomes salient enough to outcompete the background noise, DA is released and interacts with NE to enhance synaptic strength (**Fig. 3.8**). This would be necessary to tag specific synapses recruited by the increased glutamate signaling for future memory consolidation, given that DA seems to be more involved in the tagging process than NE (Kramar et al., 2004). For this reason, having two separate release mechanisms might enable more efficient signal processing and storage of new information, since DA released out of the NET would not interfere with the formation of neural representations driven by vesicular NE release (for a working theoretical model of this process, see **Fig. 3.9**).

Altogether, these observations concerning the NET's involvement in DA-dependent potentiation are in conflict with a couple of studies that also measured nisoxetine's effect on LTP in CA1 (Mlinar et al., 2015; Thompson et al., 2005). The authors of both papers found no difference in LTP when nisoxetine was present. One explanation could be that, in contrast to our methods, these reports did not use any adrenergic receptor antagonists, potentially leading to excess β-adrenergic receptor recruitment and cAMP dependent LTP enhancement (O'Dell et al., 2010). Experiments in the Thompson paper were also done in the ventral hippocampus, an area that receives much less LC innervation. In contrast, as mentioned above, our NET knockout assay was also performed in no-drug conditions and produced a massive difference in LTP. A reason for this is not immediately obvious, but we cannot rule out developmental consequences of NET deletion on normal adrenergic system function.

However, a critical observation arising from our experiments is the fact that if LC DA was vesicular in origin, then blocking NET should have the opposite effect, as nisoxetine application should lead to an increase in extracellular DA and thus stronger LTP. Along those same lines, David Sulzer's group uncovered a large subset of "silent" VTA synapses in the striatum that do not release DA vesicularly (Pereira et al., 2016). These synapses may not actually be functionally silent, but instead rely on DAT reversal to efflux dopamine.

In closing, our findings support the idea that the NET and NMDA receptors contribute to DA signaling (**Figs. 3.2-3.7**), and therefore interaction with NE signaling (**Fig. 3.8**), to regulate attention-guided memory storage in the CA1 region of dorsal hippocampus. One drawback of our methods is that LC fibers were not selectively stimulated. Instead, catecholamine release was elicited by electrical stimulation of all fibers within the range of the stimulating electrode, which could include any other neuromodulatory inputs into CA1 that might interact with the effects of NE and DA (e.g. acetylcholine or serotonin). Also, since we were stimulating with bursts of 100 Hz, this could unnaturally overload LC terminals since their usual maximum firing rate is <20 Hz, leading to DA release out of the NET that would not occur under normal physiological conditions. Future studies may employ specific optogenetic activation of the LC to study this question with greater precision. It will also be necessary to utilize the recently developed genetically encoded fluorescent DA (Elsersy et al., 2004; Nolan et al., 2004) and NE (Dhanrajan et al., 2004) sensors to probe the dynamics of LC catecholamine co-release in greater detail. In conclusion, although our evidence is indirect, it presents

a vital first step toward elucidating the complex interplay between glutamate activity and catecholamine release, not only within the hippocampus, but in all LC terminal fields throughout the central nervous system.

Materials and Methods

Animal Approval

All animal procedures performed were approved by the animal care and use committee (IACUC) at the University of Texas Southwestern Medical Center and comply with federal regulations set forth by the National Institutes of Health.

Tyrosine hydroxylase-Cre mice were purchased from The Jackson Laboratory (Bar Harbor, ME) (B6.Cg-Tg (TH-Cre)1Tmd/J; #008601). Floxed NMDA-NR1 subunit mice were also obtained from The Jackson Laboratory (Bar Harbor, ME) (B6.129S4-*Grin1*^{tm2Stl}/J; #005246). Norepinephrine transporter knockout mice were a generous gift from Dr. Marc G. Caron, and creation of these mice can be found in Wang et al. (1999).

Ex vivo slice preparation

Coronal slices (300 µm thick) containing dorsal hippocampus were made from male, wild type, C57BL/6J mice (6-12 weeks old) in low-light conditions to prevent photooxidation of catecholamines. Animals were anesthetized under 1.5-2% isoflurane, after which brains were removed and blocked following rapid decapitation. Slices were prepared using a Leica VT1000S vibratome (Wetzlar, Germany) in ice-cold NMDG ringer solution containing (in mM): 5 NaCl, 90 NMDG (N-Methyl-d-Glucosamine), 37.5 Na-Pyruvate, 12.5 Na-Lactate, 5 Na-Ascorbate, 2.5 KCl, 1.25 NaH_2PO_4, 25 $NaHCO_3$,

25 Glucose, 10 $MgSO_4.7H_20$, 0.5 $CaCl_2.2H_20$. The pH was set between 7.3 and 7.4 using 12 N HCl, the osmolarity was adjusted as needed to ~315 mOsm using glucose, and the solution was continuously bubbled with 95% O_2 and 5% CO_2 gas during slicing. Slices were then transferred and maintained for up to 6 hours, while protected from light, in artificial cerebrospinal fluid containing (aCSF; in mM): 120 NaCl, 3 KCl, 1.25 NaH_2PO_4, 1 $MgCl_2$, 2 $CaCl_2$, 25 $NaHCO_3$, and 11 dextrose continuously bubbled with 95% O_2 and 5% CO_2 gas.

Field recordings

After at least 1 hour of recovery in aCSF, slices were transferred to a submersion recording chamber and perfused with aCSF at a rate of 2-3 ml/min at 31-32ºC. Extracellular voltage recordings from the stratum radiatum field of dorsal CA1 were acquired using a borosilicate glass electrode (1-2 MΩ, Sutter Instrument (Novato, CA)) filled with normal aCSF. A bipolar stimulating electrode (FHC, Inc. (Bowdoin, ME)) was also placed in the stratum radiatum of CA1 within ~300 µm of the recording electrode (see **Fig. 2.1A**), and stimulus strength was controlled with a stimulus isolator unit (World Precision Instruments, Sarasota, FL). Stimulus strength was set to produce a baseline excitatory field postsynaptic potential (fEPSP) slope (**Fig. 2.1B**) that was ~50% of the slope measured following the first appearance of a population spike. This method led to a typical baseline stimulation current of 20-30 µA, while stimulus duration was set to 0.2 ms. Schaffer collateral stimulation was given once every 30 seconds and the average of every two consecutive stimuli was taken. For the DA and NE synergy experiments, a stable 15 minute control baseline was obtained, followed by another 15 minute baseline

with drug washed in. At the end of the 15 minute drug wash, a weak theta-burst tetanus was applied consisting of 5 bursts (given at 5 Hz), with each burst containing 5 spikes at 100 Hz (25 total spikes). Baseline stimulation then resumed as described above for 45 minutes. For the NET blockade experiments, the entire experiment was run in the presence of various antagonists. A 15 minute baseline was obtained, followed by a strong theta-burst tetanus containing 15 bursts (given at 5 Hz), with each burst containing 5 spikes at 100 Hz (75 total spikes). Baseline stimulation then resumed as described above for 60 minutes. All experiments were performed in low-light conditions to avoid photooxidation of catecholamines. Data was acquired using a Multiclamp 700B amplifier and pCLAMP 10 software (Molecular Devices, San Jose, CA). The signal was low-pass filtered online at 2 kHz using the Multiclamp 700B Commander software, and then digitized at 20 kHz using a Digidata 1440A (Molecular Devices, San Jose, CA).

For **Figures 3.2, 3.3,** & **3.5**, a different electrical stimulation protocol was used to elicit LTP at Schaffer collateral synapses. This stimulation consisted of a single train of 100 pulses given at 100 Hz.

Whole-cell recordings

Slices were transferred to a submersion recording chamber and were perfused with aCSF at a rate of 2-3 ml/min at 30-32 ºC. A borosilicate glass electrode (3-5 MΩ), pulled with a Sutter P-97 horizontal pipette puller, was filled with Cs-methanesulfonate pipette solution (in mM): 110 $CsMeSO_3$, 15 CsCl, 8 NaCl, 2 EGTA, 10 HEPES, 2 ATP and 0.3 GTP adjusted to 295 mOsm and pH 7.3. Whole-cell stratum radiatum interneuron recordings from area CA1 were acquired, and a constant current was

applied to cells using a Multiclamp 700B amplifier (Molecular Devices, San Jose, CA) that was about equal to their rheobase current. Data was acquired using P-Clamp 10 (Molecular Devices, San Jose, CA).

Contextual Fear Conditioning

Procedural diagram found in Figure 3.6A. Wild type male mice, aged 10-12 weeks, were subject to the following 3-day experimental regimen: Acclimation to context and injection on Day 0, contextual fear conditioning training on Day 1, and contextual memory testing on Day 2. More specifically, on Day 0, mice were removed from their home cages and put into the context that the fear conditioning training would take place in (Context A, see **Fig. 2.8D**). Each mouse was allowed 5 minutes to acclimate to the novel context. Then, the mice were returned to their home cages until the same time the next day. Importantly, mice that we planned to inject with drug on Day 1 also received an acclimating intraperitoneal 0.9% saline injection on Day 0 to prevent excess stress from the injection process on the first training day that might interfere with catecholamine signaling.

Twenty-four hours later on Day 1, mice were either injected with vehicle or drug 30 minutes prior (amounts can be found in **Fig. 3.6B**) to being put back into the same context that they were acclimated to the day before (Context A, **Fig. 2.8D**). Mice were then subject to a tone/shock pairing protocol, presented a total of 4 times during the trial (schematic found in **Fig. 3.6A**). During the 4 tone/shock pairings, behavioral freezing data was measured within a 1 minute epoch after each tone presentation. Data is shown as a percentage equal to the time a mouse spends “frozen” in fear divided by the

total epoch time (1 min). All data were generated automatically using the EthoVision program that tracks an animal's movement over time and quantifies freezing behavior as a percent of total measurement time in a given epoch (Noldus, Leesburg, VA).

Finally, on Day 2 (24 hours later), the same mice were put back into the same context (Context A), and freezing behavior was measured. Data was analyzed as the total percent freezing during the first minute in Context A. The quantification of this data is displayed in **Figure 3.6B**.

Staining and imaging

Dorsal hippocampal sections, 30 µM thick, were cut with a cryostat and stored in 4% PFA in 1X PBS overnight. They were then transferred to a 30% sucrose + 1X PBS solution for cryoprotection. Free floating sections were washed 3x with 1X PBS and treated with a H_2O_2 solution (PBS + 10% methanol + 1.05% H_2O_2) for at least 1 h. Sections were washed, blocked for 2 h in 10% normal donkey serum + 1X PBS + 1% Triton X-100 (blocking solution) and then were treated overnight at 4ºC with primary antibody diluted in blocking solution containing the following 2 antibodies: mouse monoclonal NET primary antibody, 1:200, Invitrogen/Thermo Fisher Scientific, (Waltham, MA); rabbit polyclonal NR1 primary antibody, 1:100, Alomone Labs, (Jerusalem, Israel). The following morning, slices were washed and incubated for 2h at RT covered with secondary antibody diluted in blocking solution containing the following 2 antibodies: donkey anti-mouse Alexa Fluor 488, 1:2000, Invitrogen/Thermo Fisher Scientific, (Waltham, MA); donkey anti-rabbit Alexa Fluor 594, 1:1000, Invitrogen/Thermo Fisher Scientific, (Waltham, MA). Sections were next washed,

mounted on gelatin covered slides and coverslipped using PermaFluor (Thermo Fisher Scientific, Waltham, MA) to preserve fluorescence for long-term storage at 4ºC. Images were taken on a custom built 2-photon microscope at 20X magnification.

Drugs

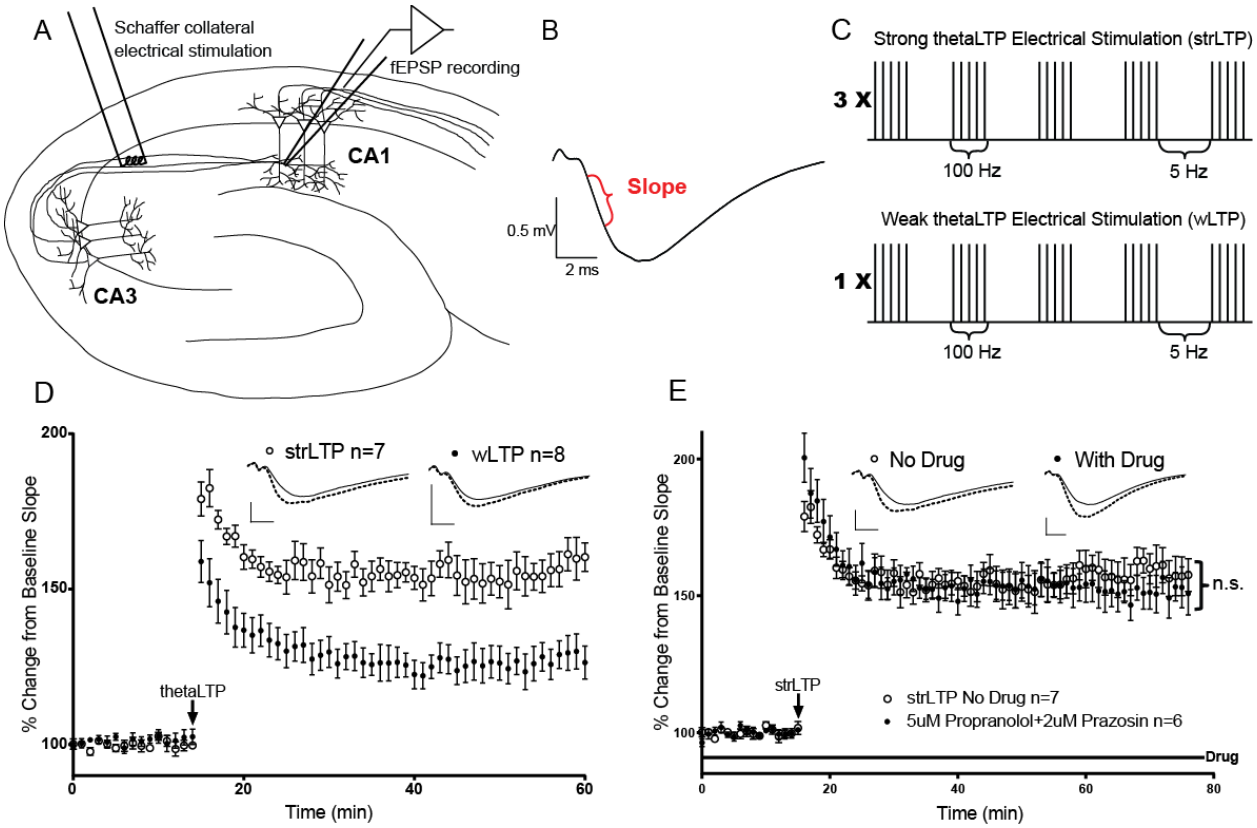
Where indicated, the following drugs were used: (-)-norepinephrine (NE; 20 μM), dopamine (DA; 20 μM), prazosin (α1-adrenergic antagonist; 2 μM), propranolol (β-adrenergic inhibitor; 5 μM), SCH23390 (D1-like receptor antagonist; 1 μM), nisoxetine (norepinephrine transporter blocker; 5μM), RS 79948 (α2-adrenergic antagonist; 5 μM). All drugs were purchased from Tocris Bioscience (Minneapolis, MN).

Statistical analysis

All electrophysiological data points are represented as the mean ± SEM. Field recordings were analyzed using a two-way repeated measures ANOVA with time as an independent variable. Electrophysiology data points are presented as occurring every minute, but are an average of every two slope recordings taken every 30 seconds. Most ANOVAs were run over the last 30 minutes of recording after LTP stimulation. However, in **Figure 3.8A** an additional ANOVA was run over the 15 minutes that norepinephrine was present in panel A, and ANOVAs were run over the last 15 minutes of recording after LTP stimulation since recording only lasted 45 minutes after wLTP stimulation. Also, in **Figure 3.4C** another ANOVA was run over the first 30 minutes after LTP. All analyses were performed using GraphPad Prism 7 software (San Diego, CA).

Figures

Figure 3.1 *Establishment of weak and strong long-term potentiation (LTP) protocols.*



A, Diagram of a hippocampal slice with electrodes in place. The stimulating electrode (left) is placed in contact with Schaffer collateral axons from the CA3 region about 400 µm from the recording electrode. The recording electrode (right) measures the extracellular field excitatory postsynaptic potential (fEPSP) in stratum radiatum dendrites of CA1. **B,** Example fEPSP from CA1. Data is taken as the initial slope of the voltage trace as shown in red. Scale bars represent the 0.5 millivolt amplitude and two millisecond duration in all of the following figures. **C,** Weak (wLTP) and strong (strLTP) Schaffer collateral thetaLTP stimulation protocols (see methods for more details). **D,**

Weak versus strong thetaLTP time course. The black arrow represents the moment that either LTP stimulation was given after a 15 minute baseline. Insets are representative traces before and after each stimulation protocol. The solid lines represent an average of baseline traces from 0-15 minutes before LTP stimulation, while dotted lines represent an average of traces from the last 5 minutes of the recording after LTP stimulation. **E,** strLTP (open circles) is not blocked by the addition of prazosin and propranolol to the bath (closed circles), $F(1, 17) = 0.06472$, $p=0.8022$, 'n.s.' stands for 'not significant'. All data points are represented as mean +/- SEM. Tests for significance were done using a two-way repeated measures ANOVA over the last 30 minutes of strLTP.

Figure 3.2 *Adrenergic signaling exerts bidirectional control over LTP magnitude in dorsal CA1.*

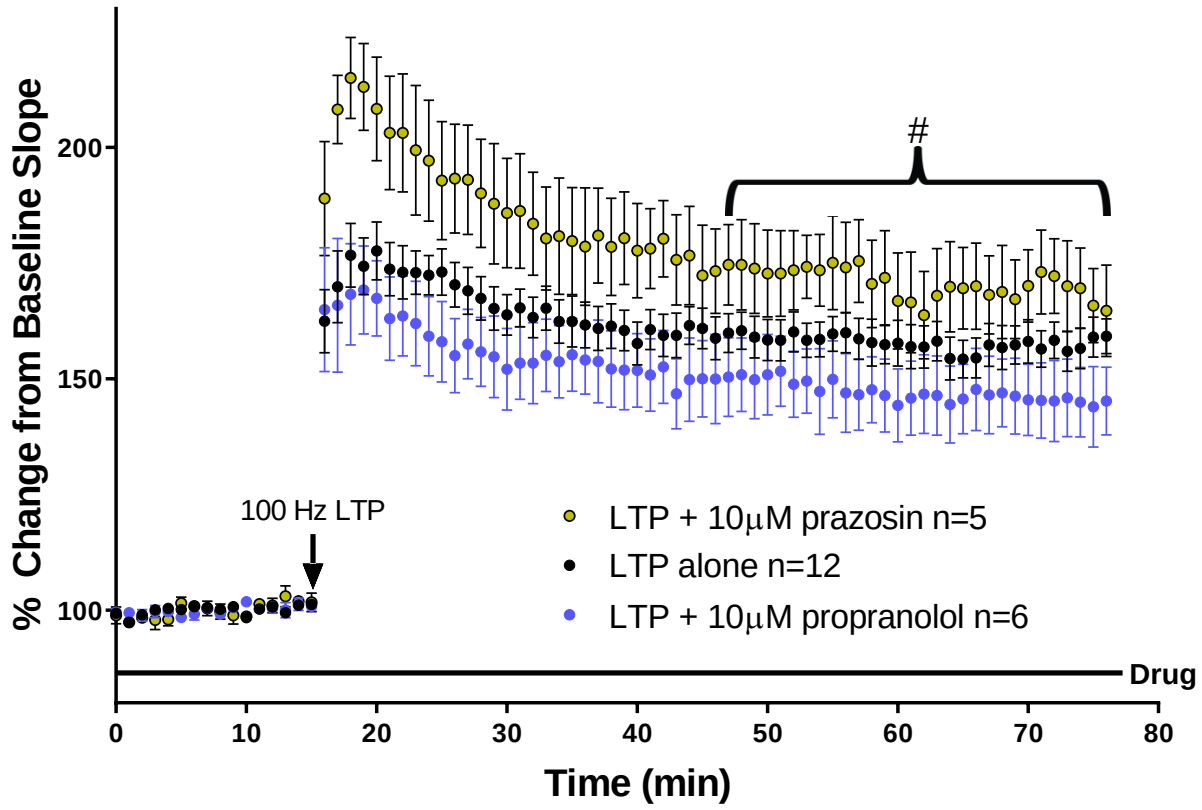


Figure 3.2, A different LTP protocol was administered (100 Hz for 1 second) with the addition of adrenergic receptor antagonists in slices from wild-type control mice. Application of the α1-adrenergic receptor antagonist, prazosin, alone showed a trend toward increased LTP magnitude (gold circles, $F(1,15) = 2.300$, $p=0.1502$). In contrast, application of propranolol, a β-adrenergic receptor antagonist, alone produced a trend toward decreased LTP magnitude after 1 hour (blue circles, $F(1,16) = 1.751$, $p=0.2043$). All data points are represented as mean +/- SEM. Tests for significance were done using a two-way repeated measures ANOVA over the last 30 minutes of 100 Hz LTP. Hashtag represents non-significant p-values for both comparisons.

Figure 3.3 *Addition of SCH 23390 reduces LTP to a greater extent than propranolol alone, and this seems to be dependent on protein synthesis.*

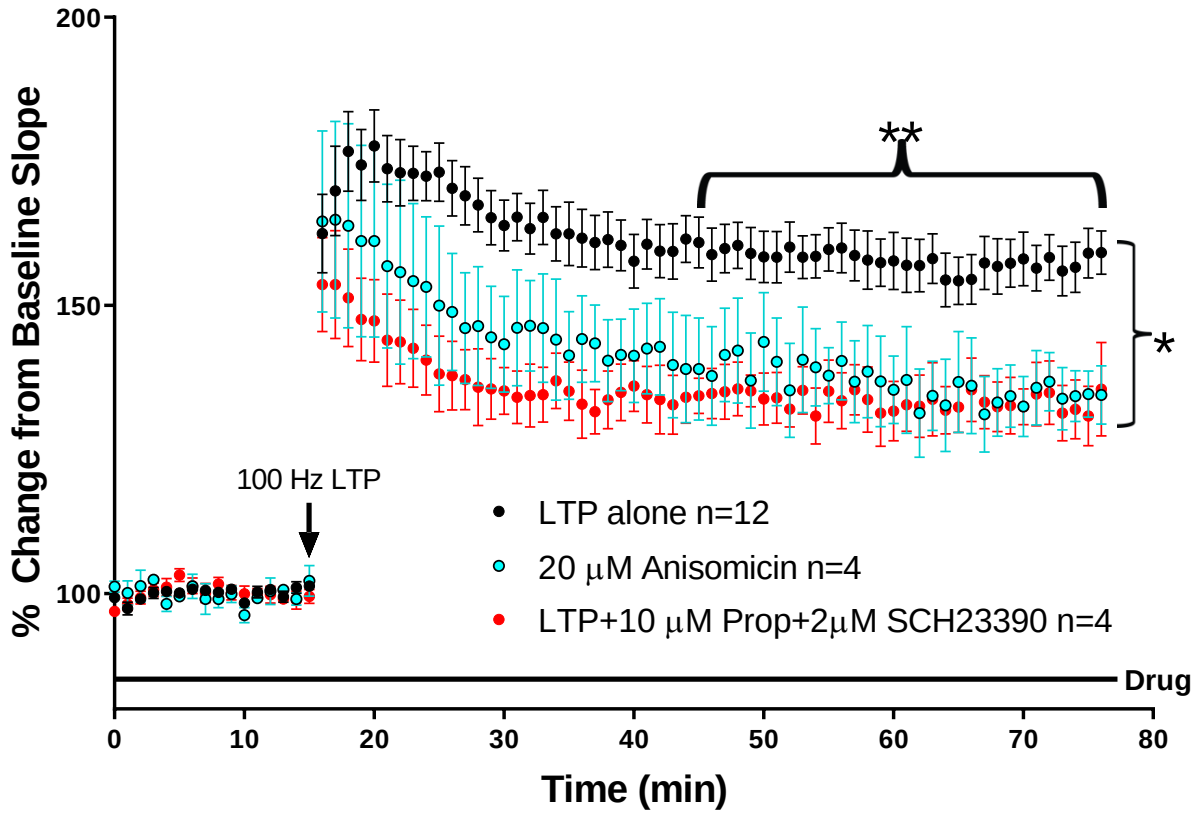
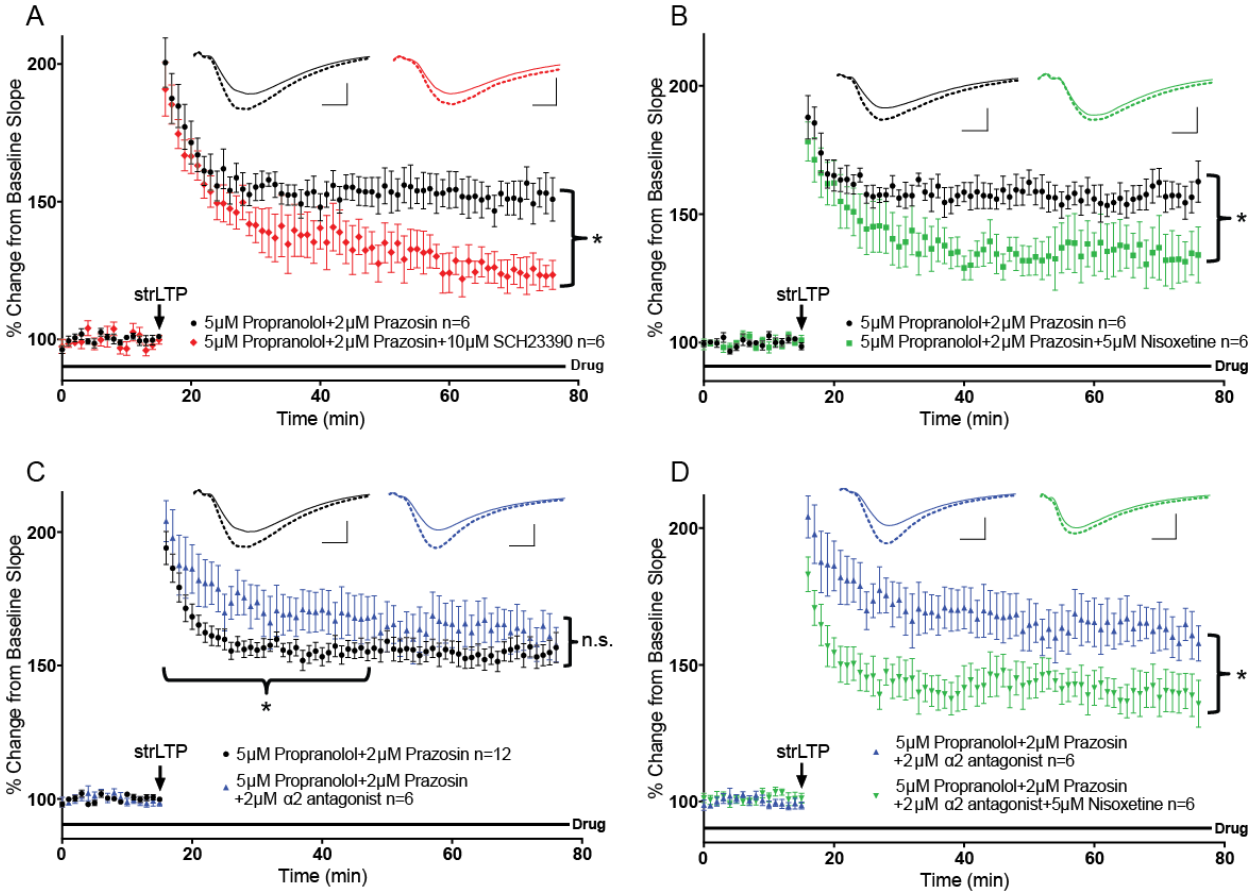


Figure 3.3, A similar protocol as described in **Figure 3.2** was applied, except that SCH 23390 was added to the bath with propranolol before LTP stimulation (red circles). Having both drugs together in the bath produced a significant reduction in LTP magnitude. $F(1,14) = 10.15$, $p=0.0066$, double asterisk on top). Similarly, application of anisomycin, a protein synthesis inhibitor, alone also decreased LTP magnitude after 1 hour (cyan circles, $F(1,14) = 6.926$, $p=0.0197$, single asterisk on right side). All data points are represented as mean +/- SEM. Tests for significance were done using a two-way repeated measures ANOVA over the last 30 minutes of 100 Hz LTP.

Figure 3.4 *The norepinephrine transporter (NET) contributes to dopamine-dependent long-term potentiation.*



A, The previously established strong LTP protocol (strLTP, black arrow, see **Fig. 3.1C&D**) was not blocked by application of antagonists for β- and α1-adrenergic receptors, propranolol and prazosin, respectively (black circles). However, application of SCH 23390, a dopamine D1-like receptor antagonist, along with β- and α1 blockers was enough to significantly reduce the last 30 minutes of LTP (red circles), $F(1, 10) = 9.265$, $p=0.0124$. **B,** Similar to **A**, but the D1/5 receptor antagonist was replaced with the NET blocker nisoxetine (green squares), which was sufficient to attenuate the dopamine-

dependent LTP, $F(1, 10) = 5.028$, $p=0.0488$. **C,** Blockade of ALL adrenergic receptors (by adding an α2 autoreceptor antagonist) selectively increased the *first* 30 minutes of LTP (blue triangles) compared to β- and α1 blockers alone, $F(1, 16) = 4.963$, $p=0.0406$. **D,** Even with all adrenergic receptors blocked (blue) the application of nisoxetine was still able to significantly reduce LTP (green triangles), $F(1, 10) = 5.521$, $p=0.0407$. All data points are represented as mean +/- SEM. Tests for significance were done using a two-way repeated measures ANOVA over the last 30 minutes of strLTP, or during the first 30 minutes as shown in panel C. Asterisks represent p-values <0.05, while 'n.s.' stands for 'not significant'.

Figure 3.5 *Knocking out the norepinephrine transporter (NET) also reduces the magnitude of LTP in dorsal hippocampus.*

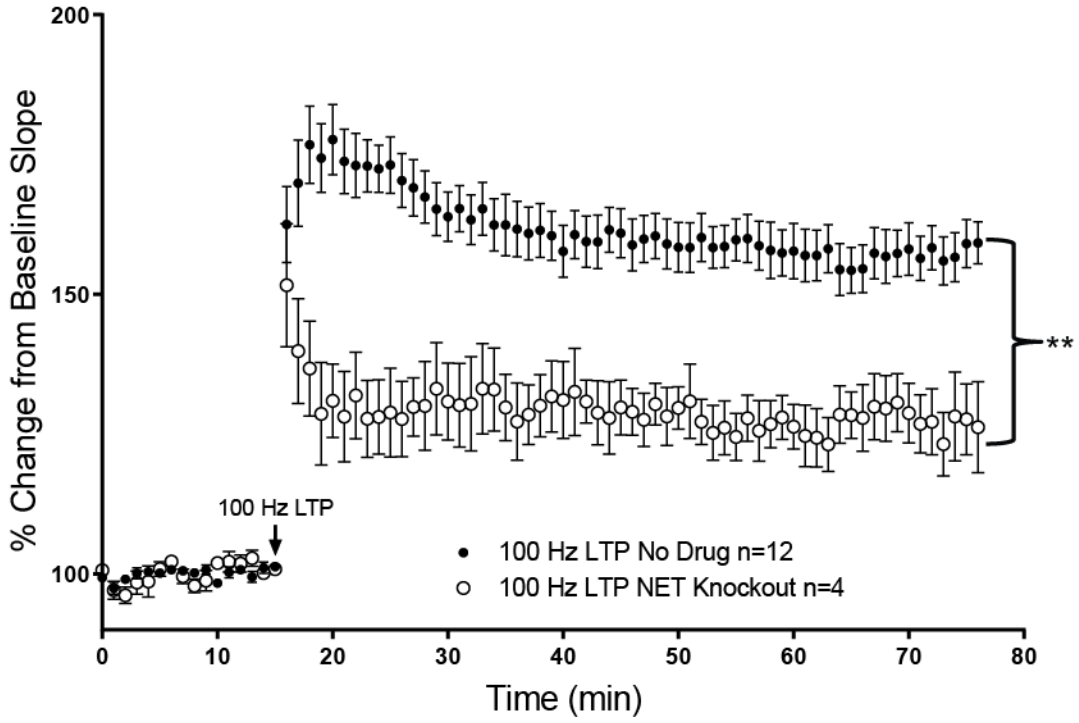
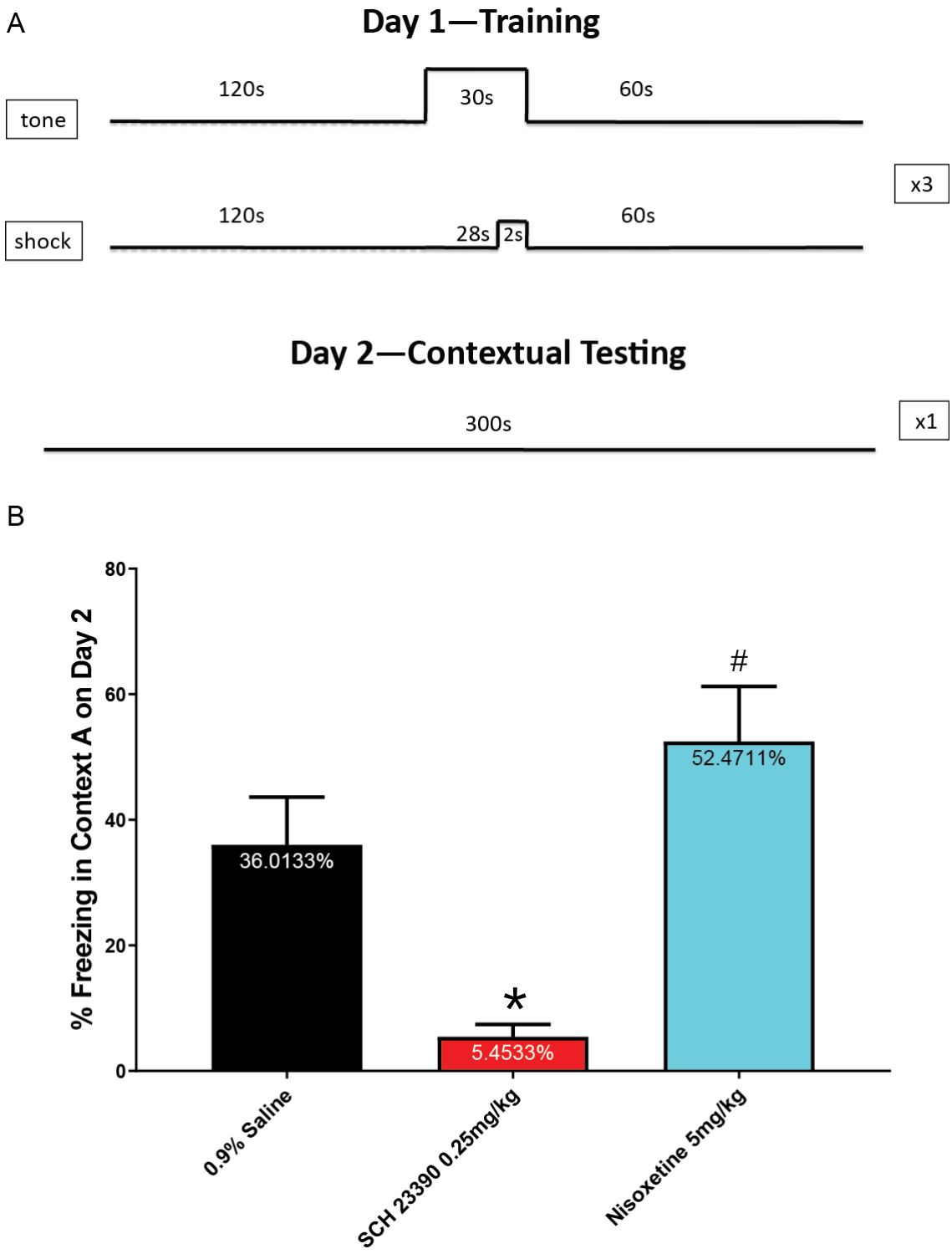


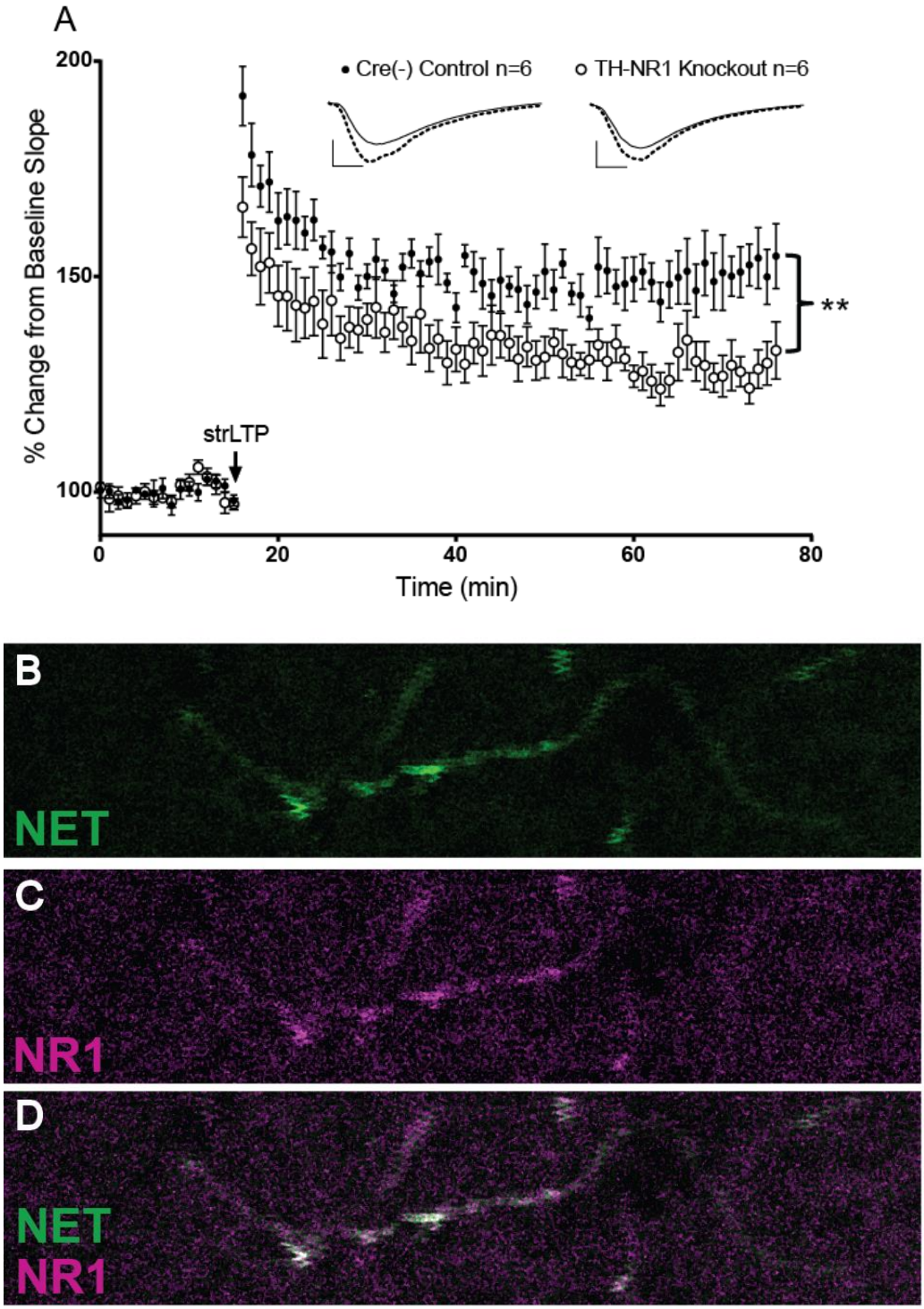
Figure 3.5, A different LTP protocol was administered (100 Hz for 1 second) without the addition of any adrenergic receptor antagonists in slices from Cre(-) control mice (black circles) and NET knockout mice (open circles). All data points are represented as mean +/- SEM. Tests for significance were done using a two-way repeated measures ANOVA over the last 30 minutes of 100 Hz LTP. Double asterisk represents $p<0.01$, $F(1, 14) = 15.59$, $p=0.0015$.

Figure 3.6 *Intraperitoneal injections of nisoxetine in mice do not block dopamine-dependent contextual fear conditioning.*



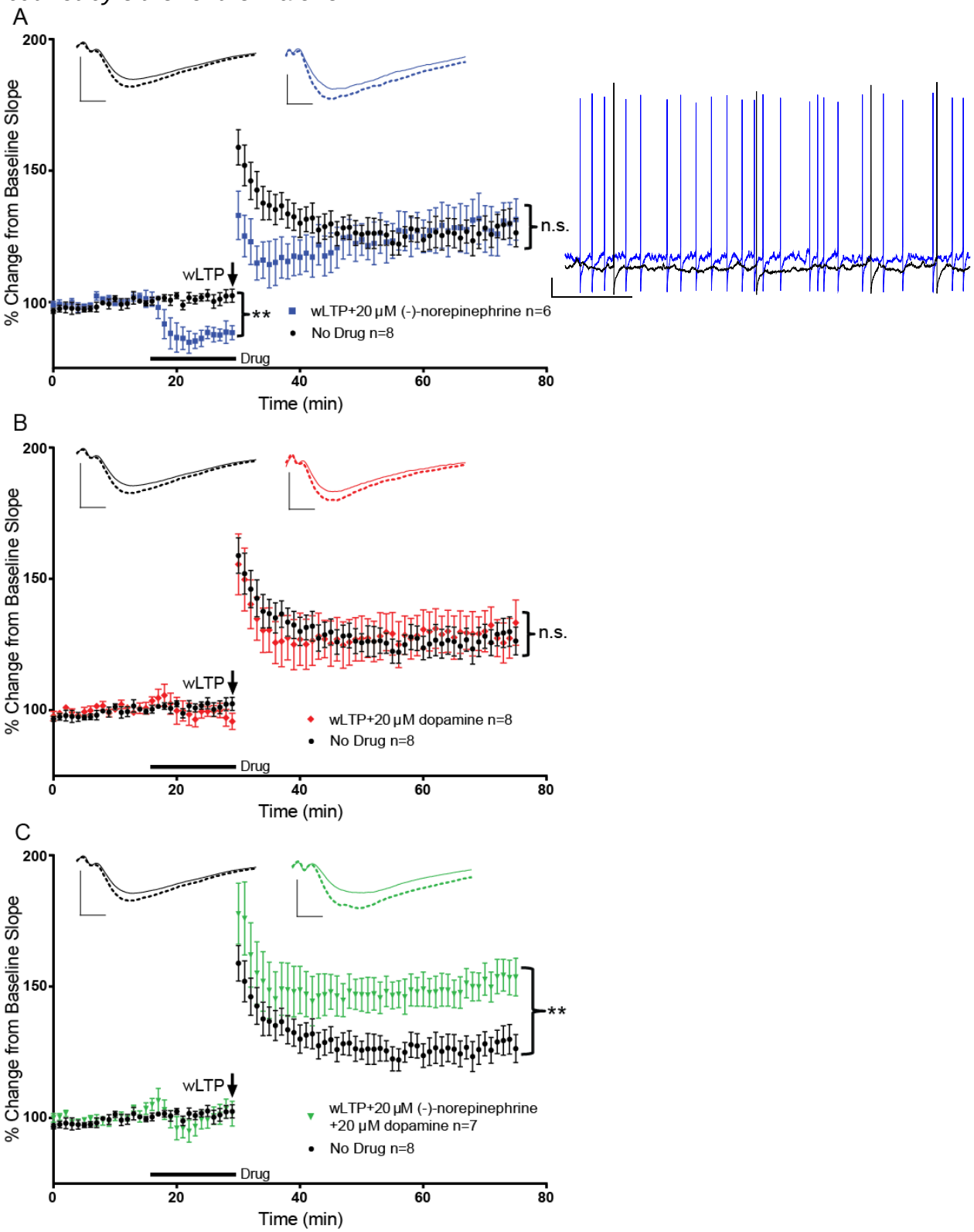
A, Schematic of contextual fear conditioning paradigm showing time timing of tone/shock pairings in on each day in Context A (see **Fig. 2.8D**). Dotted lines represent times when the animal was placed in the cage before tone/shock pairing started. **B,** Percent freezing over the first minute of a 300 second learning trial which took place on day 2 in Context A, the same context as Day 1. Blocking D1/D5 receptors prior to training essentially eliminated all memory of the association between Context A and a foot shock (red bar) compared to a saline control (black bar). However, blocking the norepinephrine transporter with nisoxetine (teal bar) trended toward increasing the memory of the fearful association. After a one-way ANOVA and a Dunnett's multiple comparisons test, the asterisk represents an adjusted p-value of p=0.0124 and the hashtag represents p=0.1987.

Figure 3.7 *Knocking out NMDA receptors from catecholamine neurons reduces the magnitude of dopamine-dependent LTP in dorsal hippocampus.*



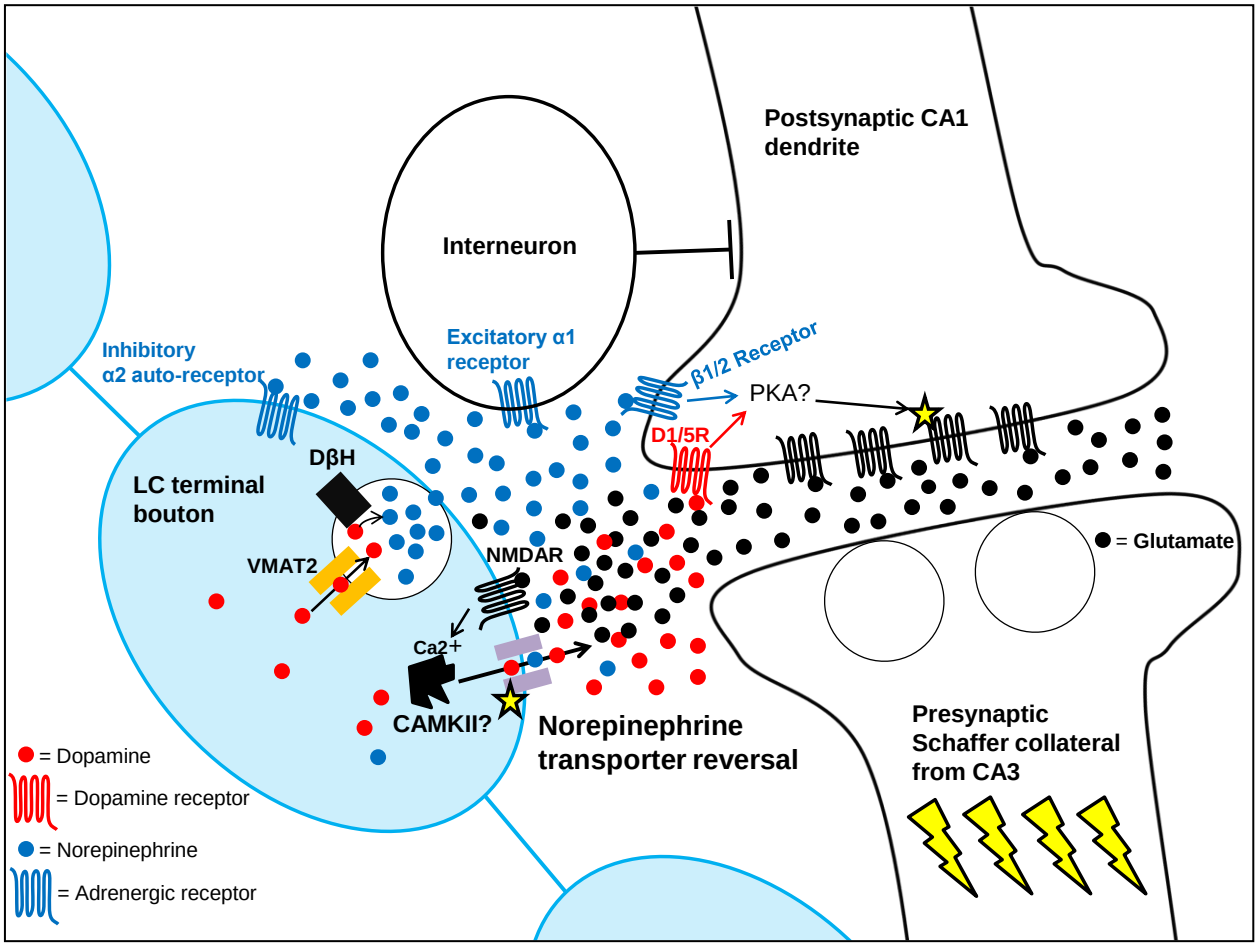
A, The same strong LTP protocol used previously (strLTP, black arrow) was administered in slices from Cre(-) control mice (black circles) and NMDA-NR1 subunit knockout mice (open circles). All data points are represented as mean +/- SEM. Tests for significance were done using a two-way repeated measures ANOVA over the last 30 minutes of strLTP. Double asterisk represents a significant difference <0.01, $F(1, 10) = 13.24$, $p=0.0046$. **B,C,D,** Immunnostaining of LC fibers in the CA1 region of the dorsal hippocampus showing co-localization (**D**) of the norepinephrine transporter (NET, green, **B**) and presynaptic NMDA receptors (NR1, magenta, **C**) on LC terminals.

Figure 3.8 *Weak LTP (wLTP) is enhanced by dopamine and norepinephrine together but not by either of them alone.*



A, Left, wLTP (black arrow, black circles) is not enhanced with bath application of 20 μM norepinephrine alone (blue squares, 61-76 min), $F(1, 12) = 0.05005$, $p=0.8267$. Instead, the application of norepinephrine after a 15 minute baseline significantly reduced the size of the baseline field potential slope (blue squares, 16-30 min), $F(1, 12) = 18.87$, $p=0.0010$. Right, example excitability traces from a CA1 stratum radiatum interneuron before (black) and 10 minutes after (blue) the addition of NE to the bath. **B,** Bath application of 20 μM dopamine (red diamonds) was also unable to enhance wLTP, but did not show a similar decrease of baseline slope, $F(1, 14) = 0.1202$, $p=0.7339$. **C,** Application of 20 μM norepinephrine and 20 μM dopamine together (green triangles) produces a significant increase of wLTP, $F(1, 13) = 9.318$, $p=0.0080$. All data points are represented as mean +/- SEM. Tests for significance were done using a two-way repeated measures ANOVA over the last 15 minutes of wLTP, or during 15 minutes of drug application as shown in panel A. Asterisks represent p-values < 0.05, double asterisks represent p-values <0.01, while ‘n.s.’ stands for ‘not significant’.

Figure 3.9 *Working model of the local LC-hippocampal circuit at CA1 synapses during selective attention and synaptic potentiation.*



CHAPTER FOUR: Closing Discussion and Future Directions

Closing Discussion

This dissertation comprises the first set of experiments aimed at determining the role of LC-dependent dopamine transmission in synaptic plasticity. Our results build from years of speculation regarding the source of DH dopamine, but in doing so, raise many more questions about catecholamine interactions in the region (Duszkiewicz et al., 2019; McNamara and Dupret, 2017). Upon completing this body of work, it seems clear that LC-DA influences plasticity and memory formation in dorsal CA1. We showed that LC-DA release likely only occurs during phasic bursting of LC neurons. This phasic LC firing is only observed when an animal is experiencing something salient in their environment (e.g. frightening, dangerous, exciting, or necessary for survival), suggesting that LC-DA release is only able to modulate synaptic strength and memory formation at CA1 synapses if a stimulus is important enough and worth remembering.

However, some reports describe findings contradictory to ours. In one such study, Lemon and Manahan-Vaughan (2012) exposed mice to a novel environment or stimulated the locus coeruleus while recording CA1 fEPSPs *in vivo*. With this paradigm, they saw significant long-term depression of the CA3-CA1 synapse that positively correlated with enhanced learning, and then blocked the LTD and memory with D1-like receptor antagonists. This is the opposite of our observations, and it is not immediately obvious why such a disparity exists. One explanation could be that electrical stimulation in slices activates nearly all CA3 axons and strengthen most synapses in CA1. Contrarily, in the 2012 study they were not giving electrical CA3 stimulation, and only measuring the more natural plasticity in CA1 after mice were exposed to a novel

experimental environment. It could be that normal *in vivo* learning only recruits a small subset of CA3-CA1 synapses to represent a stimulus, and the rest of the synapses would be dampened by α1-receptor activation on interneurons. If the majority of synapses are dampened, it could overpower the potentiation from the minority of highly active glutamate inputs involved in the representation, and resemble LTD at the global circuit level.

One theory that may help to connect the LC's role in hippocampal learning to more global LC control of network activity is the "network reset" theory proposed by Susan Sara's group (Bouret and Sara, 2005). According to this, short-lived phasic LC activity and subsequent NE release in the prefrontal cortex causes an animal to disengage from what it is currently doing and shift attention to a new stimulus, presumably one that is more immediately salient. Our results indicate that this phasic LC activity will release DA, marking the most recently active hippocampal synaptic representations for long lasting potentiation and memory storage. This fits well with network reset theory, as representations that an animal was just manipulating in prefrontal cortex could be transferred to the hippocampus for consolidation and association with recent past or future stimuli that are also deemed worthy of consolidation.

In line with this, when mice are placed within a novel context *after* the acquisition of new information, they are able to retain the past information more reliably. This increased consolidation is dependent on both LC firing and D1-like receptor activation in dorsal CA1 (Takeuchi et al., 2016). An interesting *ex vivo* experiment that could shed

light onto this phenomenon is similar to the optogenetic experiments done in **Chapter 2**. Instead of giving the optogenetic LC stimulation before and during the weak LTP high-frequency electrical stimulation, weak LTP could be given first, followed by phasic optogenetic LC stimulation 30 minutes later. I would expect that this phasic LC stimulation given *after* weak LTP would either prevent the decrease in fEPSP slope due to weak potentiation, or even promote the fEPSP slope to increase and reach a level similar to that seen when strong LTP stimulation is given. A result similar to this would further link LC activity to the control of temporally associated learning.

Retroactive potentiation by DA has also been observed in CA1 of the hippocampus in the context of spike-timing dependent potentiation (STDP) (Brzosko et al., 2015). Importantly, the authors found that NMDA receptors are required for this STDP enhancement by DA. On the other hand, our results here support a more proactive role for LC-DA in memory formation. In combination, the temporal nature of this LC-DA modulation indicates that it operates over a specific time-window to consolidate associations occurring before or after the current stimulus. The length of this time-window remains unknown, and will be an important point of study in this field going forward.

Within this dissertation, a couple of experiments seem to contradict each other at first glance. For instance, results from **Figure 3.1** showing that β-adrenergic receptor blockade does not reduce LTP clash with the interpretation of **Figure 3.8** that both DA and NE need to be present for LTP enhancement. With no knowledge of the electrical stimulation paradigms, one would expect β-adrenergic receptor blockade to reduce LTP

since NE needs to be present alongside DA to enhance it. However, strong (**Figure 3.1**) and weak (**Figure 3.8**) LTP stimulation likely produce different physiological and biochemical effects at CA1 synapses, with different dependences on glutamatergic signaling through NMDA receptors. It is plausible that weak LTP stimulation is only sufficient to barely phosphorylate AMPA receptors, but not to insert new ones into the postsynaptic density or initiate protein synthesis (Reymann and Frey, 2007). The application of NE and DA are therefore necessary to reach some threshold for stronger synaptic augmentation. Contrarily, the strong LTP protocol already stimulates a large amount of glutamatergic activity and probably does not rely as heavily on catecholamines, but more on NMDA receptor activation. Our assumption is that the weaker LTP protocol, being less intense, is more natural as it requires neuromodulation by catecholamines similar to *in vivo* learning.

Future Directions

One possible mechanism that we have not ruled out is canonical neurotransmitter release from LC vesicles. Vesicular release could occur if the enzyme dopamine β-hydroxylase (DBH) becomes saturated with higher-frequency LC firing and fails to convert all DA into NE inside of vesicles. In our case, blocking the NET may not have necessarily blocked the reversal of neurotransmitter uptake, but rather led to lower excitability in LC terminals due to less influx of sodium ions during reuptake. In fact, catecholamine transporters are known to resemble ligand-gated ion channels because the uptake of NE or DA accompanies a depolarizing inward current (De Felice, 2017). It is possible that blocking the NET blocked this inward current that would normally be

quite large after phasic LC stimulation and NE release. This may have attenuated a positive feedback loop that usually leads to LC terminal depolarization and more rapid vesicle release, a condition under which DA may not be fully converted into NE.

A second possibility is that some LC boutons do not express DBH at all and only produce dopamine. Checking for vesicular DA release would require the specific elimination of vesicle fusion in LC axons while performing the same NET experiments as above. Recent genetic technology could allow for this type of manipulation with a photoactivatable botulinum toxin that could be genetically targeted to LC cells (Liu et al., 2019). Likewise, reserpine could be used to deplete CA1 vesicles of catecholamines (Yaffe et al., 2018), after which only NET signaling would remain. However, both approaches could lead to a huge imbalance in the usual catecholamine concentration gradient from the extracellular space to the cytosol, causing unnatural NET reversal.

A final set of experiments is needed to advance our understanding of how DA+NE interaction at single neurons imposes the circuit-level effects that we saw in our fEPSPs. It will be important to untangle the functions of DA and NE together or separately within individual CA1 neurons. This information could provide insight into the "Why?" question of DA and NE co-release from LC terminals. As touched on in **Chapter 3**, I predict that NE would have more of a network tuning effect by directing glutamate signaling through the most active inputs, while suppressing the least active ones, leading to an increased signal-to-noise ratio and less overlap in environmental representations. Norepinephrine could impose this type of effect on hippocampal circuits using a mechanism similar to that described with oxytocin signaling (Owen et

al., 2013), after which dopamine would help consolidate the most important synapses into stable, and less generalized, memory traces. Excitability and synaptic activity of pyramidal cells and stratum radiatum interneurons in the CA1 region should thus be recorded in the presence of DA alone, NE alone, and both together.

Closing remarks

In summary, this and other recent work have firmly established the LC as a DA-secreting nucleus in multiple brain regions. This realization has major implications not only for information processing, but also for treatment or diagnosis of neurodegenerative disease pathology (Betts et al., 2019). Locus coeruleus neuronal dysfunction or death has been increasingly correlated with symptom development in Alzheimer's disease (Kelly et al., 2017). Therefore, our conclusions could lead to new therapeutic strategies for Alzheimer's and related diseases.

In terms of a more real-world, everyday use arising from these conclusions, the molecular connection between novelty and enhanced learning could be implemented in school curricula as a way to help students retain information (Ballarini et al., 2013). More controversially, nootropic drugs may try to exploit these mechanisms for cognitive and memory enhancement. No matter the application, we hope that our initial findings can pave the way for a more detailed understanding of the complex interplay between glutamatergic information processing and its modulation by catecholamines.

BONUS CHAPTER: Examining the effects of the adenosine system on synaptic transmission in health and sleeping sickness

Intro/Results/Methods/Conclusion

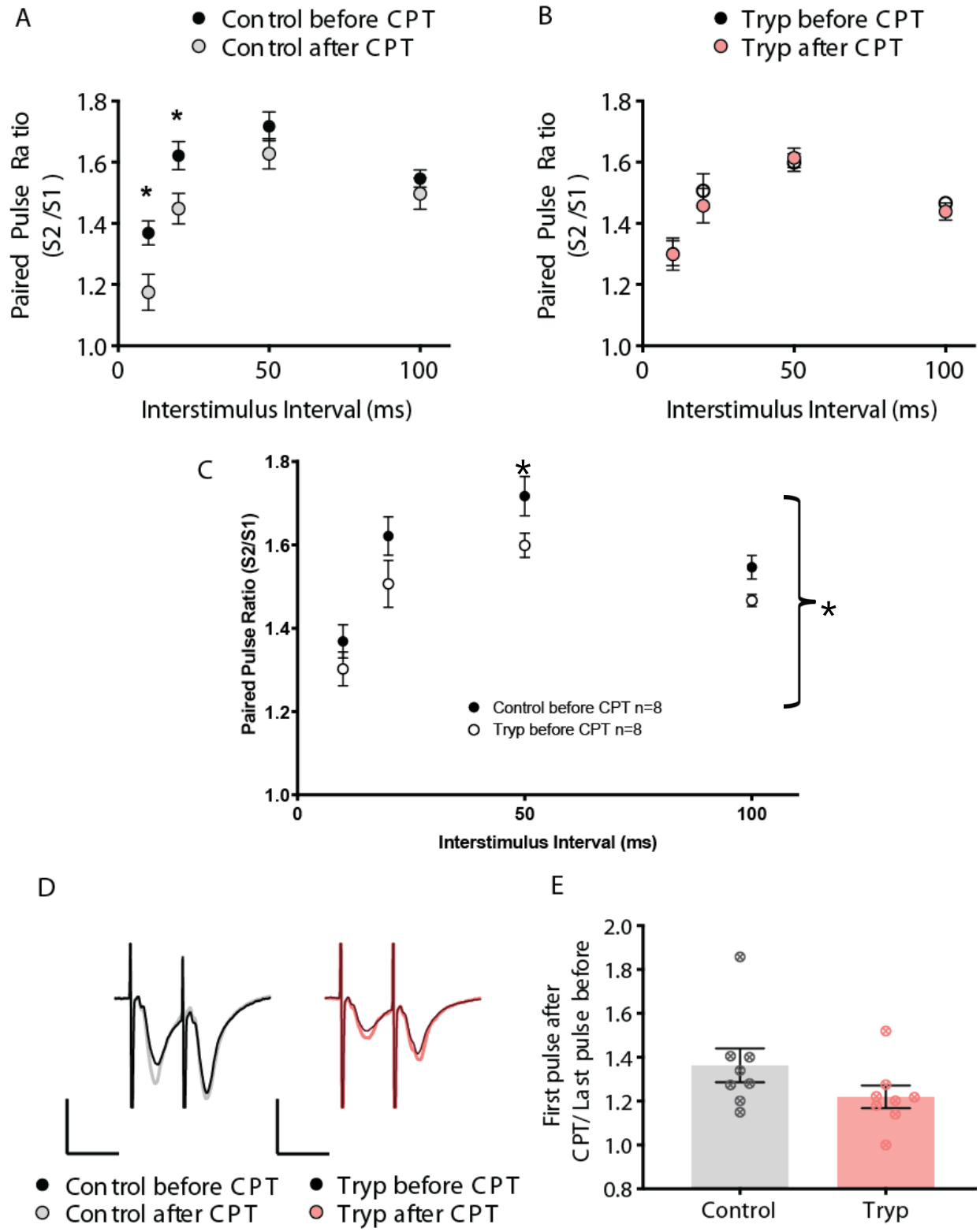
Infection with the parasite *Trypanosoma brucei* eventually leads to the development of sleeping sickness, a disease characterized by a highly disrupted circadian rhythm and excessive sleep loss (Rijo-Ferreira et al., 2018). If left untreated, sleeping sickness can cause coma and even death, although antiparasitic medications such as suramin are available and are quite effective (Steverding, 2010). The mechanism behind sleeping sickness's pathophysiology is unknown, but a severe lack of homeostatic sleep response indicates at least a partial involvement of the adenosine system (Bjorness et al., 2009; Huang et al., 2011). So, we went after the idea that sleeping sickness may exert its effects by disrupting adenosine A1 receptor signaling, a major receptor involved in the homeostatic sleep response (Bjorness et al., 2016).

Activation of adenosine A1 receptors in hippocampus leads to a lower probability of neurotransmitter release at CA3-CA1 synapses. Under normal conditions in wild-type mice, this should cause an increase in paired-pulse ratio at Schaffer collateral synapses in slices. Conversely, blocking A1 receptors with a potent antagonist, 8-Cyclopentyltheophylline (CPT), will decrease paired-pulse ratio by reducing the constitutive signaling at these receptors in our control animals (sham cranial injections without the *Trypanosoma brucei* parasite) (**Bonus Fig. 1A**). To test whether Trypanosome-infected mice exhibited deficits in adenosine signaling, we infected mice with the *Trypanosoma brucei* parasite (Rijo-Ferreira et al., 2018). After allowing 85 days

for sleeping sickness to progress, mice were subject to the same slicing (see methods from previous chapters) and Schaffer collateral paired-pulse procedure as control mice. Interestingly, slices from infected mice did not respond to CPT application with decreased paired-pulse ratios (**Bonus Fig. 1B**). Comparing control mice to Tryp infected mice before CPT application revealed significantly lower baseline paired-pulse ratios at 3 out of 4 interstimulus intervals (**Bonus Fig. 1C**).

This small set of experiments implicates adenosine A1 receptor signaling deficits in the pathophysiology of sleeping sickness. Although, the results say nothing about the downstream signaling of A1 receptors, which are GPCRs coupled to G_i effectors (Bjorness et al., 2009). It is possibly that the disruption manifests downstream of these receptors, for example at cAMP, kinase, phosphatase, or ion channel signaling. More biochemical work is needed to tease apart the underlying molecular mechanism.

Bonus Figure 1 *Trypanosome infected mice display deficits in adenosine receptor signaling.*



A, Paired-pulse ratios using field recordings from the CA1 of the hippocampus of control animals (injected with vehicle but not the parasite) before and after treatment with an adenosine A1 receptor antagonist, cyclopentyltheophylline (CPT). Controls demonstrate a decrease in paired-pulse facilitation after CPT treatment at 10 and 20 millisecond paired pulse interstimulus intervals (ISI) (2-way ANOVA; main effect of treatment $F(3,56)= 25.10$; $p<0.0001$; asterisks indicate significant differences [$p<0.05$] after multiple comparisons). **B**, Tryp Infected mice show no response to CPT treatment. **C**, Control versus Tryp infected mice before the addition of CPT. A 2-way repeated measures ANOVA revealed a statistically significant overall difference between groups ($F(1,7)= 5.122$, $p=0.0482$, denoted by asterisk on the right side with bracket). A Sidak's multiple comparisons test revealed a statistically significant difference between the groups at 50ms ISI ($p=0.0444$, denoted by asterisk above the 50ms point). **D**, Representative traces from control and Tryp infected mice taken from recordings in **A** and **B**. **E**, First pulse after CPT divided by the last pulse before CPT should give a rough estimate of the immediate effect of CPT on overall synaptic transmission. Direct comparisons using this metric between control and treated animals failed to reach statistical significance ($p=0.1443$), but suggest that CPT increases basal transmission to a lesser extent in Tryp infected mice than in Control mice. All data points are shown as mean +/- SEM.

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