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Differential Cholinergic Modulation of Prelimbic and Thalamic Input to the Basolateral Amygdala

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DIFFERENTIAL CHOLINERGIC MODULATION OF PRELIMBIC AND THALAMIC INPUT TO THE BASOLATERAL AMYGDALA

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DEDICATION

This manuscript is dedicated to my family. To Hudson, thank you for your constant brotherly love and encouragement. While we don't see each other as often now, I know that I can always count on you to listen and provide rational, thoughtful support. Mom and Dad, thank you for three decades of unwavering love and support. From early days of reading Bob Books, spending hours upon hours to flip through math flashcards and grammer lessons that never seemed to end, being at the sidelines of school and sports competitions, to later years of providing listening ears when I needed them and encouraging me to pursue my goals and never give up, you both have shaped who I am today.

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ABSTRACT

The basolateral amygdala (BL) is critical for emotional memory acquisition and expression. It receives afferent projections from both cortical and subcortical regions that send glutamatergic transmission to the BL. This input conveys information necessary for survival, including information about one's behavioral state and environment. How this information is integrated and processed by the BL, however, remains largely unknown. Interestingly, the BL receives the densest amount of cholinergic innervation from the basal forebrain. This acetylcholine (ACh) can modulate emotional memories, but how it modulates specific afferent inputs to the BL is unexplored. To answer this question, we used brain slice field and whole cell electrophysiology, optogenetics, and pharmacological tools to investigate how released endogenous ACh modulates afferent input to the BL. We found that endogenous ACh suppresses cortical input to the BL through muscarinic receptors. We then further explored this modulation by optogenetically activating prelimbic (PL) and thalamic (THAL) input to the BL and pharmacologically activating muscarinic ACh receptors to examine pathway-specific regulation of glutamatergic transmission from these inputs. Muscarine, by acting on M3 and M4 receptors at PL synapses and M3 receptors at THAL synapses, suppressed glutamatergic input from both regions. However, muscarinic receptor activation inhibited the prelimbic input to a significantly greater extent than the thalamic. Furthermore, in examining the mechanisms underlying this inhibition, it was found that muscarinic inhibition of these two pathways occurs through distinct mechanisms. At PL input muscarinic receptors inhibit

glutamatergic transmission through an endocannabinoid independent mechanisms whereas they inhibit thalamic input through an endocannabinoid-dependent mechanism. Additionally, muscarinic receptors displayed frequency-dependent regulation of glutamate transmission. When the PL and THAL inputs were stimulated at low frequency trains (1Hz), muscarinic inhibition was consistent throughout the train. However, when PL and THAL were stimulated at gamma frequency trains (40Hz), muscarinic inhibition remained intact throughout the train at THAL inputs, but was relieved at PL inputs. Taken together, these findings suggest differential modulatory mechanisms during enhanced cholinergic tone in the BL, such as during exercise or unexpected stimuli. However, this inhibition is frequency dependent. During behavioral states in which the PL is oscillating at gamma frequency, that muscarinic inhibition could be overcome. Together, these results indicate unique modulatory mechanisms conferred upon the PL and THAL input to the BL and could serve as rich avenues for pharmaceutical manipulations in future behavioral studies.

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LIST OF ABBREVIATIONS

2-AG 2-arachidonoylglyceral (2-AG)

AB .....Accessory Basal Nucleus

ACh .....Acetylcholine

AChE..... Acetylcholinesterase

AEA .....N-arachidonoyl-ethanolamine

BL .....Basal/Basolateral Amygdala Nucleus

BL .....Basolateral Amygdala

BNST .....Bed Nucleus of Stria Terminalis

BOAT..... Bed Nucleus of the Accessory Olfactory Tract

CAMP ..... cyclic AMP

CB1R.....CB1 Receptor

CB2R.....CB2 Receptor

CeA ..... Central Amygdala

ChAT.....Choline Acetyltransferase

ChR2 ..... Channelrhodopsin

Co.....Cortical Nucleus

CS.....Conditioned Stimulus

DAG lipase.....Diaglycerol Lipase

DSI ..... Depolarization Induced Suppression of Inhibition

eCBs.....endocannabinoids

fEPSP ..... (field) Excitatory Postsynaptic Potential

fIPSP ..... (field) Inhibitory Postsynaptic Potential

GDP ..... Guanosine Diphosphate

GPCR ..... G Protein Coupled Receptor

GTP ..... Guanosine Triphosphate

IL ..... Infralimbic Cortex

mACHR ..... Muscarinic ACh Receptor

nAChR ..... Nicotinic ACh Receptor

NAT ..... N-acyltransferase

NLOT ..... Nucleus of the Lateral Olfactory Tract

NMDA ..... N-methyl-D-aspartate

PAC ..... Periamygdaloid Cortex

PL ..... Prelimbic Cortex

PLC ..... Phospholipase C

PT ..... Paratenial Nucleus

PVT ..... Paraventricular Nucleus

REM ..... Rapid Eye Movement

SI ..... Substantia Innominata

Te1 ..... Primary Auditory Cortex

THAL ..... Midline Thalamic Nuclei

US ..... Unconditioned Stimulus

VAChT ..... Vesicular Acetylcholine Transporter

vSUB ..... Ventral Subiculum

CHAPTER 1

GENERAL INTRODUCTION

1.1 SIGNIFICANCE

Persistent, intrusive and excessive feelings of worry and fear affect the 40 million, or 18% of, Americans who suffer from a form of anxiety disorders (Kessler et al., 2005). Anxiety and fear disorders, including post-traumatic stress disorder (PTSD), are characterized by dysregulation of the neural fear circuitry in the brain. Thus, an understanding of how the neural regions within the fear circuit are connected and regulated is required in order to develop treatments for anxiety disorders such as PTSD. One of these regions, the basolateral amygdala (BL), is heavily implicated in emotional disorders. Fear circuit regions that relay afferent glutamatergic projections to the BL include the prelimbic cortex (PL), the midline thalamic nuclei (THAL) and the ventral subiculum (vSub). Interestingly, the BL receives the densest amount of cholinergic projections from the basal forebrain, suggesting a potential to exert modulatory influences by acetylcholine (ACh) on afferent inputs to the BL. Behavioral studies have shown that ACh is involved in learning and memory, and that disruption in the cholinergic system leads to impairments in fear acquisition and fear extinction. Yet despite the importance of ACh in emotional memory and the role of the BL in emotional regulation, the mechanisms by which ACh regulates neurotransmission to the BL remain unknown. Such an understanding would elucidate

targets for pharmacotherapies to pair with behavioral interventions such as exercise in an effort to alleviate symptoms of anxiety disorders.

Our goal is to fill the void in understanding how BL functioning is modulated by exploring how ACh receptors regulate afferent input to the BL from regions involved in the fear network. Such a comprehensive understanding of the fear circuitry would enable more targeted and effective behavioral and pharmacological interventions for emotional disorders. This study provides novel insight into how ACh receptor subtypes modulate different inputs to the BL and will augment the development of interventions and therapies for anxiety disorders.

1.2 ACETYLCHOLINE

1.2.1 ACETYLCHOLINE: THE FIRST NEUROTRANSMITTER DISCOVERED

We owe much of what we know about acetylcholine to Englishman Henry Dale and German Otto Loewi. While testing sympathomimetic effects of various compounds extracted from a rye fungus, Henry Dale noticed that one of his extractions reversed the sympathetic effects of adrenaline. Dale performed further experiments to conclude that the physiological effects he was seeing were due to actions "on the sympathetic myoneural junctions," building upon Thomas Elliott's previous proposal that adrenaline could be released upon nerve stimulation (Dale 1906). Around this same time, scientists Hunt and Taveau from the United States Public Health Service found certain cholinergic compounds reduced blood pressure in a parasympathetic manner more potent than the sympathetic one of adrenaline (Hunt and Taveau 1909, Fishman 1972). Keeping Hunt and Taveau's findings in mind, Dale noticed that application of one of his rye fungus extracts evoked

responses similar to those seen by his American counterparts, and commenced detailed studies of cholinergic effects on different organs. By 1914, it seemed that Dale had found the parasympathetic "ying" to the sympathetic "yang" in the autonomic nervous system, as he concluded that acetylcholine and adrenaline have effects that "are in many directions at once complementary and antagonistic" (Dale 1906, Fishman 1972). However, at that point, it was not yet known if acetylcholine existed endogenously in the body. In 1921, Otto Loewi's elegant experiments conducted in Graz, Austria that confirmed the hypothesis that chemicals called "neurotransmitters" were responsible for mediating nerve impulses from one nerve to another. Separating a frog heart in solution with an intact vagus nerve from another frog heart with a severed vagus nerve, Loewi stimulated the first heart's intact vagus nerve, collected the surrounding solution, and added it to the fluid surrounding the second heart. When he did this, he found the second heart started beating as if its missing vagus nerve were stimulated. Thus, Loewi concluded that an actual, chemical messenger he termed Vagusstoff, was released from the vagus nerve and acted on other tissue. This proved to be the seminal finding that Vagusstoff, later found to be acetylcholine, was the first discovered neurotransmitter.

1.2.2 ACETYLCHOLINE SYNTHESIS, STORAGE, SECRETION AND DEGRADATION

As the very first neurotransmitter discovered, ACh has been extensively studied in both the periphery and in the central nervous system. ACh is synthesized in the cytoplasm of select neurons by the enzyme choline acetyltransferase (ChAT). ChAT is synthesized in the soma but is transported down neuronal axons to the terminals where it synthesizes

ACh from choline and acetyl-coenzyme-A by transferring an acetyl group from acetyl coenzyme-A to choline. ChAT is selectively expressed only in cholinergic neurons and thus can be used as a biomarker of cholinergic neurons. The choline from which ACh is synthesized in the liver, is introduced by diet or is recycled choline from former ACh molecules. As it is not naturally produced in neurons, cells that synthesize ACh must also express the high affinity choline uptake transport protein in order to bring in choline from the extrasynaptic space to the cell to be synthesized into ACh. Once ACh is synthesized, it is loaded into vesicles by vesicular ACh transporter (VAChT) that pumps ACh in while pumping hydrogens (H+) out. Thus, another marker commonly used to determine cholinergic activity in neurons in VAChT. Once an action potential depolarizes a nerve terminal and Ca2+ influx results, ACh is released and can bind to its receptors located both presynaptically and postsynaptically. Cholinergic signaling is terminated by the degradation of ACh by the enzyme acetylcholinesterase (AChE) (and to a smaller extent, by butyrylcholinesterase) into choline and acetate. The choline that is created is then reuptaken into terminals by high affinity uptake transport proteins and ACh synthesis can begin anew.

Peripherally, ACh is largely known for its rapid effects at the neuromuscular junction to evoke contraction of muscles. However, ACh is one of the most influential neurotransmitters in the autonomic nervous system, and thus also affects many peripheral physiological functions associated with parasympathetic stimulation including heart rate, bladder control, sexual behavior, perspiration and digestion (Tiwari et al., 2013). Centrally, ACh can act as a neuromodulator to regulate many aspects of neuronal functioning by altering neuronal excitability, affecting probability of neurotransmitter

release from presynaptic neurons, modulating postsynaptic physiological characteristics that raise or lower the threshold for responsiveness, inducing synaptic plasticity, and more recently explored, by affecting cortical plasticity by regulating Ca^{2+} levels in astrocytes (Dannenberg et al., 2017; Picciotto et al., 2012; Araque et al., 2002; Takata et al., 2011).

1.2.3 ACETYLCHOLINE FUNCTION

An abundance of studies have found neuromodulatory effects of ACh to be foundational for ACh's importance in healthy cognitive functioning. Normal cholinergic modulation is important for memory, learning and attention. The vital role of ACh in memory has been illustrated in pharmacology studies that antagonized cholinergic receptors in rodents and saw a resulting impairment on performance in working memory task (Levin et al., 2002; Wirsching et al., 1984). Blocking cholinergic receptors in humans with the FDA-approved scopolamine impairs memory, reduces learned verbal associations in humans and reduced neural activity in brain regions important for encoding (Ghoneim and Mewaldt 1975; Schon et al, 2005; Atri et al., 2004). Pharmacologically increasing the amount of ACh by administering AChE inhibitors has been shown to improve cognitive impairments and is a current treatment avenue for patients suffering from Alzheimer's Disease (Hasselmo & Sarter, 2011; Aigner and Mishkin, 1986; Thal et al., 1983; Bartus, 1979). Blocking cholinergic receptors in the dorsal hippocampus seems to cause impairment in the acquisition of spatial memory (Dannenberg et al., 2017; Blokland et al., 1992; Riekkinen et al., 1997; Herrera-Morales et al., 2007). However, elevating cholinergic levels in specific subregions of the hippocampus do not affect acquisition of spatial information but instead block retrieval of that information, suggesting phase-

specific effects of varying levels of ACh whereby elevated ACh is involved in encoding and decreased ACh involved in retrieval (Giocomo & Hasselmo, 2007; Rogers & Kesner 2003). Taken together, pharmacological, behavioral and neuroimaging studies in humans suggests healthy cholinergic modulation of brain regions and neural circuits is important for learning and memory.

Through receptor-mediated mechanisms to be discussed later, ACh is thought to affect attention, learning and memory through presynaptic and postsynaptic effects on pyramidal neurons as well through effects on interneurons. ACh can alter the dynamics of information flow to selectively inhibit one pathway while sparing another. The functional consequence of such a dynamic regulation is to increase the signal-to-noise ratio of information within one pathway while filtering out extraneous information not necessary for a goal or survival (Hasselmo & Giocomo 2006; Thorn et al., 2016).

1.2.4 ACETYLCHOLINE RELEASE IS STATE-DEPENDENT

ACh levels have been shown to fluctuate in response to behavioral states and demands. Microdialysis experiments have found ACh levels in the cortex, hippocampus and thalamus to be elevated during quiet waking (when an animal is not participating in exploratory activities), active waking (exploratory behavior) and REM sleep (with the greatest amount of ACh increase in the hippocampus than the cortex during REM) (Williams et al., 1994; Marrosu et al., 1995; Ruivo et al., 2017).

While the mnemonics can be argued as to whether it is “attention,” “novelty,” “saliency,” or “unexpectedness,” there appears to be a clear increase in ACh during states when one needs to predict what will happen in the environment in order to respond to

potentially threatening or life-saving stimuli like a predator or food, respectfully (with an exception being an increase of ACh during REM sleep). Such states would include the early stages of learning when one doesn't know what will follow a stimulus, when an organism is shifting from sleep into a high arousal state, when an organism is in a new environment, during an attentionally demanding scenario, etc. Intriguingly, these are the types of states in which ACh has been shown to be elevated in both animal models and humans.

The ability to determine if a stimulus in the environment, or the environment itself, is novel or familiar is crucial for survival. Evidence supports novelty or "unexpectedness" as being a key stimulus for ACh release. Interestingly, the confrontation with a novel stimulus seems to elevate ACh in the cortex and other brain regions regardless of the stimulus modality. Novel objects, environments, auditory stimuli, painful stimuli, even gustatory stimuli have been shown to elicit the highest ACh release when presented initially and elicit decreasing amounts of ACh when presented again. Giovannini et al. (1998) showed that if an animal is placed in a new environment, their ACh levels increase almost two-fold in the hippocampus (200%) and by 64% in the cortex, whereas placing them in the new environment again results in only 37% increase in the cortex and only 51% increase in the hippocampus. In a study by Acquas et al. (1996) to determine ACh release in response to stages of fear learning, ACh release appeared largest when a novel object is the most unexpected, as cholinergic tones in the prefrontal cortex and in the hippocampus were the greatest after a paired auditory stimulus and light, but decreased over the course of repetitive presentations (Acquas et al., 1996; Pepeu and Giovannini 2004). In this study, rats were divided into three groups: one that received a prior training session of auditory

tones played with light, a second group that received the auditory tone and light followed by a footshock, and a third group that received neither auditory tones nor light. On a following day, all three groups were subjected to the tone and light. In agreement with the role of ACh in novelty, the group that was "habituation to," or received prior exposures of auditory tones and light, showed no increase in ACh in response to the second round of presentations to these tones, whereas the other two groups showed increased in ACh in their frontal cortices and hippocampi (Acquas et al., 1996). Survival doesn't just rely on detecting a novel stimulus that predicts a noxious or harmful stimulus, but also one that predicts an appetitive stimulus, as retrieving food, mates, etc. are equally necessary for survival. Ruivo et al. (2017) used electrochemical biosensors with a resolution less than a second to measure ACh release and found ACh is released in a tonic manner when changing states of attention and arousal and in a phasic manner when performing a task that demands working memory. The ability to distinguish a novel and a familiar taste is also vital to survival. Thus, not surprisingly, ACh has been found to play a role in novel versus familiar tastes. In the insular cortex, the primary gustatory center, ACh levels increase in response to a novel taste (in their study, saccharine) but decreased over repeated presentations of the novel taste (Miranda et al. 2000). ACh in this gustatory context appears to be directly involved in novelty detection, as a separate study blocked muscarinic ACh receptors in the insular cortex and found when a taste was administered a second time (ie it was no longer "novel"), muscarinic blockade seemed to reduce the memory that the reintroduced taste had been tasted before (Noar & Dudai 1996).

1.2.5 EXERCISE AND ACETYLCHOLINE

It should come as no surprise that evolutionarily conserved behaviors like walking and running have been shown to influence many aspects of the cholinergic system across the lifespan, given the important of ACh for cognitive behaviors that support survival. Both short, acute bouts of physical activity and longer, chronic exercise regimes have been shown to affect cholinergic tone in the brain. In both young adult (3-4 months) and aged rats (26-29 months), walking at a moderate speed for 30 seconds to 5 minutes produced a significant increase in hippocampal and cortical ACh that peaked during exercise and returned to baseline after exercise cessation (Kurosawa et al., 1993; Uchida et al., 2006). Acute running at a moderately intense speed of 60ft/min also elevates ACh in the hippocampus compared to when the animal was sedentary, but levels declined to baseline after exercise cessation (Dudar et al., 1979). Another mechanism by which acute physical activity can influence the cholinergic system is by resulting in inhibition of acetylcholinesterase (AChE), thereby increasing synaptic levels of ACh. Taskaris et al. (2006) subjected rats to either a 2-hour swimming session or a 5-hour swimming session and found that whole-brain levels of AChE was inhibited by 30% and 45%, respectively. However, it has been shown that stress can reduce the activity of AChE in the hippocampus, thereby indirectly increasing cholinergic tone (Rao & Raju, 2000), so the findings that forced swim reduced AChE activity may be confounded by the stress of the prolonged swimming session. Effects of acute exercise bouts on the cholinergic system seem to be short-term responses to exercise as opposed to lingering adaptations, as increased ACh levels observed in the previously summarized studies all declined once exercise terminated and AChE levels were not measured further out than 24 hours post-

exercise. Overall, there seems to be a short term response to acute bouts of physical activity on the cholinergic system throughout various brain regions.

In addition to short bouts of physical activity, regular training has also been shown to affect the central cholinergic system as well. Middle-aged rats (18 months of age) that underwent a chronic swimming regime of 2hrs/day, 5days/week for 24 weeks displayed a significant reduction in AChE activity in both the medial prefrontal cortex and hippocampus. The same exercise protocol induced an upregulation of the muscarinic ACh receptor m1 protein expression and mRNA levels in the mPFC and hippocampus. These findings taken together suggest that in older individuals, a chronic exercise regime holds the potential to increase cholinergic function in various brain regions, as chronic exercise increased synaptic ACh and the expression of M1 receptors upon which that ACh can act (Abhijit et al., 2017). Chronic exercise affects the uptake of ACh as well. In a separate study, a chronic exercise regime for 14 weeks (1 hr/day at 75% of VO2max, 5 days/week, 14 weeks) resulted in a decrease in the choline reuptake transporter in the hippocampus, whereas acute exercise in the same study resulted in an increase of hippocampal ACh reuptake transporter, suggesting that there may be different underlying mechanisms of acute versus chronic exercise on cholinergic functioning (Fordyce & Farrar 1991). Similar to the findings by Abhijit et al. (2017) that running upregulates expression of M1 receptors in the hippocampus, Fordyce & Farrar found that chronic running, but not an acute bout of exercise, significantly upregulated muscarinic receptors in the hippocampus. Interestingly, this may be reflecting of chronic exercise activating mechnisms that elicit long-term changes, as chronic exercise training seems to exert adaptations to exercise that can be detected only after two weeks post-exercise. In their 2016 study, Hall and Savage induced

a loss of cholinergic basal forebrain neurons in a rat model of thiamine deficiency. Diseased and healthy (control) rats were assigned to either a sedentary group or a voluntary exercise group provided with a running wheel and allowed ad-libidum access to running for two weeks. Rats were then subjected either 24 hours or 2 weeks after the last day of exercise to an attentionally-demanding task and ACh levels in the hippocampus were measured with dialysis. Interestingly, it was found that ACh efflux in the hippocampus was highest after two weeks post-exercise in both the healthy and diseased groups than 24 hours later, suggesting that there is exercise-induced adaptations that occurs in the cholinergic system in trained vs sedentary rats. Equally as interesting, in the diseased animals with reduced basal forebrain cholinergic neurons, exercise seemed to increase the number of cholinergic neurons in the basal forebrain, and this effect was also only observed after a 2-week adaptation period (Hall & Savage 2016). Overall, both acute and chronic physical activity exert influences on the cholinergic system in the brain in a manner that causes an overall increase in ACh release and cholinergic signaling. Taken together, these studies suggest potentially different mechanisms induced by acute bouts of physical activity versus chronic training, and highlight the need to understand how ACh is functioning in the brain in order to determine the consequences of physical activity's influences on the cholinergic system.

1.2.6 ACETYLCHOLINE RECEPTORS

ACh, when released, has a multitude of effects. The effects of ACh are mediated by the specific receptor to which it binds. ACh binds to two classes of receptors: nicotinic ACh receptor (nAChR) and muscarinic ACh receptor (mAChR). Peripherally, nAChRs are heavily expressed in muscles and tissues involved in the autonomic nervous system and

mAChR are expressed in tissue in many different organs, including cardiac, bladder, etc. Centrally, nAChR and mAChR are expressed in nearly every brain region.

1.2.7 NICOTINIC RECEPTORS

Since its discovery at the end plate of neuromuscular junctions, nAChRs have become one of the most-studied ligand-gated ion channels. As their name implies, nicotinic ACh receptors selectively bind the agonist nicotine and are ligand-gated ion channels that, when bound by ACh, nicotine, or other agonists, open and allow positive cations through. nAChRs can be composed of four different subunits, α, β, δ, and γ (Albuquerque et al., 2009). Different compositions of these subunits combine into a pentamer to give rise to different nAChRs with different physiological properties; muscular nAChR consist of α1 subunits and one β, δ, and γ and ε subunits (Albuquerque et al., 2009). In other tissue, there appears to be a much more complex combination of subunit possibilities for nAChRs; 7 different forms of the α subunit (α2, α3, α4, α5, α6, α7, α9, and α10) and 3 different forms of the β subunit (β2-β4) exist in neurons in mammals (Albuquerque et al., 2009). Importantly, these subunits display distinct expressions in the body; α1 is expressed distinctly in muscles whereas α7 is expressed nearly distinctly in neural tissue. The composition of the pentamer has important functional consequences like receptor sensitivity, affinity and desensitization. For example, nAChRs composed of the α4 and β2 subunits (α4β2 nAChRs) tend to display the highest affinity for nicotine, α3β4 nAChRs have a much lower affinity for the agonist, the presence of β2 is necessary for nAChRs to be upregulated, the existence of the α5 subunit can affect receptor functioning without directly affecting the ligand binding site, and the α7 subunit-containing neuronal nACh

receptors rapidly desensitize, for example (Dani 2015, McCallum et al., 2006; Fenster et al., 1997; Albuquerque et al., 2009). In terms of cation permeability, there are subunit-specific differences; for example α7 receptors are more permeable to calcium than sodium ions (Castro & Albuquerque 1995).

nAChR in the brain have multiple functions depending on their location and subunit composition. For example, in CA1 of the hippocampus, α7 nAchRs are usually located postsynaptically on both neurons and interneurons, α4β2 nAChRs tend to be localized on GABAergic terminals synapsing onto cell bodies of pyramidal neurons or dendrites of interneurons, and α3β4 have been located on pyramidal neuron terminals that project to interneurons (Albuquerque et al., 2009). The presence of nAChRs on pyramidal neurons or interneurons would cause different effects in a circuit or region; nAChR activation on pyramidal neurons would elicit excitation whereas nAChR activation on interneurons would depolarize interneurons that would then elicit inhibitory effects. Regardless of the type of neuron on which they are located, nAChRs located on presynaptic terminals possess the ability to enhance neurotransmitter release upon activation, and the type of neurotransmitter would determine if the resulting synaptic transmission was excitatory or inhibitory.

1.2.8 MUSCARINIC RECEPTORS

mACHRs are G protein-coupled (GPCR) metabotropic receptors and thus compared to the faster ionotropic nAChRs, elicit slower responses to synaptic transmission. Similar to other GPCRs, mAChRs are composed of seven transmembrane-spanning domains with an associated heterotrimeric G protein complex (composed of three protein

subunits α, β and γ) (Huang & Thathiah 2015; Rosenbaum et al. 2009). Ligand, or ACh, binding induces conformational change in the receptor that then induces the substitution of a guanosine triphosphate (GTP) in the place of a released guanosine diphosphate (GDP) on the α subunit (Oldham & Hamm 2008). The association of GTP with the α subunit destabilizes the heterotrimer, and the βγ subunits dissociate from the GTP-α subunit. Each newly dissociated complex can then interact with their respective downstream proteins; βγ tends to remain near the membrane and thus interacts predominantly with membrane-bound effectors whereas the GTP-α subunit tends to affect cytosolic effector proteins (Thiel 2013). Heterotrimeric proteins are grouped into a family based on the specific type of α subunit it has. Three of the main types are $G\alpha_s$, $G\alpha_I$, and $G\alpha_q$ (Oldham & Hamm 2008). The signaling is aborted when the GTP gets hydrolyzed to GDP.

The three different types of Gα proteins, $G\alpha_s$, $G\alpha_i$, and $G\alpha_q$, interact with specific downstream effector targets whereby Gα activates adenylyl cyclase, G_i inhibits adenylyl cyclase, and G_q activates phospholipase C (PLC). The βγ also activates downstream targets, including certain types of inward rectifying channels and GPCR-associates with potassium, sodium and calcium channels, including presynaptic calcium channels (Hamm 1998).

Five different mAChRs exist in the body and are classified according to which G protein they couple to. M1, M3, and M5 are considered "M1 type" and preferentially couple to the G_q protein. These "odd" mAChRs, through their coupling with G_q, activates PLC which hydrolyzes phosphoinositol-1,4,5-biphosphate (PIP2) into inositol triphosphate (IP3) and diaglycerol (DAG). IP3 then causes an overall excitatory effect in the cell by elevating intracellular levels of calcium via release from the endoplasmic reticulum and

activating calcium-calmodulin, whereas DAG enables downstream phosphorylation by activating protein kinase C (PKC) (Thiel 2013; Felder 1995). The remaining mAChRs, M2 and M4, are called "M2 type" and preferentially couple to G_i proteins. As such, the "even" mAChRs, upon activation, couple to G_i and inhibit adenylyl cyclase which ultimately reduces cyclic AMP (Caulfield & Birdsall 1998; Thiele 2013; Brown 2010). An occasional exception is the ability of M2 and M4 receptors to stimulate PLC through their $\beta\gamma$ subunit , but with significantly less potency than M1-M5 receptors (Felder 1995). The eventual downstream result of reduced cyclic AMP is a decrease in phosphorylation by protein kinase A and overall inhibitory effect on the cell. However, G_i coupled mAChRs M2 and M4 also elicit responses that are the result of membrane-association interactions with other channels through interactions with their $\beta\gamma$ subunit, including activation of inward rectifying potassium channels (GIRKS), and voltage-gated calcium channels (Thiel 2013). Taken together, the differing downstream signaling cascades catalyzed by differing effector targets separates muscarinic receptors into generally excitatory M1, M3 and M5 receptors and generally inhibitory M2 and M4 receptors.

1.3 MUSCARINIC MODULATION OF GLUTAMATERGIC TRANSMISSION IN CORTICAL STRUCTURES

1.3.1 MUSCARINIC MODULATION OF GLUTAMATERGIC TRANSMISSION IN THE HIPPOCAMPUS

In the hippocampus, ACh has been shown to affect cholinergic receptors located both at synapses and elsewhere (Dannenberg et al., 2017). If ACh is released in the hippocampus in a diffuse manner, then the location of receptors plays a vital role in

determining cholinergic modulation of networks, as different amount of ACh release have been proposed to exert different modulator effects (McQuiston et al., 2014). mAChRs are differentially expressed in different circuits and cellular regions of the hippocampus, so that activation of select receptors would elicit certain modulatory effects of circuit functioning in the hippocampus, whereas activation of other receptors would elicit other distinct effects. M1, M2 and M4 have been heavily documented in the hippocampus, and have distinct localizations. M1 receptors are predominantly on cell bodies and dendrites of pyramidal neurons and granule cells (Thorn et al., 2017; Levey et al., 1995; Yamasaki et al., 2010; Dannenberg et al., 2017). M4 receptors exist presynaptically on glutamatergic neurons (Thorn et al., 2017; Sanchez et al., 2009; Shirey et al., 2008). M3 has also been identified, to a lesser degree, as existing on presynaptic terminals in the hippocampus as well (de Vin et al. 2015; Thorn et al., 2017). These presynaptic M4 mAChRs function to inhibit presynaptic release of glutamate from intrinsic connections in the hippocampus, while failing to inhibit glutamate from afferent input to the hippocampus (Thorn et al., 2017; Hasselmo et al., 2006). This selective inhibition serves to enhance the signal from afferent input while dampening noise from intrinsic connections, thus shifting the influence to that of external input (Hasselmo et al. 2006; Dannenberg et al., 2017). Thus, when ACh levels are elevated, presynaptic M4 mAChRs would enhance the influence of afferent input while suppressing intrinsic connections; when ACh is low, the suppression of these intrinsic circuits would be alleviated (Hasselmo et al., 1999; Dannenberg et al., 2017; Thorn et al., 2017).

Hasselmo and others have extensively studied cholinergic modulation of glutamatergic transmission in the hippocampus and found that this cholinergic inhibition

of internal connections, but not afferent pathways, holds behavioral and cognitive consequences; ACh shifts pathway dynamics in the hippocampus to enhance the encoding of new information (ie, by allowing afferent input to the hippocampus to occur unaffected) while suppressing pathways (ie internal recurrent connections) that represent aspects of an already-stored memory (Dannenberg et al., 2017; Thorn et al., 2017; Hasselmo & Wyble 1997; Knierim & Neuneubel 2016; Rolls & Kesner 2006).

1.3.2 MUSCARINIC MODULATION OF POSTSYNAPTIC NEURONS IN HIPPOCAMPUS

Hippocampal mAChRs, in addition to affecting presynaptic glutamatergic release, also affect the excitability of both postsynaptic neurons and interneurons via M1 receptors (Gulledge and Kawaguchi 2007; Dasari & Gulledge 2011; Thorn et al., 2017). Muscarinic agents induce a slow depolarization in pyramidal neurons accompanied by increase spiking mediated by mAChR inhibition of leak potassium channels, voltage-dependent potassium channels and the M-current (Dannenberg et al., 2017; Brown and Adams 1980; Cole and Nicoll 1984). This increase in excitability could serve to make the postsynaptic neurons that possess cholinergic receptors more sensitive to certain afferent input.

On interneurons, effects of cholinergic receptor activation tend to be much more varied that neurons in the hippocampus. mAChR modulation of interneurons in the hippocampus have resulted in depolarizing responses, biphasic hyperpolarizing and depolarizing responses, and hyperpolarizing responses (Bell et al., 2013; Dannenberg et al., 2017).

The differential expression of mAChRs on different neuronal compartments is important to consider when examining ACh's varying effects, as focused ACh release would only activate mAChRs in the vicinity of release whereas tonic ACh could diffuse to activate receptors further from the site of release. This has been demonstrated in the hippocampus in studies comparing local ACh puffs with greater amounts of ACh released that could diffuse further distances (Dasari & Gulledge 2011; Gulledge et al., 2007). These studies highlight the important consequences that differential mAChR expression and differential ACh release have in brain circuits.

1.3.3 MUSCARINIC MODULATION OF GLUTAMATERGIC TRANSMISSION IN THE CORTEX

Similar to the hippocampus, ACh in the cortex is largely thought to enhance the signal to noise ratio of incoming stimuli from afferent layers and other brain regions. Numerous studies by Hasselmo and colleagues have found that ACh, or cholinergic agonists, differentially modulate afferent versus intrinsic synapses in the cortex. In the piriform cortex, Hasslemo & Bower found that application of muscarinic agonists and ACh inhibited internal recurrent projections within layer Ib via presynaptic mAChRs, but did not inhibit projections from afferent layer Ia that terminated in layer Ib (Hasselmo & Bower 1992). A similar selective enhancement of afferent information is also observed in the auditory cortex, where intrinsic intracortical projections or afferent thalamocortical projections were stimulated and responses recorded in the auditory cortex (Hsieh et al., 2000). Cholinergic agonists applied in high concentration selectively inhibited intracortical transmission but allowed afferent thalamocortical transmission to occur.

These effects were blocked by mAChR antagonists, showing differential muscarinic modulation of inputs whereby afferent input was spared but intrinsic cortical connections arising from the same region were inhibited (Hseih et al. 2000).

The neuromodulatory effects of mAChRs in cortical regions suggests an overall modulatory pattern whereby mAChRs, when activated by high ACh, change the circuit to allow afferent input into a region but dampen intrinsic processing of information. In theory, as discussed by Giocomo & Hasselmo, such actions would allow for mAChR activation to enhance the processing of novel, incoming information while reducing noise associated with older information (Giocomo & Hasselmo 2007).

1.4 ENDOCANNABINOID SYSTEM

Endocannabinoids (eCBS) are endogenous lipid-based compounds that bind to cannabinoid receptors (CBRs) both centrally and peripherally. Two eCBs have been found in humans and mammals, N-arachidonoyl-ethanolamine (AEA/anandamide) and 2-arachidonoylglyceral (2-AG). While both are present in the brain, 2-AG is the more common endocannabinoid centrally (Piomelli 2003). Both eCBs are derived from arachidonic acid, though they are synthesized differently. Anandamine (AEA) is produced when intracellular calcium levels increase and cAMP-dependent PKA phosphorylates the enzyme NAT (N-acyltransferase). NAT then catalyzes arachidonic acid to be transferred to phophatidylethanolamine to form N-arachidonoyl-phosphatidylethanolamine, the precursor that is then cleaved into AEA by phospholipase D (Cadas et al. 1996; Piomelli 2003; de Fonesca et al. 2004). 2-AG, on the other hand, is synthesized from the precursor 1,2-diacylglycerol (DAG) (Stella et al., 1997). This process begins when intracellular

calcium rises and PLC catalyzes the hydrolysis of PIP2 into IP3 and DAG. DAG can then activate PKC and its respective downstream targets. One of these targets is diaglycerol kinase (DAG kinase) which exerts negative feedback on DAG, and another target is diaglycerol lipase (DAG lipase), which hydrolyses the production of DAG into 2-AG (Zou & Kumar 2018; Piomelli 2003).

Because the synthesis of AEA and 2-AG require rises in intracellular calcium, both eCBs can be synthesized when receptors permeable to calcium are activated or in response to downstream cascades that ultimately enhance the levels of intracellular calcium release (Piomelli 2003). Examples of such receptors that, when activated, could stimulate the release of eCBs are NMDA receptors, nAChRs and mAChRs (Piomelli 2003; Stella & Piomelli 2001; Kim et al. 2002). Whereas NMDA and nAChR activation would increase the influx of calcium directly and through voltage-gated calcium channels, mAChR and other GPCRs enhance eCB synthesis by coupling to Gq receptors and initiating the aforementioned PLC/DAG signaling cascade (Gyombolai et al. 2012; Caulfield 1993).

Once synthesized, eCBs can diffuse out of the cell and activate CB receptors. eCBs bind to two cannabinoid receptors CB1 and CB2, of which CB1 is most prevalent in the brain (Zou and Kumar 2018). Of the two receptors, CB1R is the most evolutionarily conserved between humans and rodents, whereas much more diversity exists between species in the genetics of CB2R (Liu et al. 2009; Zou & Kumar 2018). CB2Rs have been found to exist in the brain but only in very low amounts, whereas CB1Rs are quite prevalent in the central nervous system, especially in regions pertaining to emotion and homeostasis (Gong et al., 2006; Tsou et al. 1998; de Fonseca et al., 2004). The two identified eCBs, AEA and 2-AG, have drastically different affinities for CBRs. Whereas 2-AG is a full

agonist at both CB1R and CB2R, AEA predominantly binds to CB1R (Zou & Kumar 2018).

1.4.1 MUSCARINIC MODULATION OF CANNABINOID RECEPTORS

The connection between Gq-coupled mAChRs and the induction of eCB synthesis due to calcium rise and Gq receptor coupling by GPCRs has been an emerging area of investigation in the brain. The ablity of mAChR activation to increase cannabinoid synthesis and release was first demonstrated in the hippocampus by Alger and colleagues (Kim et al. 2002). Application of carbachol resulted in mAChR enhancement of eCBS, which then diffused presynaptically to reduce GABA release, the phenomena of which is termed "depolarization-induced suppression of inhibition," or DSI. Muscarinic-released eCBs acted on CB1 receptors, as application of a CB1 receptor antagonist blocked their seen effect. Mechanistically, the short-acting DSI was abolished by intracellular application of a calcium chelator, demonstrating the calcium-dependent aspect of mAChR-mediated DSI (Kim et al. 2002). Later studies using pharmacological approaches and knockout-mice determined that mAChR subtype M1 is largely responsible for enhancing eCB signaling upon activation in the hippocampus and striatum, respectively, whereas M1 and potentially M3 are responsible for endocannabinoid signaling in the periaqueductal gray (Narushima et al., 2007; Ohno-Shosaku et al. 2003; Lau and Vaughan 2008).

Interestingly, a novel putative mechanism through which mAChRs could mediate transmission through CB2Rs has recently been identified (Foster et al., 2016). Activation of M4 mAChRs (achieved via activation with an M4 PAM + muscarinic agonist) inhibited dopamine release via release of eCBs and was blocked by a CB2 antagonist AM630. This

M4-mediated CB release was not due to an increase in intracellular calcium, as neither intracellular calcium manipulations nor inhibition of IP3 prevented the eCB inhibition of neurotransmitter release, suggesting that M4 may enhance CB synthesis via DAG. Accordingly, Foster and colleagues (2016) reversed M4-mediated inhibition of neurotransmitter release by applying a DAG lipase inhibitor, suggesting a novel mechanism by which M4 mAChRs can enhance eCB synthesis via a calcium-independent mechanism that relies on the synthesis of 2-AG from DAG (Foster et al. 2016).

Overall, muscarinic-mediated signaling through eCB receptors poses yet another complex level of modulation through which mAChRs can regulate glutamatergic signaling.

1.5 BASAL FOREBRAIN

While ACh exerts a wide range of neuromodulatory effects discussed above, it arises from defined regions in the brain. These cholinergic origins can be localized to four main areas: the basal forebrain, the striatum, select neurons in the thalamus and the pedunculopontine nuclei in the brainstem (Woolf et al., 1991; Ballinger et al., 2016; Mesulam et al., 1983). Of these groups, the basal forebrain has been heavily implicated in cognitive processing, projects the most ACh to the cortex and amygdala compared to other cholinergic areas, and displays loss of cholinergic neurons in Alzheimer's Disease (Rossor et al., 1982, Mesulam et al., 1983; Ballinger et al., 2016). However, the basal forebrain is a complex cholinergic structure consisting of many different subregions that project to various parts of the brain. Prior to the early 1980's, there was no consistent demarcation in the nomenclature for referring to the different basal forebrain subregions. In 1983, Mesulam et al. demarcated different basal forebrain regions depending on their location in

the basal forebrain and their projection targets, each with the prefix “Ch” to denote “the cholinergic nature of these cell groups since they each contained neurons with high levels of both acetylcholinesterase (AChE) and choline acetyltransferase (ChAT)-like immunoreactivity” (Mesulam et la., 1983). Since then, the subregions of the basal forebrain have been referred to as Ch1, Ch2, Ch3 and Ch4 to indicate the medial septal nucleus, the vertical limb of the diagonal band, the horizontal limb of the diagonal band, and the basal nucleus (including the substantia innominata and the nucleus basalis of Meynert), respectively (Mesulam et al., 1983; Ballinger et la. 2016; Boskovic et al., 2019). 10% of neurons in Ch1 were found to be cholinergic, Ch2 contained more cholinergic neurons (70%), Ch3 has the lowest amount of cholinergic neurons (only 3%), while a striking 90% of neurons in Ch4 are cholinergic (Mesulam et al., 1983; Boskovic et al., 2019). There is also general organization of these regions according to their projection targets (Zaborszky et al., 2013; Gielow & Zaborszky 2017). Cholinergic neurons in Ch1 (medial septum) and Ch2 (vertical limb) predominantly project to the hippocampus, olfactory bulb and prefrontal cortex, Ch3 (horizontal limb) contains projections to the olfactory bulb as well in addition to the thalamic nuclei, Ch4 (substantia innominata and nucleus basalis of Meynert) tends to project mainly to the cortical regions and basolateral amygdala (Carlsen et al., 1985; Woolf and Butcher 1982; Boskovic et al., 2019; Zaborsky et al., 1986; Woolf et al., 1991; Knox and Keller 2016; Ballinger et al., 2016).

1.5.1 BASAL FOREBRAIN FIRES IN BURSTS

The manner in which the basal forebrain fires holds tremendous influence over activation of muscarinic receptors. Should the basal forebrain release ACh in large

amounts, that ACh would be able to more easily diffuse to synaptic targets and activate distal receptors. *In vivo* studies have shown the basal forebrain fires in theta bursts during active waking and paradoxical sleep, behaviors for which microdialysis studies have also shown ACh levels to be the most elevated (Lee et al., 2005). Interestingly, this burst-firing is in synchrony with theta oscillations, 4-12 Hz oscillations shown to be important for learning, memory and is high during exploratory behavior (Caplan et al. 2003; Vinogradova et al., 1995; Colom et al., 2006; Lee et al. 2005). Basal forebrain neurons fire in short, high frequency bursts. The inter-burst-interval peaks around 16 Hz (during paradoxical sleep), is about 8 Hz during active waking, and low (0.84 Hz) during slow wave sleep (Lee et al., 2005). The frequency of intra-burst spiking peaks at 107 Hz (during paradoxical sleep).

1.5.2 BASAL FOREBRAIN IN EMOTIONAL LEARNING / PAVLOVIAN LEARNING

Interestingly, most of the regions to which the basal forebrain projects (hippocampus, medial prefrontal cortex and basolateral amygdala) are in what is known as the fear network and involved in fear learning and fear extinction. Studies using the classic Pavlovian paradigm of fear learning have shown that the basal forebrain and its projections are necessary for emotional learning (Power & McGaugh 2002; Passani et al., 2001; Knox et al. 2016; Jiang et al. 2016). In these paradigms, a neutral stimulus like an auditory tone (Conditioned Stimulus, or CS), is paired with an aversive stimulus like a shock (Unconditioned US). An unpaired CS elicits no distinct behavior, but after one or more pairings with an US, the animal eventually forms a learned association between the two stimuli. Associative fear learning is shown and measured by the display of a well-defined

fear behavior to the otherwise neutral shock once the animal learned that the CS predicts the US (Blanchard & Blanchard 1969). When the association is formed in a specific environment and the animal additionally associated an environment with an aversive stimulus, the learning is known as contextual fear conditioning. Basal forebrain neurons are necessary for forming and displaying fear behaviors in response to a learned US or a threatening stimulus, as lesions to the basal forebrain or optogenetic inhibition of basal forebrain projections disrupt fear acquisition and consolidation during Pavlovian paradigms (Power & McGaugh 2002; Passani et al., 2001; Knox et al. 2016; Jiang et al. 2016).

1.6 AMYGDALA: AN OVERVIEW

Interestingly, the largest amount of ACh from the basal forebrain is sent to the amygdala, an almond-shaped structure located in the temporal lobe. The amygdala is important for the acquisition and expression of emotional learning, a role that has been uncovered through clinical studies, pharmacological ablation and lesions studies in animals, and through behavioral paradigms often involving Pavlovian conditioning.

Much of what we know linking the amygdala with emotion in humans has resulted from case studies of individuals with Urbach-Wiethe disease, a rare autosomal recessive disease in which the amygdala has been calcified. These individuals often display normal cognition but an impaired emotional processing (Siebert et al., 2003). Case studies report such individuals slightly impaired in the ability to process and differentiate facial expressions conveying “disgust” and other negative emotion from positive expressions and extremely impaired in episodic memories involving emotional saliency (Siebert et al.,

2003). Case studies involving bilateral lesions to the amygdala similarly demonstrate the involvement of the amygdala in emotional processing, as such patients are unable to completely process fearful facial expressions (Adolphs et al., 1995). Individuals with Alzheimer's disease often express altered emotional processes that manifest as aggression, inappropriate emotional responses, postmortem investigations often reveal plaques and neurofibrillary tangles in the amygdala (Unger et al., 1991).

Studies in animal models have provided more targeted and detailed understandings of the role of the amygdala in forming activity-dependent and associative-dependent emotional memories. With the advent of Pavlov's conditioning studies, scientists have been able to use aversive conditioning tasks as well as appetitive conditioning tasks to examine the circuitry involved in forming emotional memories and producing appropriate behavioral responses to stimuli. LeDoux and colleagues have extensively demonstrated that the amygdala receives cortical and thalamic input that conveys information about an aversive stimulus and an auditory cue, respectfully, to the amygdala. If evoked responses from both the cortex and auditory thalamic projections to the amygdala arrive in close temporal proximity, synaptic plasticity will occur at the amygdalar neuron onto which the inputs converge, and stimulation of the cortical input will later elicit the learned behavioral outcome even in the absence of an auditory cue (Rogan et al. 1997; Quirk et al., 1995; Tovote et al., 2015; Kwon et al., 2014). However, it is important to note that the amygdala is not restricted to negative emotional memory but also forms appetitive associations. Amygdalar neurons respond to both learned fear and learned positive associations like safety and reward (Maren 2016). Single unit recordings in rodents show subpopulations of neurons increased their firing rate in response to stimuli representing safety, whereas others

responded cues associated with fear (Sangha et al., 2013). Similar studies in primates have agreed with these findings in that separate neurons encode appetitive outcomes and other neurons encode negative outcomes (Paton et al., 2006). The amygdala not only contains neurons that response differentially to appetitive and aversive stimuli, but it also projects to external regions and receives projections from afferent regions that convey appetitive and aversive information in projectionally-defined manners (Malvaez et al., 2019).

Overall, the amygdala is intimately associated with emotional processing, the importance of which is seen in anxiety and fear disorders when amygdalar functioning goes awry.

1.6.1 AMYGDALA NUCLEI AND NOMENCLATURE

In order to study both healthy and diseased amygdalar processing, it is important to understand the organization and anatomy of the amygdala and its connected circuits. The amygdala is composed of multiple nuclei that can be subdivided according to their cytoarchitecture (Sah et al., 2003; McDonald et al., 1998). Most investigators refer to the amygdala as having a group that displays "cortical-like" characteristics and thus refers to this nuclei as the cortex-like group, the centromedial group, and the basolateral group (McDonald et al., 1998; Alheid et al., 1995; Sah et al., 2003). These three groups of nuclei can be further divided into specific nuclei. The cortex-like group consists of the cortical nucleus and the nucleus of the lateral olfactory tract, the centromedial group consists of the central nucleus and the medial nucleus, and the basolateral nucleus consists of the lateral

nucleus, basal nucleus and the accessory basal nucleus (reviewed in McDonald et al., 1998).

As the name implies, cells in the cortical-like nucleus (in other words, cells in the bed nucleus of the accessory olfactory tract (BAOT), the cortical nucleus (Co), the nucleus of the lateral olfactory tract (NLOT), and the periamygdaloid cortex (PAC)), display an organization and morphology most similar to that of cortical regions (Carlsen and Heimer 1988; reviewed in McDonald 1998; Sah et al., 2003).

The medial nuclei of the amygdala (comprised of the central nucleus (CeA), the medial nucleus (M) and the bed nucleus of stria terminalis (BNST)) is the nucleus closest to the optic tract. The central nucleus appears between the basolateral nucleus laterally and the globus pallidus dorsally. Modern nomenclature refers to the central nucleus as having four subdivisions, the capsular, lateral, intermediate and medial (Sah et al., 2003).

The basolateral amygdala, referred to as BLA, is composed of (from dorsal to ventral) the lateral nucleus (LA), the basal / basolateral nucleus (BL), and the accessory basal nucleus (AB). Unlike in the cortex-like nuclei, the pyramidal neurons and interneurons in the BL are not laminar but rather interspersed in a seemingly unorganized fashion. In some literature, the BL is subdivided into a magnocellular and a parvicellular division, and in other literature the BL is subdivided into an anterior BLa and a posterior BLp.

1.6.2 AFFERENT CONNECTIONS TO THE AMYGDALA: GENERAL BEHAVIORAL FUNCTIONS AND ANATOMICAL TERMINATION FIELDS IN THE AMYGDALA

The lateral nucleus, specifically the dorsal subdivision, is the primary "input region" of the amygdala, receiving inputs conveying a wide range of sensory modalities: auditory, visual, somatosensory, gustatory, and olfaction. Some of these sensory modalities also project to other amygdala nuclei (to be discussed below), but the lateral amgydala generally is considered to be the primary input region (LeDoux 2007; Sah et al. 2003; Pitkaenen et al., 1997).

The central nucleus is considered the primary output region conveying information leaving the amygdala; both the lateral nucleus and the basolateral nucleus project to the central nucleus, but the lateral nuclear connections to the central nucleus are much less dense than those from the basolateral amgydala to the central nucleus (LeDoux 2007; Sah et al. 2003; Pitkaenen et al., 1997). Because there not many direct projections from the lateral nucleus to the central nucleus, it is thought that the pathway that sensory input takes to get to the central nucleus (and eventually affect autonomic behaviors mediated by the spinal cord and other downstream structures) is from the lateral nucleus to the basolateral nucleus then to the central nucleus (LeDoux 2007; Sah et al. 2003; Pitkaenen et al., 1997). The central nucleus then projects to downstream structures that elicit behavioral responses including autonomic responses like changing respiratory rate (brainstem, hypothalamus) (LeDoux 2007).

Following the organization put forth by Sah et al., afferent input to the amygdala can be best understood when divided into afferents arising from regions implicated in cognition and afferents arising from regions implicated in autonomic and endocrine

responses (Sah et al., 2003). Thus, one can think of amgydalar inputs in terms of those that arise from cortical regions and subcortical regions involved in cognition and sensory input (such as the cortex, thalamus and hippocampus) and those that arise from regions involved in autonomic and endocrine responses (like the hypothalamus and brain stem) (Sah et al., 2003; McDonald et al., 1998).

Auditory information does not project from the primary auditory cortex (Te1) to the amygdala directly, but rather the flow of auditory information seems to be relayed from the medial geniculate nucleus of the thalamus to Te1, from Te1 to cortical auditory association areas Te3 (auditory association area), Oc2L (also referred to as Te2D by McDonald 1998), and lightly to Te2, with the main amygdalar targets from these secondary cortical regions being the dorsolateral LA, magnocellular basal nucleus, and lateral Ce (Mascagni et al., 1993; Shi & Cassell 1997; McDonald et al., 1998). A similar pattern of terminations is seen, as summarized in McDonald 1998, in projections arising from additional auditory association areas Te2D and Te3R. In addition to cortical projections, the amygdala, specifically the LA, receives direct projections from auditory thalamic nuclei, including projections from the medial geniculate nucleus of the thalamus and the posterior intralaminar thalamic nucleus. Both the auditory cortical projections and the auditory thalamic projections converge on dendritic spines on LA neurons, suggesting that these inputs may synapse on the same amygdalar neurons (LeDoux et al., 1991). LeDoux and colleagues (1996) tested this hypothesis and found that both thalamic and cortical projections can synapse onto the same amygdala neurons. However, a latency period exists between cortical and amgydala transmission such that after an auditory tone, the cortical auditory inputs take longer and thus arrive at the same time as the inputs that have to pass

through the MGN and then go to the amygdala; this latency period may serve as enabling plasticity at these converging inputs as LeDoux et al. found that NMDA receptors, a receptor governing long term potentiation and learning at the molecular level, affected thalamic input but not cortical input to the LA, suggesting the thalamic inputs will depotentiate the LA neurons while the cortical input can be potentiated at those synapses (LeDoux et al. 1996). Overall, auditory information seems to take two main pathways to the LA, one from the cortical auditory association areas and another from the auditory thalamic nuclei. Importantly, these both seem to project predominantly to the LA as well as to the Ce but largely spare the BL nucleus.

Aversive somatosensory information conveyed to the amygdala is thought to arise from brainstem regions, medial geniculate nuclei of the thalamus and cortical regions. Neurons in the medial geniculate nucleus, a region that projects to the amygdala, have been shown to respond to somatosensory stimuli (Bordi & LeDoux 1994). Both the pontine parabrachial nucleus and the periaqueductal grey directly project to the amygdala. The pontine parabrachial nucleus sends the most direct projections to the CeA and fewer to the posterior basal nucleus (BLp) and the anterior basomedial nucleus (Bernard et al., 1993). Interestingly, Bernard et al. (1993) showed the pontine parabrachial nucleus also sends a dense amount of projections to the substantia innominata of the basal forebrain – a region that our lab has shown sends fewer cholinergic projections to the BLp. The periaqueductal grey is thought to convey somatosensory information about a noxious US. This pathway projects directly to the CeA in the amygdala and indirectly communicates with the BL by means of midline thalamic nuclei, including the paraventricular nucleus, the reuniens nucleus, and rhomboid nucleus (Rizvi et al., 1991; Krout & Loewy 2000).

Hippocampal projections to the amygdala have been shown to be necessary for an organism to experience reinstated fear in response to a familiar context (Ji & Maren 2007; Xu et al. 2016). Contextual information largely exits the hippocampus through efferent projections arising in the ventral subiculum, running through the angular bundle and terminating in the posterior basomedial nucleus most densely and the posterior basolateral nucleus, cortical nucleus, intercalated nuclei and the NLOT less densely (Canteras & Swanson 1992; Pitkaenen et al., 2000).

Projections from the medial prefrontal cortex (mPFC) to the amygdala are important for various processes involved in fear behaviors. The mPFC contains four subregions, two of which are the prelimbic cortex (PL) and the infralimbic cortex (IL). These two regions are important for fear acquisition and fear extinction, respectively (Milad & Quirk 2002). Tract tracing studies and optogenetic viral expression patterns and show that the prelimbic cortex projections to the amygdala densely innervate the basal nucleus and the lateral capsule of the central amygala (McDonald et al., 1996; Vertes et al. 2004; Huebner et al. 2014; Adhikari et al., 2015). The projections to the basal nucleus are restricted to just the anterior basal nucleus (BLa) (McDonald 1996). Interestingly, the densest termination field in the basolateral complex for infralimbic projections is in the ventromedial LA and the accessory basal nucleus, two regions where the PL does not project. The incredibly sparse IL projections that were seen in the basal nucleus were localized mainly in the BLp (referred to as the parvicellular subdivision by McDonald et al. 1996) (McDonald et al; 1996). Both the prelimbic and infralimbic prefrontal cortices, however, project to the lateral capsular subdivision of the central nucleus (McDonald and Mascagni 1996; Pinard et al. 2012). The IL also projects to the SI whereas the PL does not

(Vertes 2004). This differential innervation of the SI opens the physiological possibility that the IL is capable of feedback control to the cholinergic center of the brain whereas the PL is not. Also noteworthy are projections from the IL to the ITCs in the amygdala (Pinard et al., 2012). The excitation of these ITC's may affect downstream structures that give rise to extinction behaviors, a concept that is supported by electrophysiology, behavior and immunohistochemistry (Quirk et al. 2003; Amano et al. 2010; Pape & Pare 2010; Pinard et al. 2012).

Once thought to only relay sensory information, the midline thalamic nuclei (THAL) that project to the amygdala are now known to be involved in more varied processes affecting cognition and survival. The midline thalamic nuclei are often grouped in the literature into dorsal nuclei that generally target limbic subcortical structures (nucleus accumbens and amygdala) and a ventral group that generally targets limbic cortical structures (sensory cortical areas) but also send lighter projections subcortically (Van der Werf et al. 2002). The dorsal nuclei include the paraventricular nucleus (PVT), paratenial nucleus (PT) nucleus and intermediodorsal nucleus. Tract tracing studies have shown that of these nuclei, the anterior PVT nucleus sends the densest projections to the lateral central amygdala and "moderately dense" projections to the rostral basal nucleus, caudal basal nucleus, and basomedial nucleus (Vertes & Hoover 2008). Posterior PVT nucleus terminations in the amygdala showed the same topographical pattern as anterior PVT terminations but were much more dense (Vertes & Hoover 2008). PT projections were the heaviest in the medial LA and medial BLA and densest in the more caudal amygdala. Interestingly, unlike the auditory thalamic input to the amygdala, the midline thalamic input largely avoids the LA (with the exception of the PT medial LA terminations) and

predominantly terminates in the BL. This termination pattern raises the possibility that THAL are involved in BL-mediated behaviors. Accordingly, behavioral studies have suggested THAL is implicated in aspects of fear learning involved error prediction and threat detection; blocking the midline thalamic nuclei during Pavlovian conditioning blocks predictive fear learning and activating the ventral midline thalamic nuclei reduces an organism's saliency to a potential predator (Sengupta & McNally 2014; Salay et al., 2018).

To summarize the above projections, afferent input largely arrives at the amygdala in the LA nucleus and the BL (both BLa and BLp) nucleus. Pathways terminating in the LA including the infralimbic cortex conveying information necessary for extinction learning and from cortical regions conveying auditory, gustatory, somatosensory, olfactory and visual information. The BL is composed of both a BLa (consisting mainly of magnocellular cells and thus also referred to as the magnocellular division) and a BLp (consisting of parvocellular cells and called the parvocellular division in some literature). Pathways terminating in the BLa include the prelimbic cortex conveying higher order information necessary for fear acquisition, midline thalamic input conveying error prediction and influencing survival behaviors in response to threat, and ventral subiculum conveying contextual information. Pathways terminating in the BLp include midline thalamic nuclei, cortex-amygdala transition zone conveying olfactory information, pontine parabrachial projections conveying ascending somatosensory information and the anterior insular cortex conveying visceral sensations. Thus, there appears to be a topographical organization to the different afferent inputs to the amygdala.

In terms of a behaviorally relevant context like fear, information conveying the CS stimulus (an auditory tone, for example) and the US stimulus (a shock, for example) both converge predominantly in the dorsal LA (Bordi & LeDoux 1992). The temporally coinciding input will potentiate the synapse onto which the two stimuli arrived. Thus, if the CS arrives alone in the future, that auditory information conveyed will arrive on a potentiated synapse and be able to elicit the behavioral response that the US did before (Ledoux 2007).

Putting this topographical organization into a dynamic context, behavioral studies and tract tracing studies have developed models of information flow through the amygdala. The general flow of information, as reviewed by Pitkaenen et al. (1997) posits information *generally* enters the amygdala in the LA and *generally* leaves via two main output regions, the central nucleus and the amygdalohippocampal area.

Elegant studies and a review article by Pitkaenen et al. 2006 reveal that information flows within the LA nucleus in a one-way manner. The dorsal LA projects to the ventral LA and the medial LA, but the ventral LA and the medial LA do not densely project to each other nor do they project back to the dorsal LA (Pitkaenen 2006). Likewise, there are very few reciprocal connections within the dorsal, ventral and medial divisions of the LA (Pitkaenen 2006). Unlike the LA, however the BL has many more reciprocal connections within both the BLa and the BLp (Savender et al. 1995). Whereas the LA has a one-direction flow from the dorsal LA to the ventral LA and medial LA, the BL contains bidirectionality between the BLa and BLp.

So how then, does information flow from external afferents to the LA, between nuclei, and finally to the CeA and other structures? Most internuclear projections arise

from the LA, as the LA projects to the BL, the periamygdaloid complex, the medial nucleus, the cortical amygdala, the central nucleus, the amygalohippocampal area and, the densest, to the AB (Pitkaenen et al. 1995). The AB then projects to the CeA, likely resulting in downstream behaviors (Pitkaenen et al. 1995). As mentioned above, the LA also projects to the BL. However, the LA projections to the intermediate and posterial aspect are denser than the projections to the BLa, with particularly dense projections from the medial LA to the BLp (Pitkaenen et al. 1995; Stefanacci et al. 1992; Pitkaenen and Amaral 1991). Thus, once information flows into the LA, it can proceed either directly to the intermediate or posterior BL via dense projections, to the BLa via sparse projections, to the AB, CeA, CoA, MeA, PAG, or to the medial and ventral LA (Pitkaenen et al. 1995). Since the medial LA also receives hippocampal, infralimbic, olfactory as well as other inputs, the information relayed here could be further processed before leaving the LA (Pitkaenen et al. 1995).

After entering the BL, the exiting destination is ultimately determined by the subdivision of the BL it arises from. All three subdivisions of the BL (BLa, BLi, BLp) project very sparsely to the AB, PAG and the MeA. All three BL subregions project densely to the NLOT (Savander et al. 1995). The other output nucleus of the amygdala, the CeA, also received projections from the BL, but the density also depended on the specific location of origin in the BL; all rostral caudal regions of the BLa projected to the CeA, with only the middle being dense. The BLi only sent sparse projections to the CeA. On the other hand, the BLp sent very dense projections to the CeA and the capsular division of the CeA.

To summarize, sensory information enters the amygdala largely at the LA, but additional afferent inputs such as the PL, MTN, vSUB, enter the amygdala at the level of

the BL. These inputs may serve to update a preexisting association made, or influence the value assigned to an emotional given the BL's role in associative learning. However, the exact mechanisms underlying how the BL modulate these inputs remains unknown. Interestingly, the ACh that is sent to the amygdala from the basal forebrain nearly uniquely projects to the BL, raising the question of if it can modulate the afferent input (PL, THAL, vSUB) that terminates in the BLa.

1.6.3 CHOLINERGIC PROJECTIONS TO THE BL

Anatomical studies labeling for various markers of cholinergic innervation across species have consistently found that the BL has the densest labeling for ChAT and AChE compared to the other nuclei in the basolateral complex (Ben-Ari et al. 1977; Girgis 1980; Svendsen & Bird 1985; Amaral & Bassett 1989). More specifically, the magnocellular (BLa) division of the BL had the densest amount of immunoreactivity for ChAT labeling compared to the other regions (Amaral & Bassett 1989). Tract tracing, immunohistochemistry, transgenics, fiber lesioning and immunofluorescence have been used in studies spanning the last 4 decades to determine that cholinergic input to the BL arises from the basal forebrain, specifically from the substantia innominata and the horizontal diagonal band in the nucleus basalis of Meynert (Agostinelli et al., 2019; Mesulam et al. 1983; Emson et al., 1979; Woolf & Butcher 1982; Carlsen et al., 1985; Zaborsky et al., 1986).

Electron microscopy and immunocytochemistry techniques later revealed that 89% of the postsynaptic BL neurons onto which VAChT+, or cholinergic, terminals synapsed were excitatory pyramidal neurons (Muller et al. 2011). Furthermore, a majority of these

symmetrical synapses between cholinergic inputs and glutamatergic neurons were on dendritic shafts and spines. Strikingly, electron microscropy showed that cholinergic terminals were located in close proximity to glutamatergic synapses, suggesting that when ACh is released it is in an ideal position to modulate neurotransmission (Muller et al. 2011).

1.6.4 CHOLINERGIC RECEPTORS IN THE BL

While ACh exerts its effects through both nicotinic and muscarinic receptors, behavioral and pharmacological data largely supports muscarinic receptors activation mediating emotional learning and memory (Wilson & Fadel 2016). However, recent work by the Role lab has implicated nAChR in long-term plasticity of cortico-amygdalar synapses in the BL (Jiang et al. 2016).

Since the 1980's we have seen a wealth of results from electrophysiology, genetic and autoradiography studies identifying mAChRs M1, M2, M3 and M4 in the BL of rodents, nonhuman primates and humans (Sugita et al. 1991; Flynn & Mash 1993; Cortes et al. 1987; Spencer et al. 1986; Mash & Potter 1986; Bonner et al. 1988; Yajeya et al., 2000).

M1 receptors are most dense in the BL, and immunoperoxidase staining localized the majority of the M1 receptors to be on neuronal perikarya. Dual labeling experiments in the same study further identified the localization of M1 receptors to be on CaMK+ neurons, as almost all CaMK+ neurons double labled for M1, suggesting that M1 receptors are located postsynaptically on glutamatergic pyramidal neurons in the BL (McDonald & Mascagni 2010). Later studies employing electron microscopy advanced upon these findings by studying where exactly on these pyramidal neurons M1 receptors are localized;

On the M1-labeled pyramidal neurons, M1 receptors were present on 90% of dendritic shafts and 60% of dendritic spines (Muller et al. 2013). Interestingly, M1 receptors were also seen on presynaptic terminals forming asymmetrical (putative glutamatergic) synapses and symmetrical synapses. The majority of terminals containing M1 receptors on putative glutamatergic inputs synapsed onto dendritic spines, whereas the other M1+ terminals synapsed onto dendritic shafts and cell bodies (Muller et al. 2013). Strikingly, the VAChT+ terminals were in close proximity to synapses, indicative of the potential role of ACh in regulating glutamatergic transmission via M1 receptors. A separate study identified M2 receptors on both neuropil and perikarya of SOM+ and NPY+ interneurons (McDonald & Mascagni 2011). In the BL, M2+ neuropil labeling was most robust rostrally, whereas caudally it was less robust but only in the BLp. This is suggestive of M2-mediated modulation that could be specific to the BLp and not BLa. M2+ perikarya was identified in every amgydala nucleus including the ITCs (McDonald & Mascagni 2011). Successive experiments utilizing electron microscopy to more specifically localize M2 receptors found the majority of dendrites (95%) expressed M2 receptors and these M2+ dendrites also had VAChT+ projections synapsing in close proximity (although some VAChT+ terminals synapsed with M2R- dendrites forming both symmetrical and nonsymmetrical synapses) (Muller et al., 2016). Notably, there were postsynaptic M2 receptors that were "located just outside of the active zone of the synapse," suggestive that larger or smaller quantities of ACh release could differentially affect these receptors located further away (Muller et al., 2016). M2 receptors were also found presynaptically on terminals that tended to synapse asymmetrically and symmetrically with M2+ postsynaptic dendrites, although it was found that "twice as many M2R+ terminals made asymmetrical as compared to

symmetrical synaptic contacts," suggestive that M2 receptors are located on excitatory synapses and thus in a position to regulate glutamate release (Muller et al., 2016).

1.7 SIGNIFICANCE REVISITED

The basolateral amygdala is a region implicated in emotional processing and assigning emotional value to stimuli. In addition to receiving projections from other regions in the fear network, including the PL and THAL, the BL also receives the densest amount of ACh from the basal forebrain. Cholinergic projections to the amygdala have been implicated in learning and emotional processing. However, how this cholinergic innervation affects transmission from the PL and THAL to the BL remains unknown. The goal of this study is to fill the gap in the literature regarding cholinergic modulation of afferent input to the BL. The overarching hypothesis of this project is that endogenous ACh inhibits afferent transmission to the basolateral amygdala, and that muscarinic ACh receptors differentially regulate PL and THAL input to the BL.

CHAPTER 2

GENERAL METHODS

2.1 NEURONAL SIGNALING: AN OVERVIEW

In the brain, information is transmitted through and between neurons by electrical signals, such as action potentials and synaptic potentials that arise from current flow. Current flow in a neuron is due to the movement of positive or negative ions into or out of a neuron through ion channels. For any given neuron, a lipid bilayer membrane separates two sides: an intracellular side consisting of the neuron's cytosol and components, and the extracellular side. Intracellularly, neurons contain many negatively charged proteins whereas the extracellular side tends to contain many more positively charged ions. The cellular membrane is a lipid bilayer that is impenetrable to the diffusion of ions across it unless they are assisted in movement. Thus, the lipid membrane separates charges – usually a positively charged extracellular side from a negatively charged intracellular side (Figure 2.1). Because a separation of charge creates a situation in which particles can theoretically move from one side to the other releasing energy in the process, any separation of charge is referred to as a "potential" and is designated by the units of voltage. When the separation of charge is across a membrane, it is referred to as a "membrane potential." When neuroscientists quantify the charges across a membrane, they

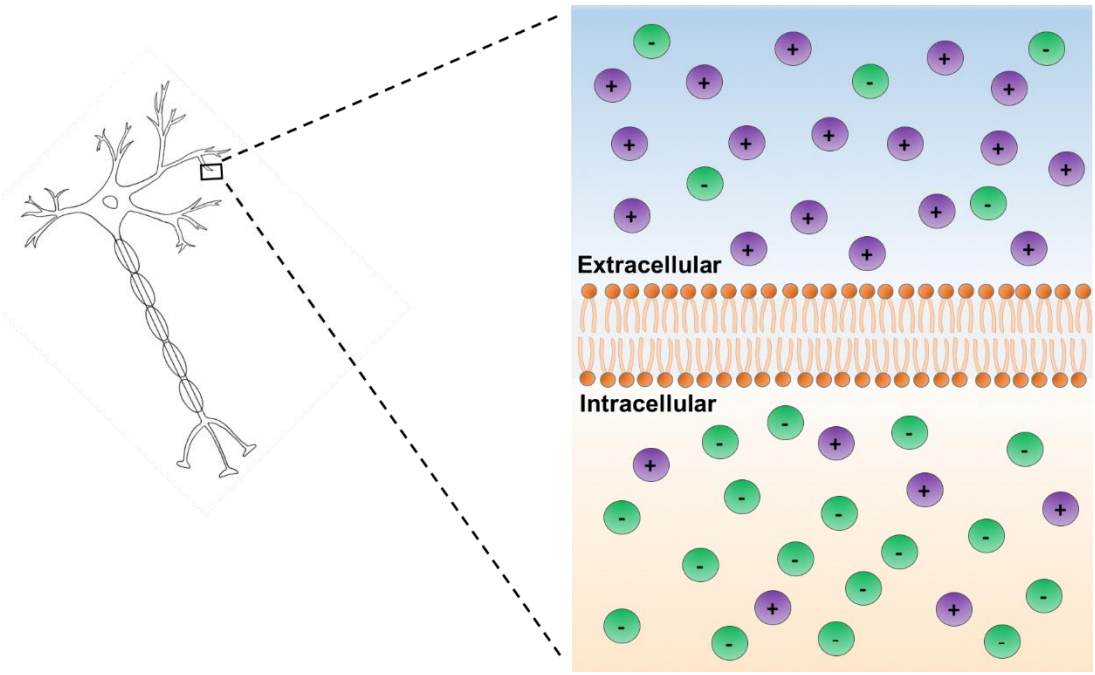


Figure 2.1. Membrane potential separated by a lipid bilayer. The internal environment of a neuron (orange) is usually more negative than the outside (blue). This difference in charge creates a negative membrane potential maintained in part due to an impermeable membrane and ATP-driven pumps that actively pump more positive ions out than in. (Image adapted from *Principles of Neural Science 5th Edition*," (p. 127), by J. Koester and S.A. Siegelbaum, 2013, New York, New York: The McGraw-Hill Companies, Inc., Copyright 2013 by The McGraw-Hill Companies, Inc.).

designate the potential outside the cell as zero and assign the potential inside the cell a quantity *relative* to the zero potential outside. Thus, as mentioned earlier, the inside of a cell tends to be more negatively charged than outside and therefore the intracellular potential is usually between -60mV to -75mV *relative* to the outside (Figure 2.2). This resting membrane potential is due to the permeability of ions as well as the electric and chemical gradients of ions on the intracellular and extracellular sides of the membrane; thus, it is closest to the equilibrium potential of the ions to which the neuron is most permeable. In the case of neurons, many K+ channels are open at rest, allowing for the flux of K+ ions out of a cell. However, once enough K+ diffuses out of a cell, the more-negative interior will electrically oppose the movement of K+ down their concentration gradient. At the same time, active transport of 2 K+ ions into the cell and 3 Na+ ions out of the cell occurs to oppose the passive movement of these two ions across membranes (Figure 2.2). Together, active and passive movements of ions across a membrane can maintain a constant membrane potential. If this potential remains constant, meaning if positive and negative ions are flowing into and out of the neuron at rates that keep the potential constant, the neuron is at rest and there is no net flow of ions across the membrane. However, if the flow of ions becomes imbalanced in the sense that more positive or more negative ions flow into or out of the neurons, the interior or the neuron becomes more positive or more negative compared to the outside; neuroscientists refer to this increase or decrease in membrane potential as depolarized or hyperpolarized membrane potential, respectively. Should a neuron be depolarized enough, a threshold will be passed and the neuron will fire an action potential. This action potential is a propagating wave of changing membrane potential (from negative to positive) down a neuron's axon.

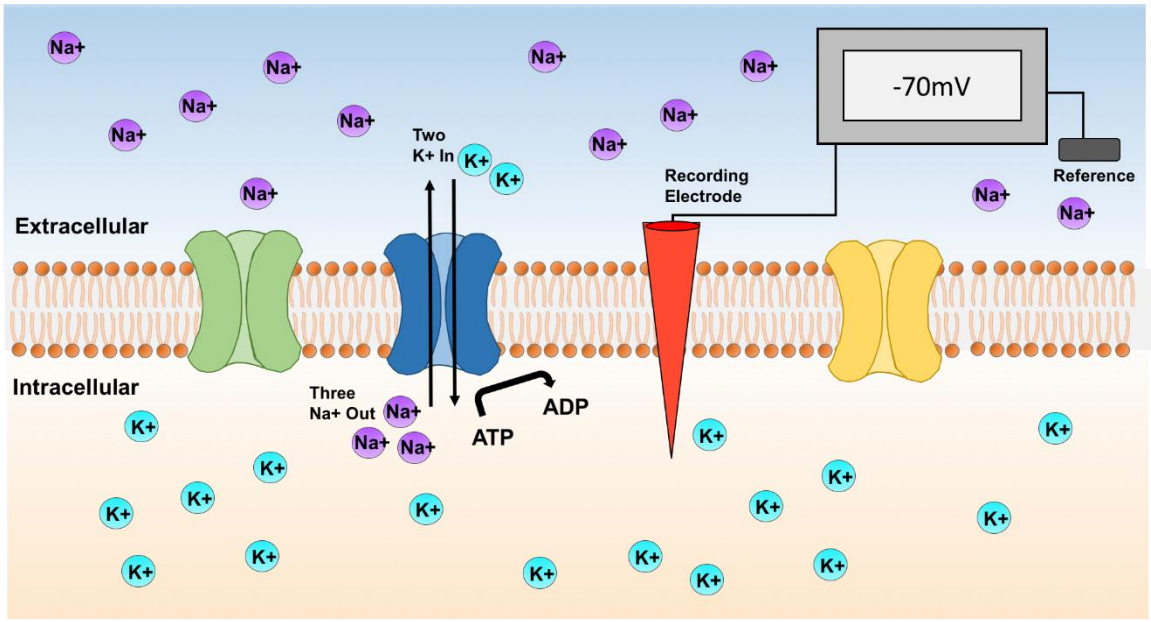


Figure 2.2. Resting membrane potential is around -70mV at rest. At rest, neurons have a negative membrane potential. Contributing in part to this potential is the sodium/potassium pump. The membrane of neurons is much more permeable to K+, so the membrane potential is closest to K+'s equilibrium potential. However, after Na+ inevitably leaks into the cell, the sodium/potassium pump pumps three Na+ ions out for every two K+ in, expending ATP in the process. In whole-cell electrophysiology, membrane potentials and ionic flux (current) can be recorded by an electrode patched onto or into the cell; it measures electrical events due to ion flow for a single neuron. (Image adapted from "Resting Membrane Potential." Mar 23, 2016, *OpenStax, Biology*. *OpenStax CNX*. Retrieved November 24, 2019, from https://courses.lumenlearning.com/wm-biology2/chapter/resting-membrane-potential/ .).

When an action potential reaches the end of an axon terminal, it depolarizes the presynaptic terminal and voltage-gated calcium channels open in response. Elevated presynaptic calcium then acts as a second messenger to mobilize neurotransmitter-filled vesicles for release. Vesicles containing neurotransmitters are immobilized by proteins called synapsins, which serve as anchors to keep the neurotransmitter-containing vesicles in reserve pools and prevent their movement to membranes and subsequent neurotransmitter release (Purves et al., 2008; Kandel, Schwartz, Jessell, Siegelbaum & Hudspeth, 2013). In the event of calcium increase, this calcium aids in phosphorylating synapsin via the calcium/calmodulin-dependent protein kinase I, thus releasing the anchor on vesicles and allowing them to move out of the reserve pool and towards the presynaptic membrane. There is another calcium-sensitive complex of proteins embedded in vesicle membranes, "SNARE proteins" that allow for the vesicle to be brought closer to the membrane (Purves et al., 2008; Kandel, Schwartz, Jessell, Siegelbaum & Hudspeth, 2013). Once the vesicle is close to the membrane, additional proteins join the protein complex, including synaptotagmin. Once calcium increases in close proximity to this complex, it binds to synaptotagmin and allows for a conformational change in synaptotagmin that facilitates the fusion of the vesicle with the membrane and ultimate release of contents outside the neuron. Thus, calcium-mediated effects of synaptotagmin are necessary for rapid neurotransmitter release (Purves et al., 2008; Kandel, Schwartz, Jessell, Siegelbaum & Hudspeth, 2013).

Once a presynaptic terminal releases neurotransmitters, those neurotransmitters diffuse across a synaptic cleft and bind to their respective receptors located on the postsynaptic neuron. If those receptors are ligand gated ion channels, they will open and depolarize or hyperpolarize the postsynaptic terminal. If the receptors are GPCRs, they

will exert effects according to their class of GPCR (see Chapter 1). The ion flow into a postsynaptic neuron generated by neurotransmission at this synaptic cleft generates a voltage difference, called a synaptic potential, between the intracellular and extracellular sides that can be measured with an electrode. Neurotransmission is the primary method by which one neuron communicates with another by converting a chemical message into an electrical one. The neurotransmission between a presynaptic and postsynaptic neuron can be modulated by various neurotransmitters, including ACh (see Chapter 1), to increase or decrease the amount of neurotransmitter released into the synaptic cleft, as well as to exert a long-term change in the strength of neurotransmission between neurons.

2.2 ELECTROPHYSIOLOGY: AN OVERVIEW

Electrophysiology is the study of a neuron or population of neurons' physiological activity by recording these electrical events. Electrophysiology can be both *in vivo* (in living animal), *ex vivo* (in slices prepared from a brain, or brain slice electrophysiology) or *in vitro* (in cultured cells, for example). The benefit of slice electrophysiology is, unlike cultured cells, connections between neurons and neural circuitry within a brain slice is *largely* intact. Using brain slice electrophysiology, one can understand neuronal functioning by examining neurotransmitter release, membrane composition, etc. Electrophysiological techniques can be used to study the electrical properties of a single neuron or of a population of neurons using patch-clamp electrophysiology or field potential electrophysiology, respectively.

Patch-cell electrophysiology involves studying the electrical events from a single cell. The advent of the voltage clamp technique by Kenneth Cole in the earlier half of the

1900's allowed for scientists to control the membrane potential of neurons and study the subsequent current flow across neuronal membrane (Purves et al., 2008). If the voltage of single neuron is held constant and glutamatergic transmission occurs which ultimately allows for positive ions to flow into the neuron, then a counter negative current will be injected into the cell to maintain the neuron at the experimentally held voltage potential. This offset current is what is measured and recorded as an indicator of current flow. This technique allowed Hodgkin and Huxley to determine the ionic permeability of membranes to Na+ and K+ during action potentials (Hodgkin & Huxley 1952). Today, the patch-clamp technique is widely used to determine receptor regulation, neurotransmission and a variety of other neural events. There are many different types of patch-clamp techniques, one of which is whole cell recording. In this technique, the membrane is broken by suction so that the contents of the cell are continuous with the recording electrode and the investigator can manipulate the cell voltage, inject current and inject compounds like chelators into the neuron (Purves, 2008).

In contrast to whole-cell, field potential recordings detect activity of a population of neurons and thus provides information about a neuronal network. This technique relies on the "volume conduction theory" of electrical potentials, or the notion that field potential recordings do not directly contact a single cell but rather measure an electrical potential field that is conducted through the extracellular space (Rutkove 2007). In other words, field potentials are measured with an electrode that is placed extracellularly (as opposed to intracellularly in whole cell) and measures the *summed* electrical field generated by all neuronal currents in the vicinity (Figure 2.3).

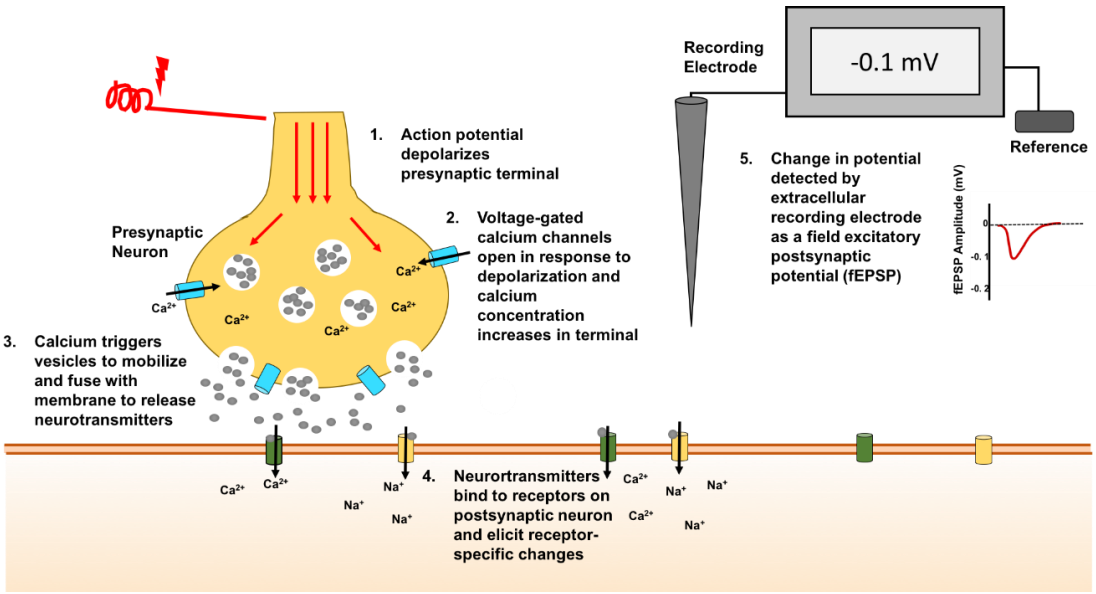


Figure 2.3 Field electrophysiology records electric events detected outside the cell. In field electrophysiology, potentials are measured in the extracellular medium as opposed to inside a cell itself. In this example, the recording electrode is positioned extracellularly. Its reading of "-0.1mV" indicates it has detected a negative membrane potential, due to ionic flow into tissue in the vicinity. The fEPSP, or negative-going waveform**,** depicts the amplitude and kinetics of this potential. It is important to note that while only one postsynaptic neuron is depicted in this schematic, the electrode will detect the summation of all electric events from neurons in the vicinity. (Image adapted from "Chemical and Electrical Synapses," Mar 23, 2016, *OpenStax, Biology*. *OpenStax CNX*. Retrieved November 24, 2019, from https://courses.lumenlearning.com/wm-biology2/chapter/chemical-and-electrical-synapses/).

2.2.1 FIELD POTENTIAL ELECTROPHYSIOLOGY: AN OVERVIEW

As mentioned briefly above, field potential electrophysiology is the study of electrical fields generated by currents that occur in the proximity of a recording electrode. It involves depolarizing a population of neurons, usually by administering a current to the tissue. The evoked depolarization causes neurons to fire and release neurotransmitters, which then affect receptors on postsynaptic neurons (either their dendrites or cell bodies). The resulting ion flow from activation of the postsynaptic neurons is either inhibitory (called an inhibitory postsynaptic potential, IPSP) or excitatory (called an excitatory postsynaptic potential, EPSP). Electrical currents across membranes can be generated in a number of ways, as elegantly reviewed by Buzsaki et al. (2012), including but not limited to synaptic currents, action potentials, and calcium and ionic spikes, and oscillations. However, in order to be detected by the recording electrode, the summed potentials must be large enough. Thus, two main principles determine the magnitude of a field response: the size of the individually generated currents and the coinciding time scale of the individually generated currents (Buzsaki et al. 2012). Thus, if a population of neurons is stimulated and synchronizes their firing, neurotransmission from those neurons should occur relatively close in time; this principal allows for synaptic currents to be one of the largest components of a field response measured in a tissue with field electrophysiology (Buzsaki et al., 2012). In the event that synaptic transmission releases glutamate, it will generate an EPSP and depolarize the postsynaptic neurons. This *active* flow of positive ions away from the extracellular space and into the postsynaptic neurons generates a negative field potential detected by the recording electrode and called a “sink” (Figure 2.4). However, because the extracellular space around the tissue is a conductive medium, it is a

complete circuit and thus if positive ions flow into a neuron at the synapse or active zone, an opposite flow of current will occur that leaves the tissue. This opposite flow of current from the intracellular space into the extracellular space is called the "source" (Figure 2.4).

If tissue is laminar, such as in the hippocampus, then the dendrites and cell bodies align and different layers are visually discernable through a microscope. In this instance, stimulating and recording electrodes can be placed such that the investigator can determine if they are recording the dendritic sink or somatic source based on electrode placement. In other areas of the brain, including the amygdala, there is no clear organization to neuronal components. Rather, it is more likely that one has to rely on pharmacological validation of components of the response to determine what they are studying.

2.3 OPTOGENETICS: AN OVERVIEW

One of the limitations of electric stimulation in electrophysiology is the nonspecific stimulation of all neurons in the vicinity of the stimulating electrode. Any neuron that is sufficiently depolarized by the electric stimulus will fire. However, this is not ideal if one wants to stimulate neurons that are sporadically scattered within other cell types. This limitation has been overcome with the development of optogenetics to stimulate neurons. As the name implies, optogenetics is the use of genetics to insert light-sensitive proteins, opsins, into specific populations of neurons (Diesseroth 2011).

Certain types of algae possess what is called an "eyespot" that detects levels of light and aid in initiating movement of flagella that propel the algae to light sources.

One of these algae, *Chlamydomonas reinhardtii* was suspected as having genes that code for a light-sensitive ion channel (an "opsin") that, when activated by light, would

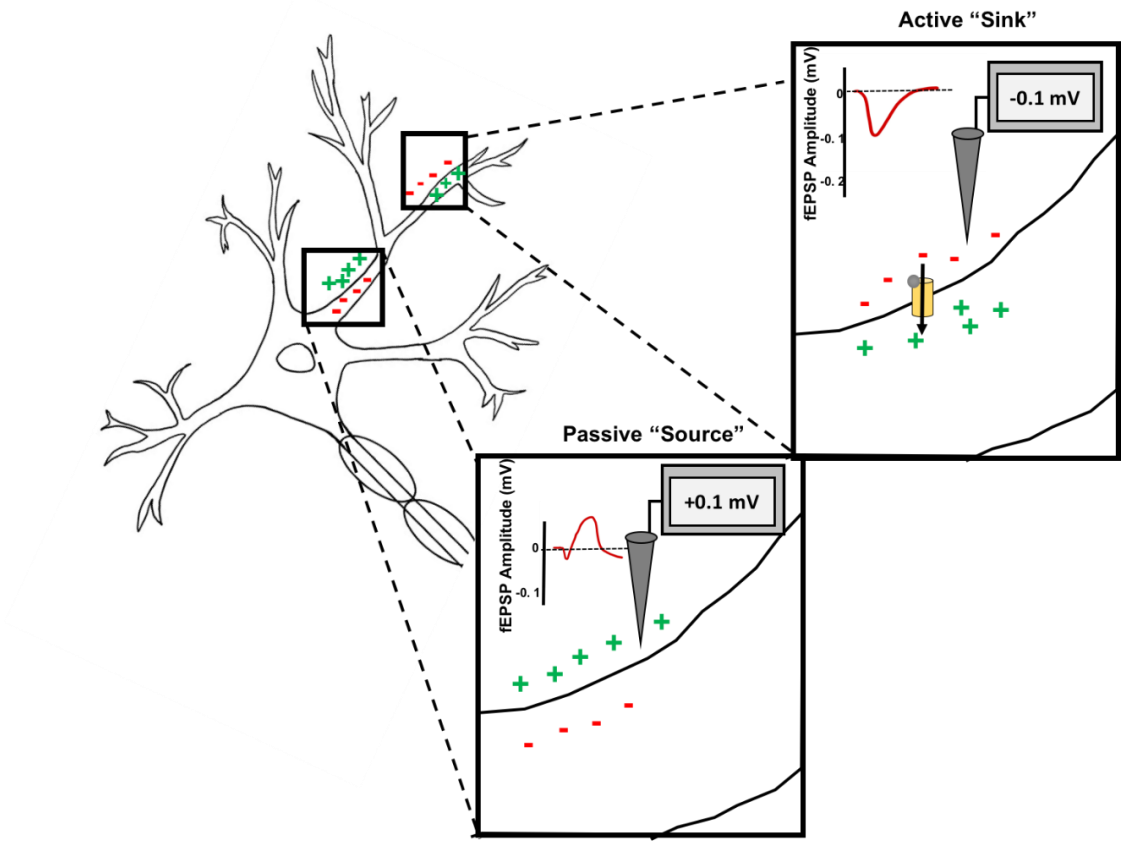


Figure 2.4 Schematic demonstration of the electric dipole generated when recording a field potential with slice electrophysiology. Neurotransmission (often of glutamate) from a presynaptic terminal onto a dendrite creates a transmembrane current of positive ions flowing into the apical dendrite. This inward current (sink) is picked up as a negative potential by extracellular electrodes (upper inset).Because the extracellular medium is a closed circuit, an opposite current flows out of the neuron (source) some distance away (lower inset) and is picked up by an extracellular electrode as an positive potential.

allow ions to pass into the cell. In 2002, German scientists Hegemann and Nagel confirmed the existence of such a gene that coded for a light-sensitive transmembrane protein, Channelopsin-1 (Chop1) that was in the membrane. They found that when Chop1 was hit by photons in the blue light spectrum, its conformational shape changed to allow cations through (Figure 2.5) (Nagel et al., 2002). The same researcher later identified another genetic sequence coding for another opsin they named Channelrhodopsin-2 (ChR2) that also responds to blue light (Figure 2.6) (Nagel et al., 2003).

Because opsins respond to light by opening channels that allow ions through, and because neurons depolarize and fire action potentials when cations enter and sufficiently depolarize them, Karl Diesseroth undertook experiments to see if opsins could be virally expressed in neurons to depolarize them in response to light. In 2004, his team from Stanford successfully spliced the *Chlamydomonas reinhardtii* ChR2 gene and a promoter gene into a viral vector and virally transfected neurons with the new construct (Boyden et al., 2005). This ability to virally insert opsins into genetically defined neuronal populations was the spark that kindled a firestorm of future studies that quickly transformed the landscape of neuroscience. It was now possible to activate neural circuits with a temporal and cell-specific resolution that was not possible with electric stimulation alone.

Since Diesseroth's fundamental study in 2004, an array of opsin genes has been discovered and spliced with various promoters. Opsins that respond to separate wavelengths of light, have different response kinetics and inactivation rates, and are permeable to depolarizing versus hyperpolarizing ions further increase the level of control scientists can exert when stimulating neurons to fire or be inhibited.

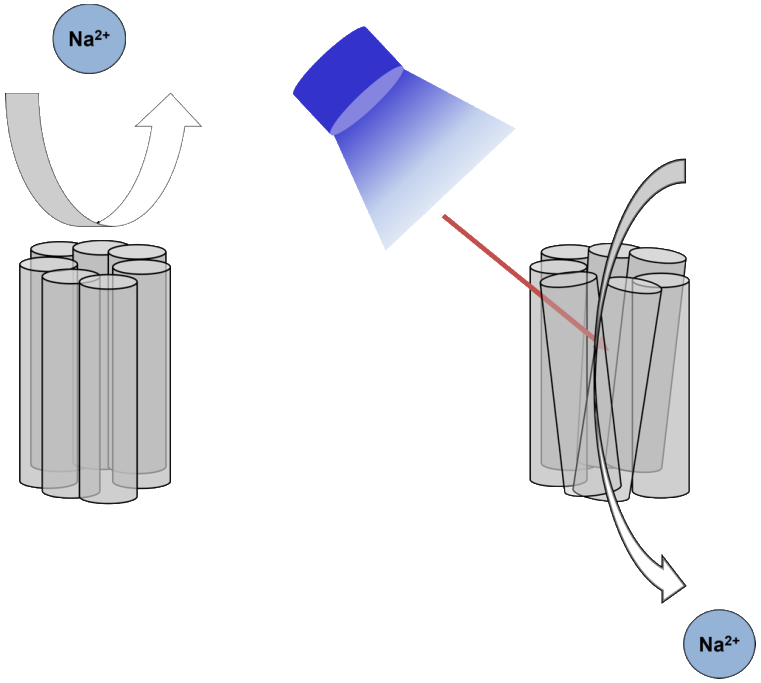


Figure 2.5 Schematic demonstrating light-induced opsin activation. Opsins are responsive to specific wavelengths of light. Here, channelrhodopsin is depicted as changing its conformational shape in response to photons of blue light (470-490nm). This light-induced physical change allows the channel to now be permeable to sodium ions. The resulting influx of sodium ions depolarizes neurons. If the expression of channelrhodopsins is high enough, the neuron will sufficiently depolarize and fire an action potential.

Lastly, the ability to splice an opsin gene behind a specific promoter allows researchers to control activation or inhibition of a multitude of cell types. For example, splicing an inhibitory opsin (one permeable to chloride ions) responsive to yellow light behind a tyrosine hydroxylase promoter would enable the transfection of the opsin genes and expression of opsins that response to yellow light into dopaminergic neurons; flashing blue light would have no effect on these cells but flashing yellow light would inactivate them. Alternatively, one could transfect cells with a virus that has a ChR2 (responsive to blue light) spliced behind a ChAT promoter to activate cholinergic neurons in response to blue light.

Yet another level of control with optogenetics was the creation of the Cre-loxP inducible system. Transgenically engineered mice can be created to express Cre recombinase, an enzyme that specifically cuts out LoxP sites in the viral vector and splices the remaining sequence together. For example, ChAT-Cre mice can be created that have a gene coding for Cre after the promoter for the gene coding for ChAT and thus express Cre in every cell that expresses ChAT, or in every cholinergic cell. A viral vector is then created that contains a promotor to drive expression, a gene of interest between the two LoxP sites, a fluorescent reporter protein, and occasionally other posttransciptional elements like the commonly seen Woodchuck posttranscriptional regulatory element. In this system, injecting a viral vector containing a gene of interest embedded between two loxP sites into a Cre animal would allow the virus to transfect any cell, but *only* be transcribed into functional proteins in those cells that actively express Cre (Tsien 2016).

2.4 FIELD RECORDINGS IN BASOLATERAL AMYGDALA: AN OVERVIEW

To date, very few studies have utilized field potential recordings in the amygdala, presumably due to the extreme difficulty in obtaining a sizeable response. Unlike the laminar hippocampus, the amygdalar neuronal dendrites and axons seem to have no clear spatial organization. As such, the field potentials recorded are small, and thus success depends largely on the ability to cut and maintain healthy brain slices.

The studies that have examined field responses in the amygdala have either electrically stimulated the LA and recorded evoked field potentials in the BL (Pu et al., 2009), electrically stimulated cortical or thalamic afferents and recorded evoked fields in the LA (Johnson et al., 2008; Sigurdsson et al., 2010) or electrically stimulated the external capsule and recorded fields in the BL (Braga et al., 2003). However, none of the studies examining cortically evoked fields in the BL validated the components of the evoked response, leaving a gap in the interpretation of the results.

In this study, glutamate from afferent inputs to the BL was released by electrically stimulating the external capsule. The evoked field responses were measured in the BL. Then, to determine how ACh regulates this glutamate from afferent inputs to the BL, mice were injected with an AAV virus containing blue-light sensitive ChR2 in the cholinergic neurons that project to the BL. Light was flashed and ACh was released from these basal forebrain projections. Then, in the presence of released ACh, glutamate was released by electrically stimulating the external capsule.

However, one limitation of electric stimulation is a nonspecific activation of neurons. Because the purpose of this study was to determine how ACh modulates

glutamatergic transmission from prelimbic, thalamic and ventral subicular input to the BL, electric stimulation does not allow for precise control over pathways stimulated. However, using optogenetics to release glutamate from the prelimbic and thalamic inputs would allow for precise control of stimulation. Thus, prelimbic, thalamic and ventral subiculum were injected with virus that transfected prelimbic, thalamic and ventral subicular neurons with channelrhodopsins that expressed in terminals projecting to the BL after a minimum of 2-3 weeks.

2.5 VALIDATION OF AMYGDALAR fEPSPs

Because field electrophysiology has not been widely used in the BL and thus far no one has used field electrophysiology to study optogenetically evoked input to the BL, one of the purposes of this study was to validate the use of fields for studying optogenetically evoked glutamatergic responses in the BL. Given the unorganized nature of BL neurons, a single spiking neuron could easily influence a field response. Thus, one of the frequent steps when validating a response was to determine if the fEPSP exhibited an "all or none" characteristics or if it gradually increased in amplitude with stimulus intensity. Should the light intensity increase gradually but the response rapidly increase in amplitude without a gradual change, it was likely due to an action potential of a single neuron and discarded. An appropriate gradual response to increasing intensity of light stimuli is depicted in Figure 2.6A.

Because the excitatory and inhibitory components of field responses in the BL are not extensively known, it was also important to examine the response in the presence of GABA inhibitors. Application of GABA receptor antagonists slightly increased the

amplitude of the response but had no effect on the directionality of the waveform (Figure 2.6B).

Lastly, to ensure that the recorded responses were indeed glutamatergic, a saturating dose of the glutamatergic antagonist, CNQX, was applied at the end of each experiment to verify via complete inhibition of the remaining fEPSP (Figure 2.6B).

2.6 ANIMAL CARE AND USE

All animal care and procedures were approved by the Institutional Animal Care and Use Committee at the University of South Carolina and performed in compliance with the guidelines approved by the National Institute of Health Guide for the Care and use of Laboratory Animals (Department of Health and Human Services). Experiments were performed in male and female transgenic ChAT-Cre (B6;129S6-Chat$^{tm2(cre)Lowl/J}$) mice (The Jackson Laboratory). Mice were housed 1-5 to a cage in a climate controlled facility with a 12/12 light/dark cycle and provided with *ad libitum* access to food and water.

2.7 SURGICAL PROCEDURES FOR VIRAL DELIVERY

Mice 1.5-3 months old were anesthetized under deep isoflurane anesthesia and placed in a stereotaxic surgery device (Stoelting, Wood Dale, IL). Bilateral injections of 0.15μL of rAAV5-CAMKII-hChR2(H134R)-eYFP-WPRE (UNC Viral Vector Core) were delivered to the prelimbic cortex (from Bregma: 1.9mm Anterior/Posterior; -0.3mm Media/Lateral; -2.0 mm Dorsal/Ventral), bilateral injections of 0.2μL of rAAV5-CAMKII-hChR2(H134R)-eYFP-WPRE (UNC Viral Vector Core) were delivered to the ventral

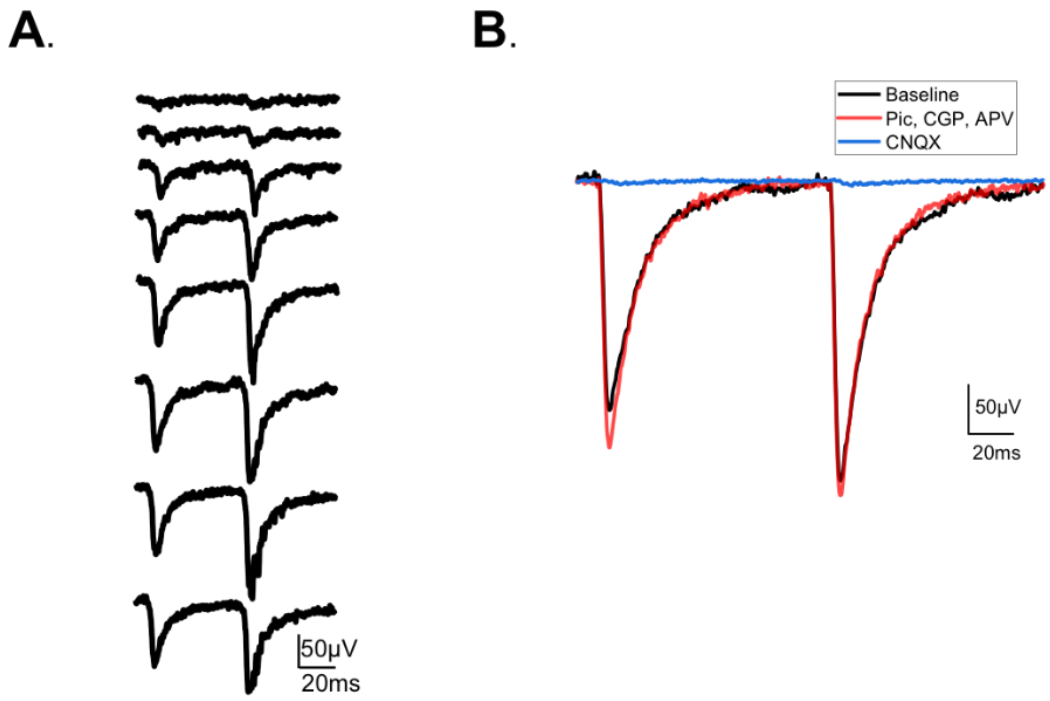


Figure 2.6 Validation of fEPSP as synaptic event versus action potential and validation of excitatory versus inhibitory components.
A. Representative traces from an input/output study showing gradual increase in amplitude of the fEPSP. Any recordings that displayed spiking behavior were not included.
B. Representative traces illustrating no large effects of GABA antagonists. Importantly, application of AMPA/Kainate antagonist CNQX at the conclusion of each experiment and complete inhibition of responses ensured the contribution of glutamate to the fEPSP.

subiculum (from Bregma: -2.5mm Anterior/Posterior; -3.2mm Media/Lateral; -5.3 mm Dorsal/Ventral) and single injections of 0.2μL of rAAV5-CAMKII-hChR2(H134R)-eYFP-WPRE (UNC Viral Vector Core) to the midline thalamic nuclei (from Bregma: -0.3mm Anterior/Posterior; 0.0mm Media/Lateral; -3.9mm Dorsal/Ventral). For experiments in which endogenous ACh was optogenetically released, bilateral injections of 0.2μL of rAAV5-EF1a-DIO-hChR2(H134R)-eYFP (UNC Viral Vector Core) was delivered into the basal forebrain targeting the substantia innominata (from Bregma: 1.2mm Anterior/Posterior; -1.3mm Media/Lateral; -5.3mm Dorsal/Ventral). Following surgeries, incisions were covered with a topical tissue adhesive (Gluture, Abbott Laboratories, Chicago, IL), placed in a recovery chamber and postoperatively monitored. Injection sites were validated and reported in Figure 2.10.

2.8 SLICE PREPARATION

Animals were deeply anesthetized with isoflurane and rapidly decapitated. The brain was quickly removed and submerged for 1-1.5 minutes in ice-cold (4°C) choline chloride-based cutting artificial cerebrospinal fluid (cutting ACSF) (in mM: 110 choline chloride, 2.5 KCL, 25 $NaHCO_2$, 1.0 NaH_2PO_4, 20 glucose, 5 $MgCl_2$, 0.5 $CaCl_2$) and superfused with 95% O_2 and 5% CO_2. To prevent glutamatergic excitotoxicity and potential potentiation of slices during the cutting procedure, 5mM kynurenic acid (Sigma, St. Louis, MO) was also included in the cutting ACSF. The brain was then transferred to a vibratome (VT1000S, Leica, Nussloch, Germany) containing the same choline-based ACSF solution and 500μm thick horizontal and coronal sections were cut. Slices were incubated at 34-36°C in an ACSF solution containing (in mM) 125 NaCl, 2.7 KCl, 25

$NaHCO_2$, 1.25 NaH_2PO_4, 10 glucose, 5 $MgCl_2$, 0.5 $CaCl_2$ (incubating ACSF) and superfused with 95% O_2 and 5% CO_2. After a minimum of 20 minutes at 34-36°C, incubation temperature was then brought to room temperature. For recording, slices were transferred to a recording chamber and continuously superfused with 32°C ACSF containing (in mM) 125 NaCl, 2.7 KCl, 25 $NaHCO_2$, 1.25 NaH_2PO_4, 10 glucose, 1 $MgCl_2$, 2 $CaCl_2$ (recording ACSF) at a rate of 4-6mL/min.

2.9 SLICE ELECTROPHYSIOLOGY RECORDINGS

Slices were submerged in a recording chamber and gently held in place by a platinum wire. Glutamate release from prelimbic and thalamic projections to the basolateral amygdala were stimulated with single or dual (50 ms apart) 2-3ms light pulses of 490nm blue LED light (ThorLabs Inc, Newton, New Jersey) to activate channelrhodopsins. A borosilicate glass electrodes with resistances between 1-3 MΩ and filled with recording ACSF was placed in the basolateral amygdala (visually identified with a light microscope) and the resulting evoked field excitatory postsynaptic potentials (fEPSP) were recorded. For frequency experiments, blue light was administered at 1Hz, 5Hz, 10Hz, 20Hz, 30Hz and 40Hz to release glutamate at various frequencies. To isolate glutamatergic neurotransmission, $GABA_A$ receptors were blocked with 10μM-100μM picrotoxin or 10μM bicuculline, $GABA_B$ receptors were blocked with 2μM CGP55845 and N-methyl-D-aspartate (NMDA) receptors were blocked with 50μM L-2-amino-5-phosphonovaleric acid (D-APV) or 10μM MK801. Glutamatergic components of field potentials were confirmed by inhibiting the response with 25μM of the potent

glutamatergic antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) at the conclusion of each experiment.

For other experiments, endogenous ACh was optogenetically released prior to electrically evoking glutamate release from cortical projections that coursed through the external capsule to the BL. Theta-burst pattern stimuli of blue LED light was administered 250ms before the external capsule was electrically stimulated. Theta-burst pattern light was defined as four 3ms pulses of light at 50Hz given four times with a burst start-to-start interval of 200ms. Responses were recorded with a Multiclamp 700B (Molecular Devices, Sunnyvale, Ca) amplifier, filtered at 1kHz and digitized (Digidata 1400) using pClamp 10 software (Molecular Devices, Sunnyvale, Ca).

For experiments in which muscarinic subtypes were studied, antagonists specific for M1, M2, M3 or M4 were bath applied (see Methods Chapters 3-5). Because electric stimulation can create a stimulus artifact due to the electric current injection and *not* the physiological response, this stimulus artifact that occurred in the presence of no glutamatergic transmission (ie, in the presence of a saturating concentration of CNQX at the conclusion of each experiment) was digitally subtracted from the rest of the experiment.

2.10 SPECIFICITY OF VIRAL VECTOR EXPRESSION IN CHAT+ BASAL FOREBRAIN NEURONS

To validate viral expression of channelrhodopsins in basal forebrain cholinergic neurons, transgenic ChAT-Cre mice injected in the basal forebrain with the floxed virus were sacrificed and perfused. Coronal brain sections were prepared and choline acetyltransferase-positive (ChAT+) neurons were labeled with goat anti-ChAT primary

antibody (Millipore, [1:500]) and Alexa Fluor 546 conjugated donkey anti-goat IgG secondary antibody (Thermo Fisher, [1:400]). Cell counts were performed to quantify the number of neurons singly-labeled by eYFP and doubly-labeled by both YFP and Td-Tomato in each z-stack image.

2.11 MATERIALS

Pharmacological compounds used in electrophysiology experiments (see Ch. 3-5) were bicuculline, D-AP5, CNQX, CGP55845 hydrochloride, MK 801 maleate, AM251, forskolin (Hello Bio, Princeton, NJ), baclofen, muscarine chloride, N-ethylmaleimide, phystostigmine (Millipore Sigma, St. Louis, MO), WIN 55, 212-2, oxotremorine M, VU10010, PD102807 (Tocris Biosciences, Bristol, UK), 4DAMP, AFDX, VU0255025, Atropine, Mecamylamine hydrochloride (Abcam, Cambridge, UK), AM630 (Cayman Chemical Company, Ann Arbor, MI), VU0467154 (StressMarq Biosciences, Victoria, BC).

2.12 DATA ANALYSIS AND STATISTICS

Electrophysiological data analysis was performed using pClamp 10 (Molecular Devices), OriginPro 2018b (Microcal, Northampton, MA) and Excel 2016 (Microsoft Corporation, Redmond, VA) software. The peak amplitude of fEPSPs was measures as the average amplitude of the steady-state peak in each condition. Between-group means were statistically compared with Student's *t* test or one-way ANOVA with a *post-hoc* Tukey test.

CHAPTER 3

INHIBITION OF EXTERNAL INPUT TO THE BL BY ENDOGENOUS ACETYLCHOLINE

3.1 INTRODUCTION

Acetylcholine (ACh) is a neurotransmitter important for learning and memory. Originating largely from cholinergic neurons in the basal forebrain, cholinergic transmission to various regions throughout the brain functions in selectively filtering neurotransmission and ultimately affect the signal-to-noise ratio of incoming stimuli while suppressing internal transmission (Hasselmo 2006). In area CA1 of the hippocampus, cholinergic receptors differentially regulate glutamatergic input from the Schaffer collaterals and the temporoammonic pathways (Hasselmo & Schnell, 1994; McQuiston 2019). Because the Schaffer collaterals project from hippocampal area CA3 to hippocampal area CA1, and temporoammonic pathway projections from entorhinal cortex, ACh differentially regulates transmission of internal input and external input to the hippocampus (Hasselmo & Schnell, 1994; Goswamee & McQuiston 2019). This cholinergic regulation of internal versus external inputs has important implications for learning as well, as animal work shows that cholinergic modulation of different pathways will differentially affect recall of previously learned memories and learning new memories

(Hasselmo and Bower 1993). What this ACh does to different pathways converging in the basolateral amygdala (BL), however, remains largely unknown.

One of the main sources of ACh is from the basal forebrain, an area from which the cholinergic projections have been shown to be important for learning and memory (Baxter et al., 1995; Muir et al., 1993; reviewed in Baxter & Chiba 1999). Basal forebrain cholinergic projections have also been implicated in emotional learning and memory as well, as lesioning the basal forebrain cholinergic neurons impairs acquisition of fear extinction and optogenetic stimulation and inhibition of cholinergic projections affects fear behaviors (Knox et al., 2016; Jiang et al., 2016). Given the role of the basal forebrain cholinergic projections in learning and memory and the role of the amygdala in emotional learning, it is surprising that our understanding of cholinergic modulation in the amygdala remains in a very nascent phase.

Only a few studies have examined how released ACh modulates the excitability of amygdalar neurons, but those that have suggest a cholinergic-evoked increase in signal-to-noise ratio (Unal et al., 2015; Aitta-Aho et al., 2018). In these studies, released ACh was found to produce neuron-dependent effects on excitation versus inhibition. In some neurons at resting potential, ACh largely produced a hyperpolarizing inhibitory postsynaptic current (IPSC) whereas in a separate study, released ACh evoked an initial hyperpolarizing current followed by a slower depolarizing current in neurons at resting potentials (Unal et al., 2015; Aitto-aho 2018). These effects were also dependent on the state of the postsynaptic amygdalar neurons; ACh inhibited neurons that were held at a slightly depolarized potential and fired at a slow rate whereas when neurons were

depolarized and made to fire at a high rate, ACh has no effect during the depolarized potential but induced an afterdepolarization (Unal et al., 2015).

In addition to modulating the postsynaptic excitability, and thus, amygdalar receptiveness to incoming stimuli, endogenous released ACh can also modulate the presynaptic inputs to effect signal-to-noise of a region and circuit. However, how this occurs in the basolateral amygdala remains largely unknown. In the only study that has examined released ACh on cortical inputs to the BL, Jiang et al. (2016) found cholinergic input arising from the basal forebrain potentiates cortical transmission to the BL (Jiang et al., 2016). When basal forebrain projections to the BL were optogenetically stimulated, basolateral neurons increased their firing frequencies. Additionally, cholinergic stimulation increased glutamatergic transmission from cortical input through presynaptic nAChRs (Jiang et al., 2016). However, this study examined cholinergic evoked changes on a long-term scale that examined long term potentiation, leaving a gap in our understanding of how to reconcile the largely inhibitory, acute postsynaptic cholinergic effects seen by Unal and Aitto-Aho with the long-term excitatory potentiation of glutamatergic input and BL firing rates seen by Jiang et al. What could aid in resolving this question is an understanding of how cholinergic inputs to the BL regulate glutamatergic input to the BL acutely.

Anatomical studies have shown presynaptic mAChRs located on glutamatergic terminals in the BL. M1 labeling was found on a subset of presynaptic terminals that also expressed VGLUT 1, an indicator of cortical terminals as well as on terminals expressing VGLUT2, an indicator of thalamic terminals (McDonald et al., 2019). Muscarinic M2 receptors were also found on presynaptic putative glutamatergic inputs to the BL that

synapsed onto dendritic spines (Muller et al., 2016). In other brain regions as well as a seminal study in the amygdala, cholinergic agonists and released ACh have been shown to inhibit glutamate release by acting on presynaptically located mAChRs (Yajeya et al., 2000; Hasselmo et al., 2006; Dannenberg et al., 2017; McQuiston 2019). However, it remains unknown how released ACh modulates cortical glutamatergic transmission to the BL in an acute manner.

To answer this question, the current study used optogenetic and electrophysiological techniques to determine how endogenous ACh regulates cortical glutamatergic neurotransmission from the external capsule to the BL. It was hypothesized that released ACh would inhibit glutamatergic transmission from cortical inputs given the role of ACh in enhancing the signal to noise of stimuli, anatomical studies demonstrating presynaptic muscarinic receptors, and pharmacological data showing muscarinic inhibition of cortical transmission to the BL. It was found that endogenous ACh inhibits cortical glutamatergic input to the BL conveyed by the external capsule, and that this inhibition is mediated by muscarinic receptors.

3.2 MATERIALS AND METHODS

3.3.1 VIRAL DELIVERY AND VALIDATION OF CHR2S TO CHOLINERGIC BASAL FOREBRAIN NEURONS

As described in Chapter 2, transgenic B6;129S6-Chat$^{tm2(cre)Lowl/J}$ mice (1.5-3 months) were anesthetized with isoflurane and injected bilaterally with 0.2 μL of rAAV5/EF1a-DIO-hChR2(H134R)-eYFP into the substantia innominata to selectively express channelrhodopsin (ChR2) in cholinergic basal forebrain neurons of transgenic

ChAT-Cre mice. To validate viral expression of ChR2s in basal forebrain cholinergic neurons, transgenic ChAT-Cre mice injected in the basal forebrain with the floxed virus were sacrificed and perfused. Coronal brain sections were prepared and choline acetyltransferase-positive (ChAT+) neurons were labeled with goat anti-ChAT primary antibody (Millipore, [1:500]) and Alexa Fluor 546 conjugated donkey anti-goat IgG secondary antibody (Thermo Fisher, [1:400]). Cell counts were performed to quantify the number of neurons singly-labeled by eYFP and doubly-labeled by both YFP and Td-Tomato in each 50μm thick z-stack image.

3.3.2 WHOLE CELL RECORDINGS

Coronal brain slices 300μM thick were cut and maintained in an incubation chamber using techniques described in Chapter 2. Voltage-clamp recordings from pyramidal neurons were conducted at a holding potential of -70mV. The series resistance was between 10-25MΩ.

To stimulate the release of endogenous ACh and record evoked cortical fEPSPs in the BLa, blue LED light (ThorLabs Inc, Newton, New Jersey) was flashed to release ACh and the external capsule was electrically stimulated. Blue light was flashed at "weak," "strong," and "theta burst" intensities, consisting of five pulses, thirty pulses and 16 pulses of blue light, respectively. The theta burst intensity was divided into four bursts of four flashes each, with burst intervals of 200 ms start-to-start and interburst frequencies of light at 50Hz.

Cholinergic currents and evoked glutamatergic currents were recorded using a Multiclamp 700B amplifier and pClamp 10 software (Molecular Devices, Sunnyvale, CA) and responses were filtered at 1kHz.

3.3.3 FIELD ELECTROPHYSIOLOGY RECORDINGS

Horizontal and coronal brain slices (500μM thick) containing the amygdala were cut and incubated using techniques described in Chapter 2. To stimulate the release of endogenous ACh and record evoked cortical fEPSPs in the BLa, blue LED light (ThorLabs Inc, Newton, New Jersey) was flashed to release ACh and the external capsule was electrically stimulated.

All field experiments were performed in which theta burst light stimuli (described above) was applied to release ACh.

3.3 RESULTS

3.4.1 COLOCALIZATION OF EYFP-LABELED CHR2S TO CHOLINERGIC NEURONS IN THE BASAL FOREBRAIN TO DETERMINE VIRAL VECTOR EXPRESSION.

In order to study cholinergic release in the BL, the first objective was to determine the efficacy of viral vector delivery of ChR2s with fused eYFP to cholinergic neurons in the basal forebrain that project to the BL. At least three weeks after viral injections, mice were transcardially perfused, sections made and immunofluorescence performed to label cholinergic neurons in the basal forebrain for ChAT with Td-tomato (Figure 3.1A). Cell

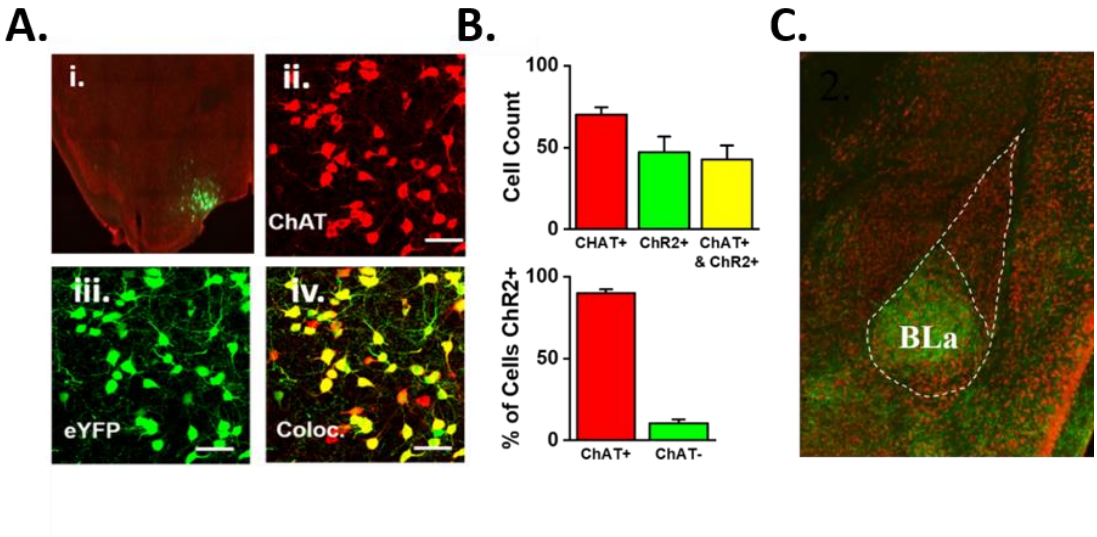


Figure 3.1. Localization of eYFP labeled ChR2s to cholinergic neurons in the basal forebrain to determine viral vector efficacy.

A. i. Injection site showing ChR2-expressing cell bodies in the horizontal limb of the diagonal band of Broca in the basal forebrain. **ii.** Confocal immunofluorescence demonstrating Td-tomato labeled ChAT+ neurons in the basal forebrain. **iii.** eyFP-labeled ChR2+ neurons in the basal forebrain. **iv.** Colocalization (yellow) of ChAT+ (red) and ChR2+ (green) neurons in the basal forebrain.

B. Top: Cell counts of basal forebrain neurons per 50µm thick coronal section that were labeled with tdTomato (red), eYFP (green) or colabeled with both (yellow). Nearly all basal forebrain neurons expressing ChR2 were labeled for ChAT+. Bottom: Percentage of neurons in the basal forebrain that express both ChR2 and ChAT versus number of neurons in the basal forebrain that express just ChR2 (n=5 mice). Nearly all (89.82%) neurons expressing ChR2 also expressed ChAT.

C. Cholinergic basal forebrain projections (green) expressing ChR2 terminating in BL.

counts taken for 50µm thick z-stack images labeled with ChAT and colabeled with ChAT and eYFP confirmed that expression of ChR2 was restricted to cholinergic neurons in the basal forebrain (Figure 3.1B). The viral infection rate was 70.2% (±4.26) for expression in cholinergic neurons (n=5 animals). Of the cells infected with the virus, 90% were cholinergic (89.81609 ± 2.44). Terminals from ChR2+ neurons that projected to the BL were visualized for eYFP (Figure 3.1C).

3.4.2 RELEASED ACH FROM THE BASAL FOREBRAIN EVOKES DIFFERENTIAL EFFECTS ON BASELINE IN BASOLATERAL AMYGDALA.

The first objective was to determine the effects of released ACh on baseline field potentials in the absence of electrical stimulation of evoked cortical input, as previous studies have shown muscarinic receptor activation can elicit postsynaptic changes in membrane potential due to cholinergic induced inward and outwards currents (Aitta-Aho 2019; Unal et al., 2015). Cholinergic evoked currents were recorded using both field electrophysiology and whole-cell electrophysiology. In response to brief (2ms) flashes of blue light, 7 out of 11 slice displayed cholinergic potentials such that light-released ACh evoked a downward field potential (Figure 3.2A pink arrow). This change in baseline potential was significantly different than in the absence of released ACh (*$p<0.01$) (Figure 3.2C). A similar potential was seen in response to released ACh in whole-cell recordings (Figure 3.2A). These findings that released ACh elicits a change in potential of amygdalar neurons agrees with findings from Jiang et al. (2016) that released ACh increases excitatory activity in BL pyramidal neurons as well as with other studies indicating that cholinergic receptor activation in the BL causes a membrane depolarization due to muscarinic

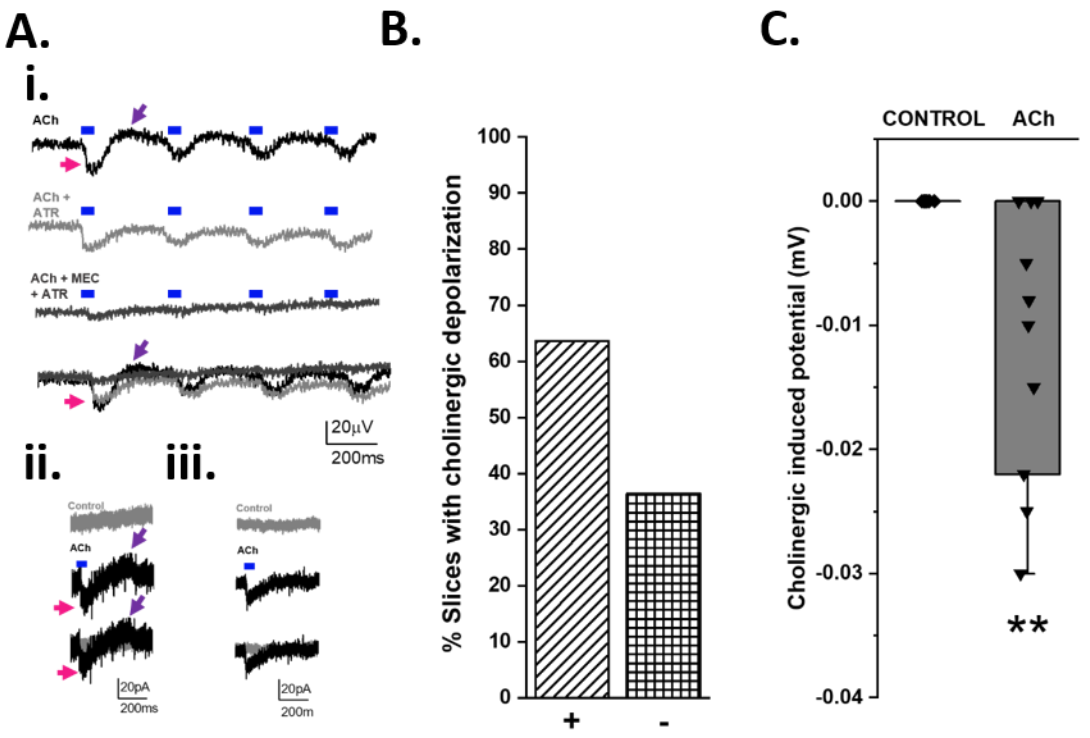


Figure 3.2. Effects of released ACh on baseline field potentials in BL neurons.
A. Representative traces showing amygdalar responses to optogenetically released ACh (blue bar). **i.** Field potentials recorded in the BL show a downward postsynaptic potential (pink arrow) followed by a slight upward potential (purple arrow) after light-released ACh. The upward potential is abolished in the presence of atropine (light grey) and the downward potential is largely abolished in the presence of mecamylamine (dark grey). **ii.** Representative traces from a whole-cell experiment recorded before (grey) and after (black) ACh release demonstrate no net current in the absence of ACh release but a depolarizing current (pink arrow) in response to ACh followed by a hyperpolarizing current (purple arrow). **iii.** Representative trace showing differential response to ACh in a separate neuron in which ACh only evoked a depolarizing current with no noticeable hyperpolarization.
B. Bar graph summarizing percent of slices that displayed potential changes in basolateral amygdalar neurons following ACh release. 7 out of 11 slices had cholinergic induced changes in baseline potential.
C. Box plot of amygdalar baseline potentials in response to no ACh (control) and light released ACh. Released ACh significantly changed the baseline potential in the BL (n=11; $^{**}p<0.05$). Variation seen in postsynaptic responses to released ACh.

inhibition of the M-current and GIRK channels (Washburn & Moises 1992; Womble and Moises 1993). However, the overall consensus for the timescale of ACh's effects on excitation of the BL pyramidal neurons remains controversial in the literature; some findings show cholinergic receptor activation in the amygdala by light-released ACh induces an inhibitory postsynaptic potential in some cells at rest but more of an excitatory effect when cells are made to fire at more depolarized currents (Unal et al., 2015). Other reports show bath application of cholinergic agonist induce only a depolarization of membrane potential but focal application of cholinergic agonist on the other hand induce a biphasic effect consisting of an initial hyperpolarization followed by a slow depolarization (Washburn & Moises 1992). Interestingly, in our study we found one biphasic postsynaptic field response to release ACh using whole cell electrophysiology that differed from previous findings in the literature (Figure 3.2Aii.). In this cell, ACh induced an inward current followed by a slower hyperpolarizing current. This effect was also robust in one slice in field electrophysiological experiments (Figure 3.2Ai.). In this experiment, the upward potential was abolished with bath application of the muscarinic antagonist atropine (5μM) while the downward potential was largely abolished with bath application of the nicotinic antagonist mecamylamine (20μM). This could be due to activation of nAChRs to depolarize interneurons and their resulting hyperpolarization of pyramidal neurons, but further experiments would be needed to further investigate this possibility.

3.4.3 ENDOGENOUS ACH INHIBITS CORTICAL TRANSMISSION TO THE BL.

We next wanted to determine the effect of ACh on evoked glutamatergic neurotransmission to the BL in baseline ACSF. To stimulate afferent cortical input to the

BL, an electrode was placed in the external capsule. Prior to evoking glutamate release, ACh was released using varying protocols to determine thresholds of cholinergic effects. It was found that ACh significantly inhibited cortical input to a similar extend regardless of the intensity of optogenetic stimulation employed to release ACh (Figure 3.3C). Weak optogenetic stimulation inhibited the evoked glutamatergic postsynaptic current by 23.2% (±6.17; n=5) strong optogenetic stimulation inhibited the evoked glutamatergic current by 25.9% (±8.11; n=4) and theta optogenetic stimulation inhibited the evoked glutamatergic current by 30% (±13%; n=4). No significant differences in the percent inhibition were found between these three optogenetic intensities. This agrees with findings from another published study that minimal stimulation to the cholinergic inputs induces robust cholinergic effects in the amygdala (Aitto-Aho 2019). Because no difference was observed between weak, strong and theta light protocols, theta-burst was used for all following experiments to release ACh, as basal forebrain neurons fire at high-frequency bursts at theta frequency during active waking, REM sleep, and after reward and punishment (Lee et al., 2005; Laszlovsky et al., 2019). In both field potential recordings and whole-cell recordings, ACh released at theta burst frequency significantly inhibited glutamatergic input to the BL (Figure 3.3 D). Furthermore, there was no difference in the extent of inhibition of cortical input between whole cell (28.9% ± 13.02; n=4) and field potential (24.95% ±7.9 n=7) recordings. Agreeing with Yajeya (2000) and Washburn & Moises (1992), these results indicate that activation of cholinergic receptors in the BL suppresses afferent glutamatergic transmission to the BL but contrast those of Jiang et al. (2016) finding cholinergic release in the BL increases glutamatergic transmission.

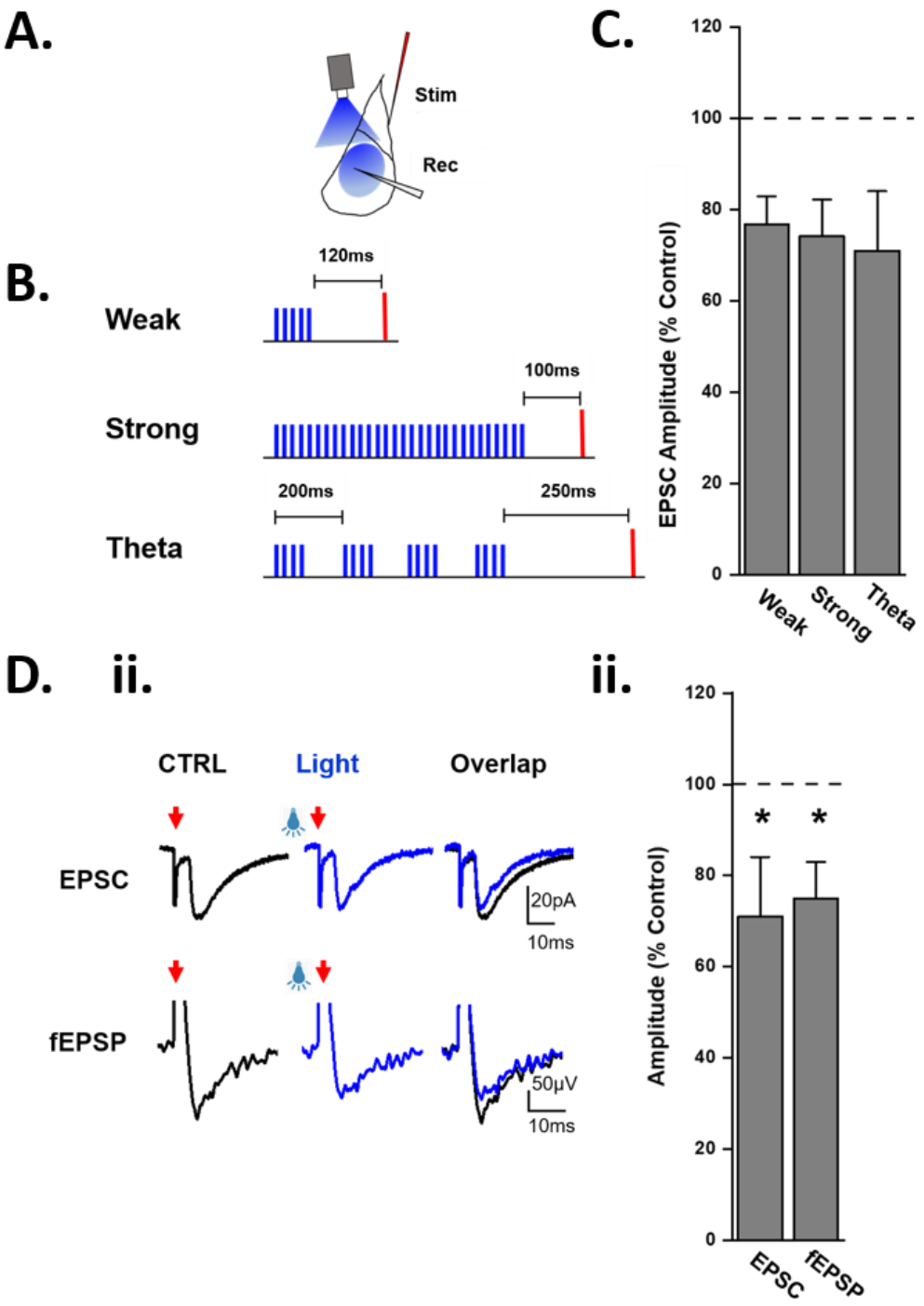


Figure 3.3. Endogenous ACh inhibits cortical transmission to the BL irrespective of strength of release stimulus.

A. Schematic illustrating optogenetic probe to evoke blue light pulses, stimulating electrode placed in external capsule to electrically evoke glutamate release and recording electrode placed in BLa to record evoked glutamatergic transmission.

B. Illustrations of weak, strong and theta light protocols. Weak light stimuli consisted of five pulses and strong stimuli consisted of 30 pulses. Theta stimuli consisted of four bursts with start-to-start intervals of 200ms. Each burst consisted of four pulses at 50 Hz for a total of 16 pulses. Following each protocol to release light, the external capsule was electrically stimulated to release glutamate.
C. Summary bar graph illustrating percent inhibition by released ACh when varying intensities of stimuli are elicited. Released ACh inhibits glutamatergic neurotransmission to a similar extent when weak, strong or theta burst patterns of released are used (n=5, n=4, n=4 for weak, strong and theta burst stimuli, respectively).
D. ii. Released ACh similarly inhibited cortical glutamate transmission to the BL in field recordings compared to whole cell recordings when theta burst stimuli was applied (n=4 and n=7 for whole cell and fields, respectively). (**i.** representative traces from Dii).

3.4.4 COMPONENTS OF CHOLINERGIC INHIBITION OF CORTICAL INPUT TO BL.

In the previous sections, it was shown that optogenetically released ACh induces both depolarizing and hyperpolarizing currents (measured with whole cell techniques) that affect baseline amygdala potentials as well as suppression of evoked glutamatergic transmission to the BL from afferent input. Additionally, as no differences were found in cholinergic effects between whole cell and field recordings, all following experiments were performed with field electrophysiology. The purpose of employing this recording technique is to examine the summed potentials evoked by glutamatergic transmission to the BL to determine how ACh regulates the overall excitability of the BL.

As previous studies have illustrated mAChRs to facilitate GABA release from interneurons, it was necessary to determine if GABA transmission is playing a role in the cholinergic inhibition of cortical input to the BL (Martin & Alger 1999). To determine if this is a possibility, afferent input from the external capsule was again evoked in the

presence and absence of ACh in control recording ACSF. Similar to our previous findings, ACh inhibited glutamatergic transmission to the BL; in the presence of ACh, the glutamatergic EPSP was only 70% of the control (70.17%±7.37, $p<0.01$, $n=6$) (Figure 3.4A). To determine if GABA was playing a role in this cholinergic inhibition, we repeated the same experiment in the presence of bath applied $GABA_A$ antagonist picrotoxin (10µM) and $GABA_B$ antagonist CGP55345 (2µM). ACh inhibited the fEPSP to the same extent regardless of whether GABA receptors were blocked (Figure 3.4B), indicating that released ACh does not inhibit evoked fEPSPs in manner that involves GABA receptors. Lastly, because NMDA receptors were also unblocked in our experimental paradigms, to ensure ACh was not somehow affecting glutamatergic responses postsynaptically by directing acting on NMDA receptor, experiments were again repeated in the presence of NMDA receptor antagonist APV (50µM). Unsurprisingly, no effect on the cholinergic inhibition was seen when NDMA receptors were blocked (Figure 3.4C). This result was not suprpsing given that we did not evoke or measure an NMDA current, and thus any effect we would have seen by ACh would have been due to ACh possibly acting directly on the NMDA receptors itself. Had we isolated the NMDA current by using lower Mg^{2+} concentrations, etc., we may have observed a cholinergic effect. These results indicate that released ACh is suppressing afferent to the BL from the external capsule in a manner independent of GABA and NMDA receptors.

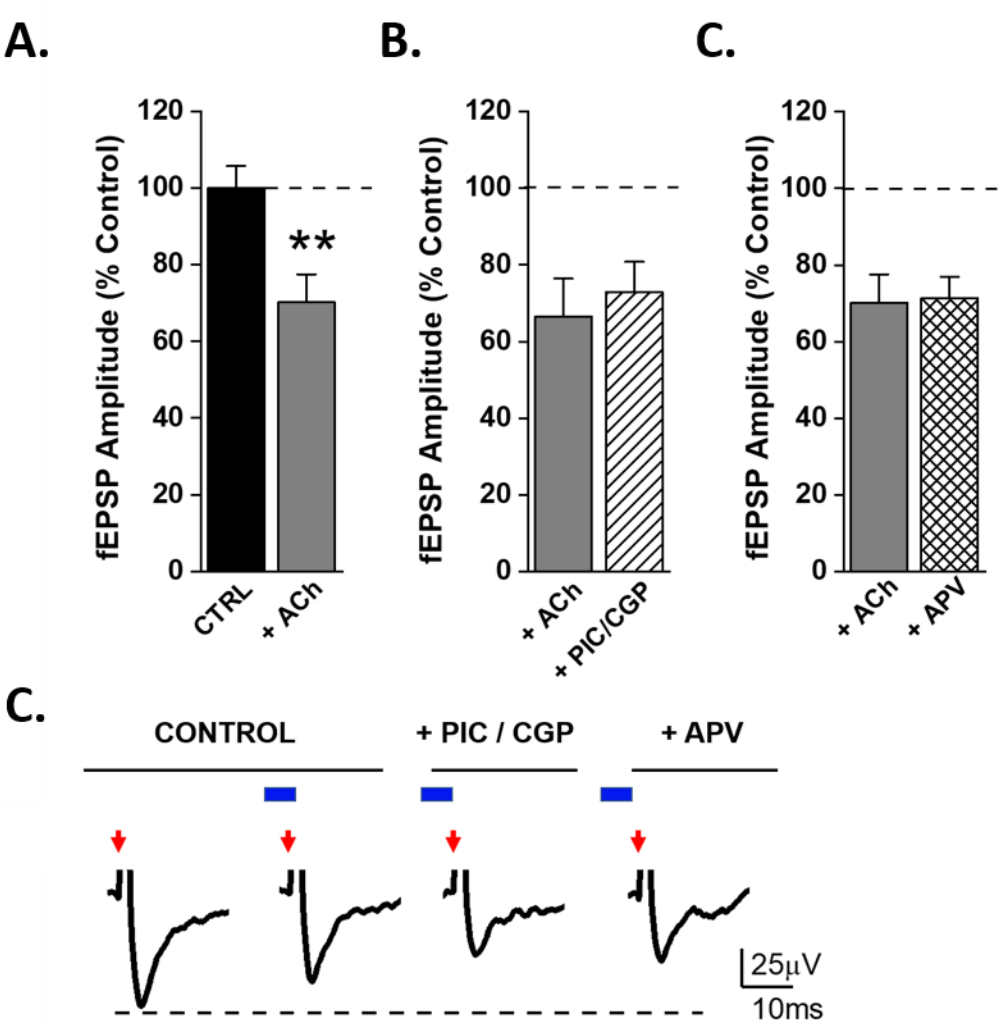


Figure 3.4. Components of cholinergic inhibition of cortical input to the BL.
A. Released ACh significantly inhibits cortical input to the BLa (70.17% ± 7.37; n=6; **p<0.01).
B. Inhibition of cortical input by endogenous ACh is not mediated in a significant manner by GABA receptors. Application of $GABA_A$ antagonist picrotoxin (10µM) and $GABA_B$ antagonist CGP55845 (2µM) had no effect on extent of inhibition (n=4).
C. Released ACh does not inhibit cortical input by acting directly on NMDA channels. Application of NMDA antagonist D-APV (50µM) had no effect on extent of cholinergic inhibition (n=6).
D. Representative traces illustrating light released (blue bar) ACh inhibited evoked (red arrow) gluatmatergic transmission in the BLa. Application of GABA antagonists (PIC / CGP) and NMDA antagonist (APV) had no effect on inhibition.

3.4.5 ENDOGENOUS ACH SUPPRESSES CORTICAL INPUT TO THE BL THROUGH MUSCARINIC RECEPTORS.

As electrophysiological studies have shown conflicting evidence of nicotinic (Jiang et al., 2016) and muscarinic (2000; Washburn & Moises 1992) mediated inhibition of glutamate release in the BL, and anatomical data indicates presynaptically located mAChRs on putative glutamatergic input to the BL (McDonald et al., 2019; Muller et al., 2011), we wanted to then determine if cholinergic inhibition in our experimental paradigm is being mediated by nAChR or mAChR receptors. Glutamatergic fEPSPs were recorded in the absence and presence of bath-applied nicotinic antagonists mecamylamine (20µM). Antagonizing nAChRs had no significant effect on the amplitude of the evoked fEPSP (Figure 3.5A, B, C, D). However, a trend was seen whereby the cholinergic inhibition was slightly increased after nicotinic receptors were antagonized, indicating nAChRs may be present and slightly facilitating glutamate release presynaptically, presumably by increasing presynaptic Ca^{2+} (Girod et al., 2000). Bath application of the muscarinic antagonist atropine (5µM) completely reversed the muscarinic inhibition (-6.91 ± 5.38; n=5) (Figure 3.5A, B, C, D). These findings were also observed in whole cell experiments (Figure 3.5 B, C,D). Interestingly, the amplitude of the glutamatergic response was larger in the presence of atropine (Figure 3.5A), suggesting either tonic release of endogenous ACh in the BL or potentiation of the response by release ACh. A tonic ACh release in the BL was also supported by a previous study that stimulated the external capsule and found application of physostigmine alone inhibited the glutamatergic response, and that this was sensitive to atropine (Washburn & Moises 1992).

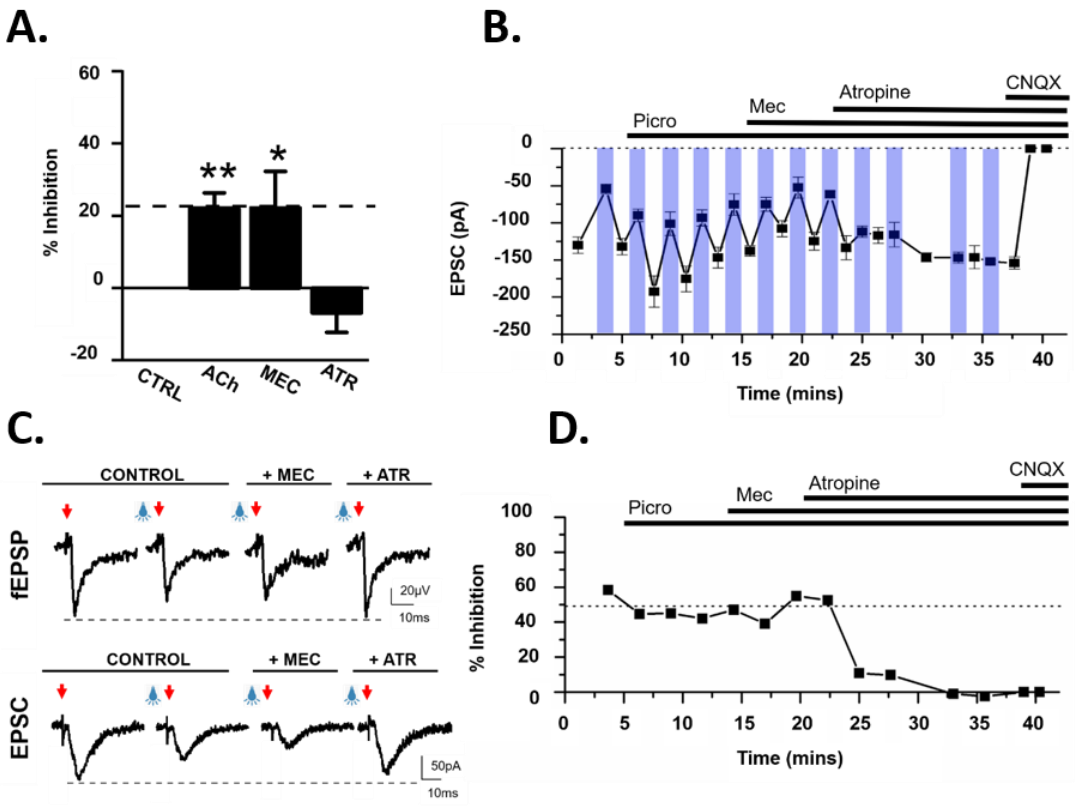


Figure 3.5. Muscarinic ACh receptors mediate cholinergic inhibition of glutamatergic input to BL.

A. Cholinergic inhibition by ACh is not affected by nicotinic receptor antagonist mecamylamine (20μM) (22.13% ±10.17; n=3) but is completely reversed by application of muscarinic antagonist atropine (5μM) (-6.91 ± 5.38; n=5), indicating muscarinic-receptors mediate the cholinergic inhibition of the evoked fEPSP (***p<0.001).

B. EPSCs evoked by stimulation of the external capsule are significantly inhibited by light-released ACh (blue bar). Application of mecamylamine (20μM) has no effect on inhibition whereas atropine (10μM) completely reverses cholinergic inhibition.

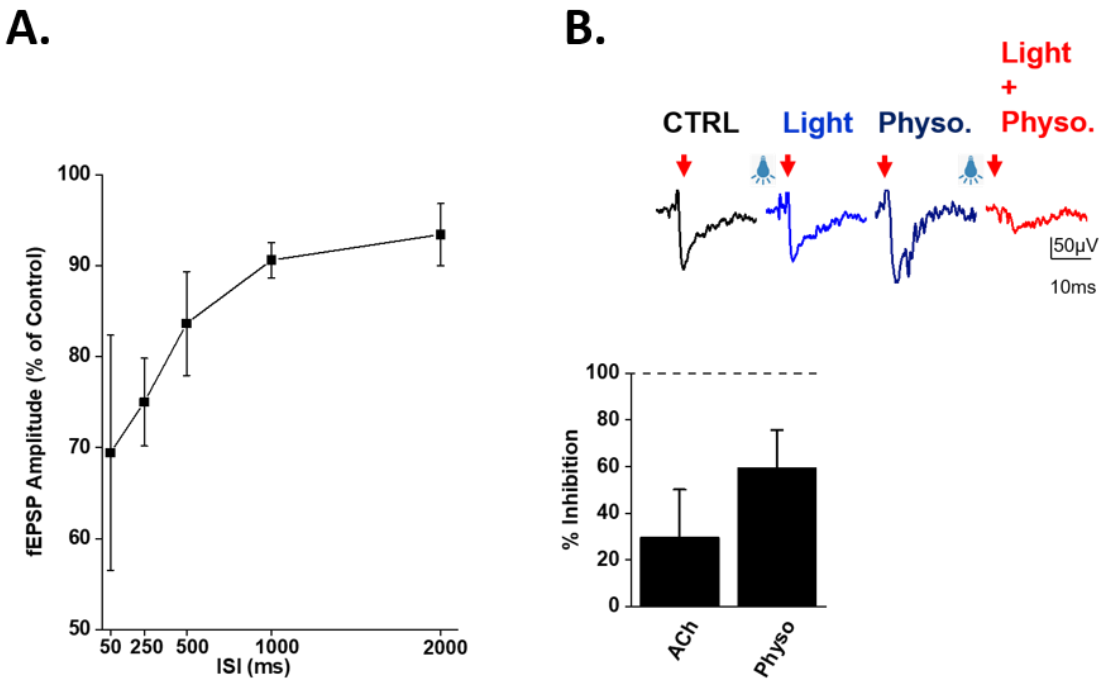
C. Representative traces illustrating inhibition of fEPSPs and EPSCs and reversal of inhibition in the presence of the muscarinic antagonist, atropine.

D. Representative experiments illustrating % inhibition of EPSCs by ACh in the presence of mecamylamine and atropine.

3.4.6. THE ACTION OF ACETYLCHOLINESTERASE LIMITS THE INHIBITORY CAPACITY OF MACHRS AT AFFERENT INPUT TO THE BL.

Given the previous findings that released ACh inhibits glutamatergic input to the BL by acting on mAChRs, and that other studies have found increasing the amount of ACh present by inhibiting acetylcholinesterase, we wanted to know if the 20% inhibition we were observing could be maximized by changing the amount of ACh that remains in the synapse or extrasynaptic space. To examine this question, we first pursued experiments that would inform the time course of the cholinergic inhibition of glutamate transmission. The external capsule was electrically stimulated to release glutamate at increasing intervals following light-released ACh. This would elucidate longer or shorter inter-stimulus-intervals (ISI) would increase or decrease the observed inhibition by ACh. As expected, the time course demonstrated that the inhibition we were observed followed the typical time course for metabotropic receptors, whereby the most effective interval between ACh release and glutamate stimulation was around 250ms (Figure 3.6A).

We next wanted to know if released ACh was rapidly degraded by acetylcholinesterase and thus limited in efficacy by the presence of this esterase. To test this, we compared the fEPSP in the presence of physostigmine, an inhibitor of acetylcholinesterase. By inhibiting the enzyme that degrades ACh, physostigmine should increase ACh in the extrasynaptic space. In these experiments, external capsule was stimulated and ACh was released, resulting in the typical inhibition of glutamate. However, application of a low dose of physostigmine (0.25µM) drastically facilitated this inhibition (Figure 3.6B top, bottom). Representative traces are illustrating in Figure 3.6B demonstrating external capsule-evoked fEPSP in control ACSF (black), slight inhibition



3.6. Cholinergic inhibition of cortical input to the BL mediated by action of acetylcholinesterase.

A. Inter-stimulus-interval (ISI) curve demonstrating decreasing suppression of ACh at longer intervals. Intervals of 1000ms and 2000ms significantly decreased inhibition by ACh.

B. Representative traces illustrating inhibition by released ACh (blue bar) is enhanced in the presence of acetylcholinesterase inhibitor physostigmine (0.25µM). Summary bar graph of inhibition by ACh is enhanced nearly twice as much by physostigmine, indicating ACh has the potential to exert larger suppression of cortical input to BL.

of fEPSP following light-released ACh (blue), external capsule-evoked fEPSP in control ACSF with added physostigmine (navy blue) and a much greater inhibition of fEPSP following light released ACh in the presence of physostigmine (red). Taken together, these results suggest that released ACh inhibits afferent input to the BL by acting on mAChRs, and that afferent input arising between 50 and 250ms to the BL following basal forebrain firing will be maximally inhibited as opposed to shorter and longer intervals.

3.4 DISCUSSION

In this study we employed optogenetics, field and whole-cell electrophysiological recordings to determine whether released ACh regulates afferent glutamatergic transmission to the BL. It was found that ACh, through its actions on mAChRs, suppresses glutamatergic fEPSPs evoked by external capsule stimulation. Furthermore, it was found that additional ACh released with an increasing number of light pulses had no effect on the extent of cholinergic inhibition. This potential maximum inhibition could presumably be due to rapid degradation by acetylcholinesterases that might serve as a limiting factor in cholinergic inhibition. The results of this study provide novel insight into the regulation of glutamatergic transmission to the BL from afferent input and build upon previous studies that have examined cholinergic regulation of glutamatergic input from the external capsule by pharmacologically activating cholinergic receptors (Washburn & Moises 1992; Womble & Moises 1993; Yajeya 2000) or examining longer-term effects of released ACh (Jiang et al., 2016).

3.4.1 ENDOGENOUS ACH RELEASED AT THETA-BURST PATTERN MODULATES GLUTAMATERGIC TRANSMISSION TO THE BL IN A MANNER OPPOSITE TO THAT OF CORTEX

Results of this study found that when ACh is released from the basal forebrain in a manner mimicking basal forebrain firing as demonstrated in in vivo studies, ACh inhibits afferent input to the BL. Studies that have used in vivo unit recordings in the basal forebrain in sleep and wake states found that during active waking basal forebrain neurons fire in bursts at theta frequency, and that the maximal basal forebrain firing correlates with "cortical arousal," or when the cortex is actively firing at higher (gamma) frequencies (Maloney et al., 1996; Lee et al., 2005). Taken together, this would suggest that when the basal forebrain is firing, the consequences on the cortex and BL are opposite;. Previous data from the cortex indicates the intrinsic recurrent activity in the cortex is suppressed for up to 5s, likely contributing to the suppression of slower oscillatory activity seen after ACh application (Dasgupta et al., 2018). In the cortex, when ACh is applied (either endogenously with optogenetic release or in vitro with slice electrophysiology), there is an overall decrease in low-frequency oscillations (Dasgupta et al., 2018). This inhibition of slow oscillatory activity in the cortex is considered necessary for the behavioral state shift from quiet wakefulness to cognitive (and even motor) arousal and allows for sensory input from other brain regions (ie thalamic) to be transmitted to, received by, and dictate activity in the cortex (Favero et al., 2012; Wester & Contreras et al., 2013; Castro-Alamancos & Gulati 2014; Dasgupta et al., 2018). Our data suggests that afferent input to the BL would be temporarily suppressed (for only 500ms-1s) but not intrinsic recurrent activity.

The results of this study suggest that when basal forebrain neurons, specifically substantia innominata and the horizontal limb of diagonal band, are stimulated at theta frequency, the released ACh will inhibit afferent input to the BL in a muscarinic receptor-mediated manner. An important consideration, however, with this conclusion is that there is heterogeneity in basal forebrain neurons, and our stimulation paradigm may be limiting which basal forebrain neurons we stimulate. For example, Laszlovsky et al., (2019) recently found basal forebrain neurons to have distinct firing properties, with some neurons able to fire in faster bursts than others (Laszlovsky et al., 2019). Additionally, the firing of these distinct populations of basal forebrain neurons were correlated with different behaviors during an auditory task involving correct and incorrection responses whereby one group was synchronized to cortical regions to predict the correct timing or responses and the other group was coupled to cortical regions to predict accuracy of responses (Laszlovsky et al., 2019). Thus, one needs to consider the possibility that when using optogenetics to activate basal forebrain neurons, the firing properties of all basal forebrain are not homogenous, and treating them as such may be biasing the selective activation of only those neurons with properties enabling them to follow the selected light stimulation. Future studies that could genetically identify different populations of basal forebrain neurons and then selectively express channelrhodopsins into the different populations would potentially allow for the ability to parse apart differential regulation of the BL by different populations of basal forebrain cholinergic neurons.

3.4.1 RELEASED ACH AFFECTS BASELINE POTENTIAL OF AMYGDALAR NEURONS

Previous studies have found the locally applied cholinergic agonist carbachol to induce biphasic changes in the membrane potential of neurons. Washburn & Moises (1992) found carbachol induced an early hyperpolarization then a later depolarization in amygdalar neurons. Data in our study builds upon this by examining endogenous, released ACh's effects on BL resting membrane potential. Interestingly, in both field and whole cell electrophysiology, we observed a biphasic response as well, but in our experimental protocol we found an initial depolarization response followed by a slower hyperpolarization. This data is interesting from multiple angles. First, the observation that released ACh causes different responses in different cells (for example, biphasic responses in one cell and monophasic in another as in Figure 3.2A) suggests heterogeneity in BL neurons, whereby there may be populations of neurons that respond one way to ACh and not another. A heterogeneous response of amygdala neurons' resting potential was also found by Sugita et al. (1990) when they applied muscarine and saw 20% of neurons displayed a hyperpolarizing potential, 42% showed a depolarizing potential, and about a third of neurons recorded from showed no change (Sugita et al., 1990). Future studies separating BL neurons based on responses to ACh, and then determining if those cholinergic-defined populations are selectively recruited during certain behaviors or emotional states could potentially identify behaviorally relevant populations of neurons. Indeed, BL neurons can be divided into different populations based on a multitude of factors, including genetic markers and projections from afferent regions onto distinct populations of BL neurons.; (Jasnow et al., 2013; McGarry & Carter 2017; Kim et al.,

2018). Some of these distinct populations demonstrate functional and behavioral relevance; for example, Jasnow et al. (2013) found activating a specific subset of BL neurons that express the genetic marker *Thy1* during extinction training facilitated consolidation of the extinction memory (Jasnow et al., 2013). It would be interesting to know if neurons that also express *Thy1*are among those we found in our study to display a depolarization to released ACh, as ACh could be released during extinction training and thus depolarization this subset of neurons and play a role in facilitating extinction learning.

Our observations with field recordings that light released ACh induces biphasic responses that seemed blocked with atropine and mecamylamine suggest different temporal involvement of nicotinic and muscarinic receptors. It is important to discuss that previous work evoking external capsule glutamatergic fEPSP and releasing ACh with light found nicotinic effects on transmission (Jiang et al., 2016). These studies used different protocols to release ACh and electrically stimulate glutamate release. It is possible that in our study, we are not able to capture a nicotinic component due to the protocol employed. For example, as illustrated in Figure 3.2A, the downward potential (putatively nicotinic, as nicotinic antagonist mecamylamine eliminated it), diminished with each theta-burst of light applied, potentially due to the desensitization of nicotinic receptors with each successive burst of ACh. This would agree with extensive literature showing nicotinic receptors are incredibly quick to desensitize (Bohler et al., 1992; Quick and Lester 2002; Geniatullin et al., 2005). Thus, by the time we evoke glutamatergic release from external capsule stimulation, the nicotinic receptors have been rendered desensitized and unable to exert a modulatory effect on glutamatergic transmission. On the other hand, this decrease in amplitude could also be neurotransmitter depletion. Thus, future studies aiming at

examining this nicotinic component would be needed to capture the full modulatory capacity of released ACh on glutamatergic fEPSPs. An alternate explanation to the putatively nicotinic potential could be that the potential is due to nAChRs on interneurons depolarizing interneurons, which then inhibit pyramidal neurons to cause the observed potential.

3.4.2 ENDOGENOUS ACH INHIBITS CORTICAL TRANSMISSION TO THE BL: FUNCTIONAL IMPLICATIONS

Results from our experiments provide novel insight into how ACh, when released in patterns mimicking those in-vivo, inhibits cortical glutamatergic transmission to the BL. These results would suggest that during periods of enhanced cholinergic tone in the BL, such as when an animal is exercising, engaging in an attentionally demanding task, or during an unexpected stimulus, glutamatergic input to the BL would be temporarily inhibited. Our experiments examining the time course of this inhibition would suggest that for a brief period of time (roughly 500ms) after ACh release, afferent cortical inputs to the BL would be suppressed to shift the influence of synaptic transmission away from external, afferent input and towards intrinsic recurrent processing within the BL. Why this would be behaviorally advantageous is still unknown, but a plausible reason can be found in the BL's function in updating preexisting models of its environment. There is a growing body of research that the BL's role is to update preexisting models of learned associations based on constantly updating information and error prediction signals (Wassum and Izquierdo 2015; Zhang and Li 2018; Campese et al., 2019). If a mouse, for example, is out foraging for food, it is in a situation where it must make choices that engage motivational conflicts to

survive. For example, it will need to decide if it should make a movement for food or remain frozen to avoid a bird of prey. Should that mouse hear or a see a stimulus that was previously associated with a threat, it will need to remember that stimulus and behave accordingly. However, if a novel stimulus appears in the environment, and that animal's BL is now flooded with ACh, the animal will need to temporarily shut off incoming afferent input while the learned model about the environment is processed and updated.

Intriguingly, this modulation of afferent input in the amygdala is different from what is known about cholinergic regulation in the rest of the brain. In the hippocampus and cortex, ACh spares afferent input but inhibits internal connections within a brain region (Hasselmo et al., 2006; Dasgupta et al., 2018). In the cortex, this selective filtering is thought to allow for selective attention to encoding of new memories without interruption by recall of previously encoded information. Given that the BL is important for fear memory and fear extinction learning, suppression of afferent input would allow for internal processing to occur in the BL without impedance from cortical regions. If the BL is important for updated preexisting associations, such a suppression of afferent input by ACh would allow this processing of new information to occur in the BL, and after 500ms, this suppression would be alleviated to allow for new information to be received by the BL and then encoded.

Taking cholinergic modulation of cortical activity into account, a potential influence of basal forebrain firing could be to suppress low-frequency cortical oscillations that enhances the receptive capacity of the *cortex* to incoming sensory stimuli while at the same time causing cholinergic suppression of incoming sensory stimuli to the *amygdala* (Favero et al., 2012; Wester & Contreras et al., 2013; Castro-Alamancos & Gulati 2014;

Dasgupta et al., 2018). This would shift the prefrontal-cortical network into a mode where the amygdala is quieted to sensory input but the cortex is "listening" to such sensory input. Temporal data that shows ACh suppresses recurrent activity for up to 5 seconds (Dasgupta et al., 2018) and our data showing ACh suppresses afferent input to the BL for up to 1s would suggests a brief, 1s period whereby the BL is silenced to sensory input but the PFC is receptive to it. Once that silenced inhibitory period in the BL is relieved, both the PFC and sensory (possibly thalamic) input could entrain the BL and synchronous oscillatory activity between the PFC and BL could occur. However, before concluding how the BL and prelimbic cortex could be coupled after basal forebrain activity, future studies will be necessary to examine specific prefrontal cortical input to the BL to determine how ACh regulates this input.

Potential limitations of electrically stimulating the external capsule must also be taken into account when discussing behavioral consequences of muscarinic inhibition of external capsule-evoked input. The external capsule is known to contain projections from a range of cortical regions. Thus, electrically stimulating this white matter bundle will ambiguously and promiscuously evoke fEPSPs from multiple regions that project to the BL. Thus, it is possible that the ability to evoke fEPSPs from specific projection regions may result in different modulatory effects by ACh. Our observations that released ACh conferred about 20% inhibition of afferent input may be misleading if we are stimulating projections that contain a mix of inputs differentially regulated. For example, it could be that a subset of projections to the BL that course through the external capsule are completely inhibited by ACh, while others are only partially inhibited or not inhibited at all. Anatomical data suggests that not all axon terminals forming glutamatergic synapses

to the BL contain muscarinic receptors; thus only distinct inputs that contain muscarinic receptors would be modulated while other input would be spared (Muller et al., 2013; Fajardo-Serrano et al., 2017; McDonald et al., 2019). Thus, if specific brain regions could be isolated and stimulated, a more clear understanding of cholinergic action on various inputs to the BL could be modeled.

CHAPTER 4

DIFFERENTIAL INHIBITION OF PL AND THAL INPUT TO BL BY MUSCARINIC ACETYLCHOLINE RECEPTORS

4.1 INTRODUCTION

An extensive body of literature exists demonstrating the amygdala's importance for emotional processing. Early observations of amygdala function found that lesions and calcifications to the amygdala produce aberrant emotional responses (Siebert et al., 2003). More specifically within the amygdala, the basolateral amygdala (BLA) is critical for processing emotional learning and memory. There is an abundance of studies using Pavlovian conditioning paradigms that show the BLA is involved in fear memory acquisition and expression of learned fear. Lesioning the BLA, chemically inhibiting the BLA, or inactivating NMDA receptors in the BLA before and after fear acquisition training impairs both cue- and context- associated fear memories (Campeau & Davis 1995; Gale et al., 2004; Fanselow & Kim 1994; Helmstetter & Bellgowan 1994; Maren et al., 1996; Anglada-Figeuero & Quirk 2005). Behavior studies combined with electrophysiology find amygdalar neurons change responsiveness and firing rates after emotionally charged events and noxious stimuli (Pelletier et al., 2005; Wolff et al., 2014; Sengupta et al., 2018). Similarly, manipulations that inhibit BLA neurons during phases of Pavlovian conditioning affect fear behaviors; for example, optogenetic inhibition of BLA neurons during extinction training improves extinction learning (Sengupta et al., 2018). However, how

incoming neurotransmission to the BLA is regulated to either increase or decrease BLA activity remains largely unknown.

The basolateral amygdala is optimally positioned to integrate information from the environment and produce a resulting behavioral response; the BLA receives information about an organism's environment, including contextual and sensory information, from various afferent regions and sends projections to downstream regions that induce physiological changes and behavioral responses. Several regions projecting to the BLA have been found to be implicated in fear behaviors, and thus are considered part of the neural fear circuit. Three of these regions, the prelimbic cortex (PL), the midline thalamic nuclei (THAL), and the ventral subiculum (vSUB), convey information to the BLA via excitatory glutamatergic neurotransmission. Afferents from the PL synapse onto both inhibitory interneurons in the BLA as well as excitatory pyramidal neurons in the BLA (Rosenkranz & Grace 2002; Huebner et al., 2014; Brinley-Reed et al., 1995; McGarry & Carter 2017). Behaviorally, the PL is generally considered important for the expression of fear, as lesioning the PL impairs the expression of acquired fear (Corcoran & Quirk 2007; Sierra-Mercado et al., 2011). The THAL, on the other hand, synapses onto mainly excitatory pyramidal neurons (Smith et al., 2000). including the paraventricular nucleus (PVT), reuniens, rhomboid and xyphoid, is important for processing internal states and regulating behaviors during motivational conflict based on those internal states (Salay et el., 2018; Choi et al., 2019). Recent research suggests that the THAL, when activated, shifts the internal state of an animal to favor saliency-promoting behaviors; in other words, when the THAL is activated, animals are more likely to approach threatening stimuli and less likely to hide (Salay et al., 2018). When the midline thalamic nuclei are chemogenetically

activated, rodents will increase tail rattling in response to a threat, and when inactivated, freeze more. Specific connections involved in these behaviors consist of the THAL xiphoid nucleus (Xi) projections to the BL, that, when activated, cause an animal to reduce is saliency and freeze (Salay et al., 2018). This study, together with additional recent data showing THAL activation to regulate behavior during motivational conflicts, suggests that the THAL is tightly involved in regulating internal states to shift an animal towards a state of increased arousal (Choi et al., 2019; Salay et al., 2018). The vSUB densely projects to the posterior BL and synapses largely onto pyramidal neurons in the BL to exert an overall excitatory effect on the PL when active (Canteras & Swanson 1992; Cenquizca & Swanson 2007; Mueller et al., 2012). The vSUB projections to the BL are also important for fear conditioning, as lesions of these projections impair acquisition and extinction during Pavlovian paradigms (Maren 1999).

Interestingly, all of these regions send glutamatergic projections to the BLA, specifically the basolateral nucleus (BL) while largely avoiding the lateral nucleus (LA). Whereas the LA is widely considered to be the amygdalar region where the primary associations form between a conditioned stimulus like a tone and an unconditioned stimulus like a shock, the role of the BL is still under speculation. Given the role of the PL in expression of fear (ie freezing in rodents) and the role of the THAL in error prediction, a possible role for their convergence in the BL is to update the preexisting association once that information arrives in the BL from the LA. However, in order to begin speculating on a behavioral function, it is necessary to first understand how this glutamatergic information from the PL, vSUB and THAL is transmitted to the BL.

The release of glutamate and the responsiveness of neurons to glutamatergic transmission is regulated by neuromodulators in the brain. In the cortex, the neuromodulator acetylcholine (ACh) regulates both presynaptic transmitter release and postsynaptic neuronal excitability. Presynaptic inhibition by cholinergic receptors occurs at intrinsic synapses between neurons in cortical layers, but does not occur at afferent synapses between incoming afferent projections, indicating that cholinergic tone in the cortex serves to enhance incoming information while quieting recurring processing within a region (Hasselmo 2006). This enhancement of externally arriving afferent transmission and dampening of internal recurrent processing is also seen in area CA1 of the hippocampus, where cholinergic receptors inhibit intra-hippocampal glutamatergic input from the Schaffer collaterals while not affecting incoming transmission from external regions conveyed via the temporoammonic pathways (Hasselmo & Schnell, 1994; McQuiston 2019). By allowing external information to arrive while suppressing transmission within a region, it is thought that cholinergic receptors increase the influence of incoming information that conveys new information to be acquired while suppressing the processing and recall of previously acquired information. However, how ACh modulates afferent versus internal information in the BL remains to be fully understood.

Radioligand binding, quantitative autoradiography, electron microscopy, immunofluorescence and immunohistochemistry studies reveal the presence of M1-M5 mAChR subtypes of mAChRs in the BL in both rodent, nonhuman primates and human primates (Cortes et al. 1987, Spencer et al., 1986; Mash & Potter 1986; Mash et al., 1988; Buckley et al., 1988; Smith et al., 1990; McDonald & Mascagni, 2010; McDonald & Mascagni 2011; Muller et al., 2013; Muller et al., 2016; Fajardo-Serrano et al., 2017).

Anatomical studies demonstrate M1 and M2 receptors to be located on a subset of axon terminals in the BL, raising the possibility that they differentially modulate distinct inputs (Muller et al., 2013; Muller et al., 2016; Fejardo-Serrano et al., 2017). Postynaptically, M1 and M2 receptors have been labeled on BL neurons and interneurons, with M1 most prevalent on pyramidal neuronal perikarya and dendrites (both shafts and spines) whereas M2 has been found on both pyramidal neurons and interneurons (McDonald & Mascagni 2010; Muller et al., 2013; Muller et al., 2016). Roughly half of BL neurons that receive input from putatively labeled cortical projections and about half of BL neurons that receive input from putatively labeled thalamic projections express M1 receptors on dendrites (McDonald et al., 2019). Lesser known is the presence of M3 and M4 receptors in the BL, due in no small part to the lack of specific antibodies and pharmacological tools. However, an earlier autoradiography study has shown putative M3 receptors in the BL, and therefore this subtype should not be ruled out of mediating muscarinic inhibition to BL (Zubieta & Frey, 1993; However, how these muscarinic receptors regulate glutamatergic transmission in the BL remains unknown.

To answer this question, we used a combination of optogenetics and electrophysiology to determine how muscarinic receptor activation regulates optogenetically released glutamatergic transmission from the PL and THAL to the BL. It was hypothesized that muscarinic receptors would regulate afferent input to the BL in a manner consistent with the cortex, such that THAL inputs, conveying external stimuli about an unexpected stimulus, would not be inhibited while PL and vSUB inputs conveying cortical information, would be inhibited. Given the anatomical data demonstrating presynaptic mAChRs M1 and M2 on axon terminals and M1 on putative cortical and

thalamic projections (Muller et al., 2016; McDonald et al., 2019) it was hypothesized that presynaptic mAChR M1 and M3 inhibit glutamatergic transmission from these afferent regions.

4.2 MATERIALS AND METHODS

4.2.1 VIRAL DELIVERY OF CHR2S TO PL, THAL, AND VSUB REGIONS

As described in Chapter 2.7, 1.2-3 month old transgenic mice ChAT-Cre (B6;129S6-Chat$^{tm2(cre)Lowl/J}$) mice) were anesthetized and injected with 0.15μL of rAAV5-CAMKII-hChR2(H134R)-eYFP-WPRE (UNC Viral Vector Core) in the prelimbic cortex (from Bregma: 1.9mm Anterior/Posterior; -0.3mm Media/Lateral; -2.0 mm Dorsal/Ventral), with 0.2μL of rAAV5-CAMKII-hChR2(H134R)-eYFP-WPRE (UNC Viral Vector Core) delivered to the ventral subiculum (from Bregma: -2.5mm Anterior/Posterior; -3.2mm Media/Lateral; -5.3 mm Dorsal/Ventral) or with 0.2μL of rAAV5-CAMKII-hChR2(H134R)-eYFP-WPRE (UNC Viral Vector Core) in the midline thalamic nuclei. As seen in Figure 4.1, injection sites were confirmed in coronal slices using a fluorescent microscope and recorded on a schematic based off of the mouse brain atlas (Paxinos and Franklin, 2001).

4.2.2 WHOLE CELL RECORDINGS

Coronal brain slices 300μM thick were prepared and incubated as described in Chapter 2. Voltage-clamp recordings from amygdalar pyramidal neurons were conducted at a holding potential of -70mV. The series resistance was between 10-25MΩ.

To stimulate the release of glutamate from the PL and record the evoked glutamatergic EPSCs in the BL, single or paired (50ms ISI) flashes (1-3ms) of blue LED light (ThorLabs Inc, Newton, New Jersey) was emitted and the EPSC was measured. The glutamatergic response was isolated with application of $GABA_A$ and $GABA_B$ antagonists picrotoxin and CGP55845, respectively and NMDA receptors were antagonized with D-APV. At the conclusion of each experiment, remaining glutamatergic currents were confirmed with bath application of CNQX.

Glutamatergic currents were recorded using a Multiclamp 700B amplifier and pClamp 10 software (Molecular Devices, Sunnyvale, CA) and responses were filtered at 1kHz. For whole cell experiments examining muscarinic effects at PL input to BL, muscarine (10μM) was bath applied.

4.2.3 FIELD ELECTROPHYSIOLOGY RECORDINGS

Horizontal and coronal brain slices (500μM thick) containing the amygdala were prepared and incubated as described in Chapter 2. To stimulate the release of glutamate from the PL, THAL and vSUB, blue LED light (ThorLabs Inc, Newton, New Jersey) was flashed (1-3ms) either singly or in pairs (50ms ISI) to release glutamate. To record the evoked glutamatergic fEPSP in the BL, glutamatergic fEPSPs were isolated by applying $GABA_A$ and $GABA_B$ antagonists picrotoxin and CGP55845, respectively and NMDA receptors were antagonized with D-APV. At the conclusion of each experiment, remaining glutamatergic currents were confirmed with bath application of CNQX.

4.3 RESULTS

4.3.1 MUSCARINIC RECEPTOR ACTIVATION DIFFERENTIALLY REGULATES AFFERENT INPUT TO BL IN PATHWAY-SPECIFIC MANNER

Coronal brain slices containing the amygdala from animals injected into the PL, THAL or vSUB display expression of ChR2 in the BL of labeled terminals. These terminals were stimulated with blue light to evoke and record glutamatergic fEPSPs in the BL (control). After acquiring a stable baseline in control condition, muscarine (10μM) in the recording ACSF was bath applied. BL field responses in the presence of muscarine to optogenetic stimulation of PL, THAL or vSUB afferents were recorded. Evoked glutamatergic responses (fEPSP mean peak amplitude) during activation of mAChR by muscarine were compared with responses recorded during control ACSF. Figure 4.2A (top inset waveforms) show optogenetically evoked amygdalar responses from PL, THAL and vSUB stimulation in control ACSF (black waveforms). Muscarinic activation significantly inhibited the glutamatergic response is PL, THAL and vSUB pathways, but to different extents. In the PL and vSUB pathways, muscarine nearly completely inhibited the amygdalar glutamatergic response (green and pink waveforms, respectively), whereas in the THAL pathway muscarinic activation inhibited by a significantly lesser extent (blue waveform). These experiments are summarized in Figure 4.2A. Overall, when mAChRs were activated, glutamatergic transmission at PL input was reduced to roughly one-fifth of that in control (17.89 ± 1.85%), vSUB to one-fourth (25.1 ± 4.28%), and THAL to half of control (45.7 ± 4.09%). Application of muscarinic antagonist atropine (5μM) reversed inhibition in all three pathways, indicating inhibition by muscarine is mediated by mAChRs. Interestingly, the lesser inhibition of thalamic inputs and stronger inhibition of

cortical inputs to a region has been shown in the auditory, visual and barrel cortices, where ACh and cholinergic agonists more strongly inhibit glutamatergic transmission from intracortical pathways than thalamocortical (Metherate & Ashe 1993; Kimura et al., 1999; Hseih et al., 2000; Oldford & Castro-Alamancos 2003). To ensure that the phenomenon we were seeing with field electrophysiology was generalizable to different electrophysiology techniques, we used whole-cell patch clamping to replicate experiments at PL input to BL. Similar to field electrophysiology experiments, PL transmission was nearly completely inhibited (11.26 ± 2.3% of control) in the presence of muscarine (10μM).

4.3.2 MUSCARINE INHIBITS PL AND THAL INPUT TO BL EQUALLY IN MALES AND FEMALES.

An extensive body of literature exists showing marked differences in the incidence of emotional disorders that involve the amygdala, with females commonly reporting higher prevalence ratios of generalized anxiety disorders, depression and emotional comorbidities (Kessler et al., 1993; Vesga-Lopez et al., 2008). Because of this sex difference, we wanted to determine if a difference in muscarinic inhibition of PL and THAL input to the BL exists in males and females. FEPSP suppression by muscarine (10μM) was compared between males and females. There was no difference in variance between males and females, and muscarine inhibited glutamatergic transmission in both sexes equally at both pathways (Figure 4.3A, B). However, there did appear to be a trending difference in the amount of inhibition between males and females at the THAL pathway, whereby mAChR activation in males produces an observably less amount of inhibition of glutamatergic transmission than females (Figure 4.3C). However, further experiments will be necessary

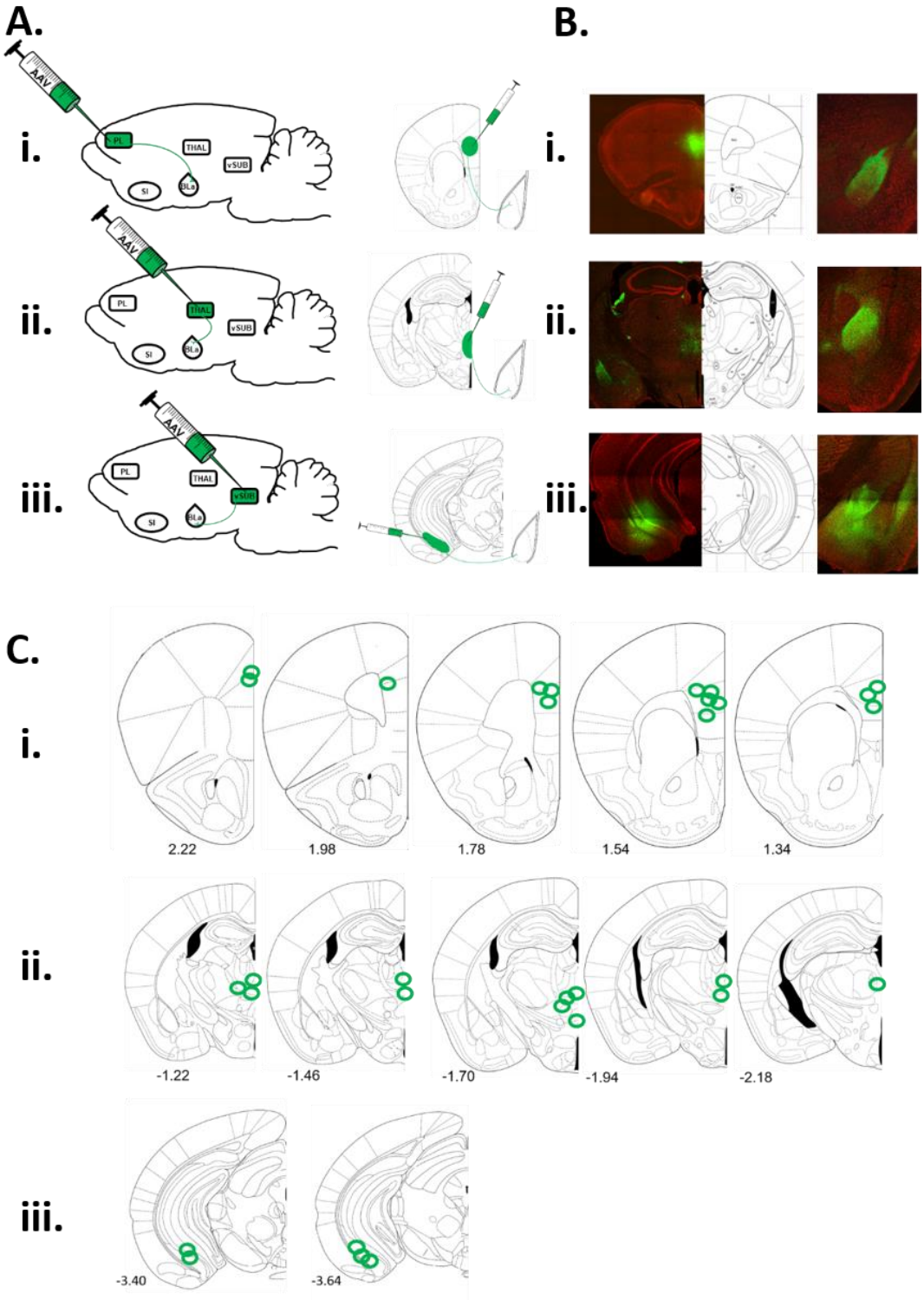

A.
B.
i.
ii.
iii.
AAV
PL
THAL
vSUB
SI
BLa
C.
2.22
1.98
1.78
1.54
1.34
-1.22
-1.46
-1.70
-1.94
-2.18
-3.40
-3.64

Figure 4.1. Viral injection sites for expressing ChR2s in afferent inputs to the BL from the PL, THAL and vSUB regions.

A. i-iii. Whole brain (left) and coronal schematics (right) illustrating injections into PL, THAL and vSUB with subsequent projections to the BL shown on the far right.
B. i-iii. Confocal images of PL, THAL and vSUB injection site with corresponding ChR2+ terminals in the BL (green).
C. i-iii. Injection sites centers (circles) based on the Franklin & Paxinos (2008) mouse atlas for PL (i), THAL (ii) and vSUB (iii) injections.
(Image adapted from *The Mouse Brain in Stereotaxic Coordinates: Compact Third Edition*, by K.B.J. Franklin & G. Paxinos, 2008, New York, NY: Academic Press. Copyright (2008) by Elsevier Inc.)

to determine if this trend is significant in a larger population. Thus, for all following experiments in this study, we combined males and females.

4.3.3 MUSCARINIC RECEPTOR ACTIVATION AT PL VERSUS THAL PATHWAY DISPLAYS DIFFERENT AMOUNTS OF SENSITIVITY AT THE TWO PATHWAYS.

We next wanted to determine the effects of increasing concentrations of muscarine on evoked glutamatergic responses at PL and THAL inputs to BL. Glutamatergic transmission from PL and THAL was again evoked with light, and fEPSPs in control and in muscarine were compared. 0.03µM, 0.3µM, 1µM, 10µM and 30µM concentrations of muscarine were bath applied and peak amplitude of the response measured. Figure 4.4 shows the magnitude of muscarinic inhibition at PL and THAL pathways for the varying concentrations of muscarine. The lowest concentration of muscarine, 0.03µM, had no significant difference on glutamatergic transmission at either pathway. The highest concentration used, 300µM, significantly inhibited both PL and THAL inputs

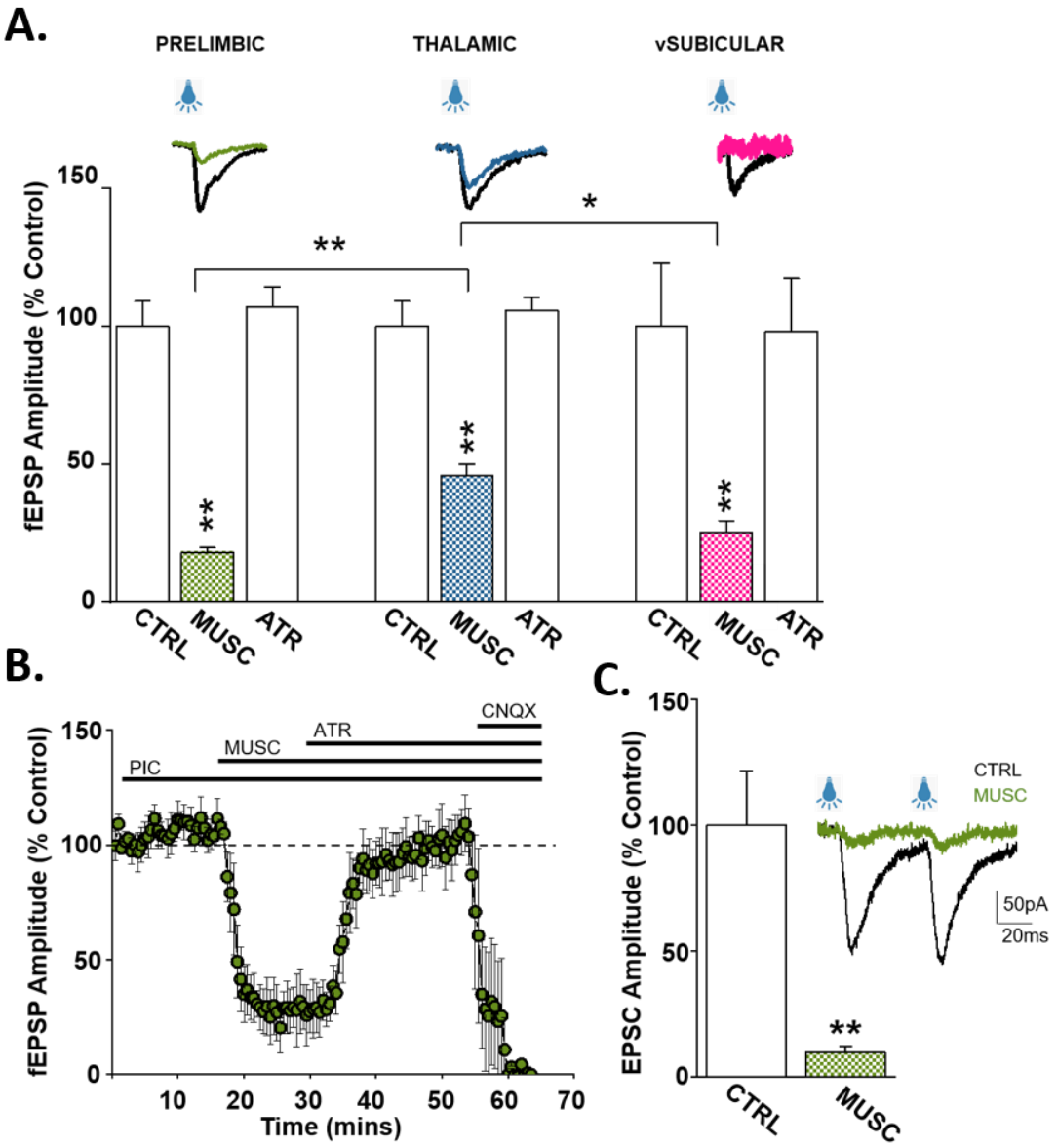


Figure 4.2. Muscarinic receptor activation differentially suppresses PL, THAL and vSUB glutamatergic transmission to BL.

A. Muscarine (10µM) strongly inhibits glutamatergic input to BL evoked by optogenetic stimulation of PL (17.89 ± 1.85, **p<0.01, n=35) and vSUB (25.1 ± 4.28, **p<0.01, n=5) whereas it inhibits glutamatergic input from THAL to a lesser extent (45.7 ± 4.09, **p<0.01, n=27). Inset: Representative traces illustrating muscarine nearly completely inhibiting glutamatergic fEPSP (green) compared to control (black) in the PL pathway, muscarine nearly completely inhibiting glutamatergic fEPSP (pink) compared to control

(black) in the vSUB pathway and muscarine partially inhibiting glutamatergic input (blue) compared to control (black) in THAL pathway.
B. Representative experiments in PL showing muscarinic inhibition of glutamatergic transmission and subsequent reversal of muscarinic inhibition by application of muscarinic antagonist atropine (5μM).
C. Muscarine (10μM) similarly inhibits PL input to BL using whole cell electrophysiological recordings (11.26 ± 2.3, *p<0.05, n=3).

compared to control, but always inhibited PL input more strongly than THAL input. This stronger inhibition of PL input was seen for every concentration of muscarine used above 0.03 μM. While future experiments implementing a concentration-response curve will allow of detailed pharmacological investigation of the pharmacodynamics of muscarinic receptors at both pathways, we can conclude that there does appear to be a difference in sensitivity to muscarine between mAChRs at the PL and THAL input to BL. This difference in sensitivity to muscarine could be reflective of different expression of muscarinic receptors at each pathway, as different receptors have been shown to posses different binding affinities for muscarinic agents (Kellar et al., 1985; Jakubik et al., 2011).

4.3.4 MUSCARINIC RECEPTOR MEDIATED INHIBITION OF GLUTAMATERGIC TRANSMISSION TO BL EXHIBITS PRESYNAPTIC LOCUS OF ACTION AT PL AND THAL SYNAPSES TO BL.

To determine effects of muscarinic receptor activation on presynaptic transmitter release, paired optogenetic stimuli at an interval of 50ms were given to either the PL or THAL input to BL (Figure 4.5A) in the presence of $GABA_A$, $GABA_B$, and NMDA receptor antagonists picrotoxin (10μM), CGP55845 (2μM) and APV (50μM) respectively. Paired pulse stimulation of PL and THAL demonstrated facilitation of fEPSPs in control

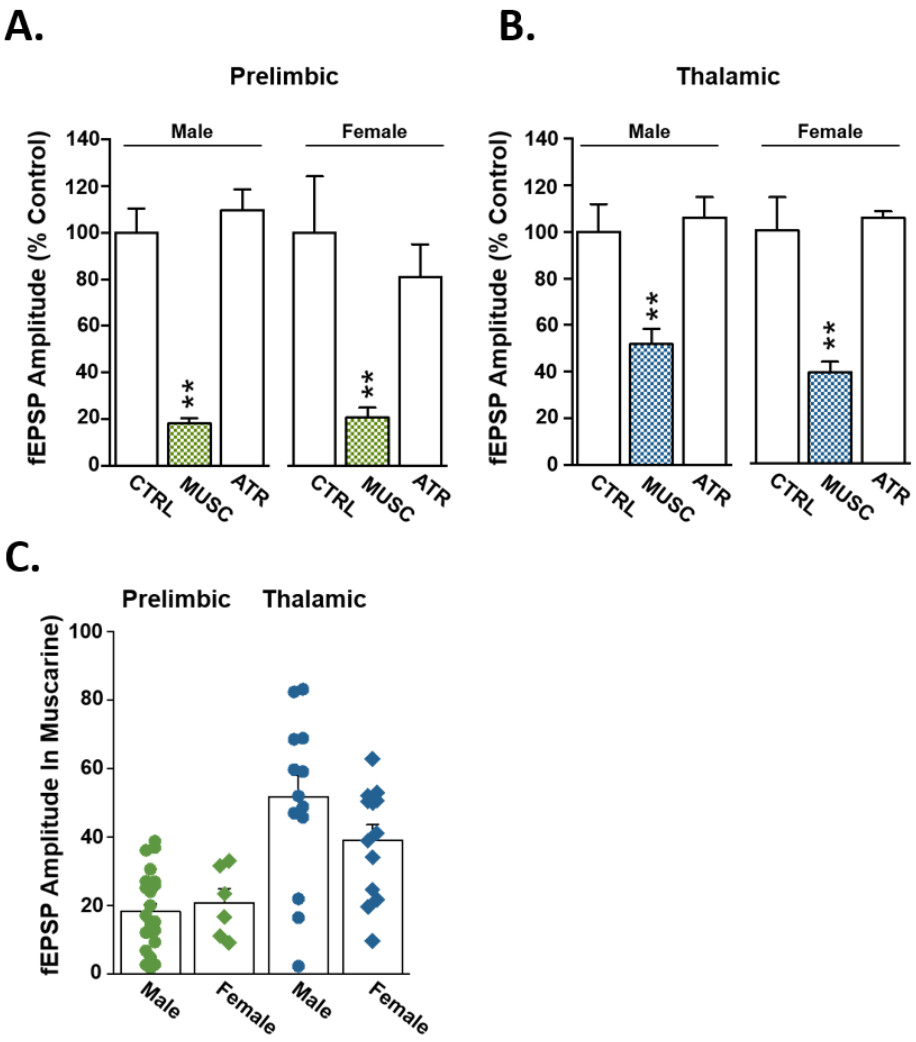


Figure 4.3. Muscarinic receptor activation inhibits glutamatergic input equally in males and females at PL and THAL pathways.

A. Muscarine (10µM) inhibits PL input in both males (18.21 ± 2.22, **p<0.01, n=26) and females (20.83 ± 4.17, **p<0.01, n=6). However, no significant difference in muscarinic inhibition was found between males and females at this pathway.

B. Muscarine (10µM) inhibits THAL input in both males (51.77 ± 6.44, **p<0.01, n=14) and females (39.16 ± 4.43, **p<0.01, n=13). However, no significant difference in muscarinic inhibition was found between males and females at this pathway.

C. Though not statistically significant, scatterplots of individual data reveal a trend in which muscarinic receptor activation suppresses thalamic input to a larger extent in females than males.

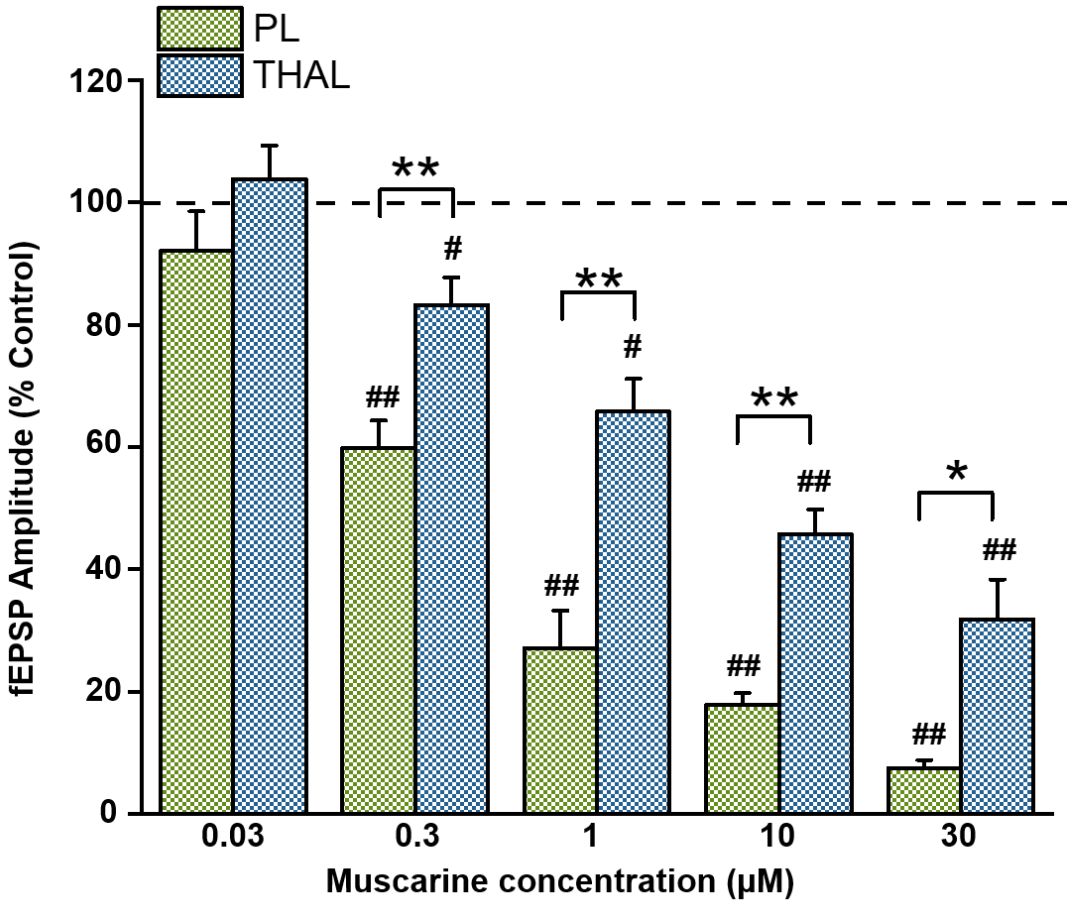


Figure 4.4. Concentration-dependent effects of muscarine at PL and THAL projections to BL.

Concentration – response summaries showing inhibition of fEPSP (as % of control) at increasing concentrations of muscarine. Inhibition for our given concentrations was maximum at the PL and THAL pathways at 30 µM muscarine, where inhibition was still significantly larger for PL input (7.44 ± 1.38% of control, n=5 and 31.80 ± 6.56% of control, n=8, at PL and THAL, respectively, *$p<0.05$). Differences in inhibition by muscarinic doses between the two pathways may reflect underlying differences in potencies of muscarine at the two pathways.

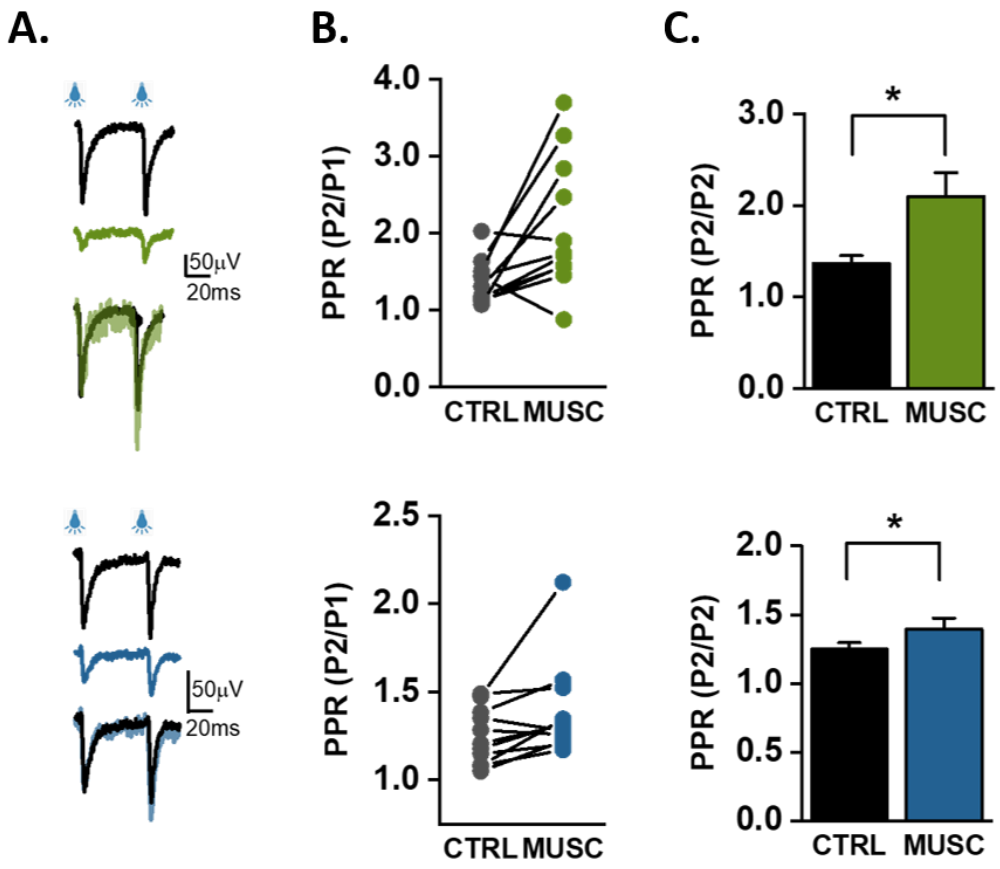


Figure 4.5. Muscarinic receptor activation exhibits presynaptic locus of action of inhibition of glutamatergic inhibition.

A. (Top) Representative traces illustrating muscarinic receptor activation (green) suppresses the PL-evoked fEPSP compared to control (black) but increases paired pulse facilitation, suggesting a presynaptic action. (Bottom) Similarly, muscarinic activation (blue) suppresses the THAL-evoked fEPSP compared to control (black) but increases paired pulse facilitation, suggesting a presynaptic action.

B. Paired pulse ratios (PPRs) of individual experiments shown for PL input in control (gray) and muscarine (green) as well as for THAL input in control (gray) and muscarine (blue).

C. Muscarinic activation (10µM) significantly increased the PPR at both the PL (green) (2.10 ± 0.26, *$p<0.05$, $n=11$) and THAL (blue) (1.40 ± 0.08, *$p<0.05$, $n=11$) pathways compared to controls.

conditions (Figure 4.5), and bath application of muscarine (10μM) significantly increased this paired-pulse ratio (Figure 4.5B,C). At PL synapses, muscarine increased paired pulse ratio from 1.36 ± 0.09 to 2.10 ± 0.26 (n=11, *p<0.05), indicative of muscarinic activation retaining transmitter in the presynaptic terminals at PL inputs to BL. Similarly, at THAL synapses, muscarine increase paired pulse ratio from 1.25 ± 0.05 to 1.40 ± 0.08 (n=11; *p<0.05), also indicating a muscarinic mechanism of inhibition of glutamate release whereby neurotransmitters are retained in the presynaptic terminal.

4.3.5 M3 MACHRS MEDIATE GLUTAMATERGIC TRANSMISSION FROM PL AND THAL PROJECTIONS TO BL

Given the anatomical data supporting the existence of different subtypes types of mAChR in the BL across species, and the presence of M1 receptors at putative cortical and thalamic inputs to the BL, we next wanted to know which specific muscarinic subtypes are responsible for the inhibition of PL-evoked and THAL-evoked fEPSPs (Cortes & Palacios 1986; Cortes et al. 1987, Spencer et al., 1986; Mash & Potter 1986; Mash et al., 1988; Buckley et al., 1988; Smith et al., 1990; McDonald & Mascagni, 2010; McDonald & Mascagni 2011; Muller et al., 2013; Muller et al., 2016; Fajardo-Serrano et al., 2017; McDonald et al., 2019). To answer this question, we first looked to see if M1 was responsible for the muscarinic-mediated inhibition given the extensive literature showing its presence in the BL. PL and THAL inputs were optogenetically evoked with blue light pulses and evoked fEPSPs in control ACSF consisting of GABA antagonists (10μM picrotoxin and 2μM CGP55845) and NMDA antagonist (50μM APV) were recorded.

Following the acquisition of stable responses in control, muscarine (10µM) was added to recording medium. As expected, muscarinic suppression of fEPSPs was seen at both inputs to BL (Figure 4.6A,B). After stable responses were recorded in muscarine, M1 antagonists telenzepine (1µM) or VU0255035 (5µM) were added to the recording ACSF. Unexpectedly, antagonizing M1 receptors at both pathways failed to significantly reverse muscarinic inhibition, suggesting muscarinic mediated inhibition of glutamatergic fEPSPs is not through M1 receptors (PL: n=9, $p>0.05$; THAL: n=7, $p>0.05$). However, we did see a trend in *slight* relief of muscarinic inhibition by M1 antagonists at both pathways, but this did not reach statistical significance. These results were surprising given the abundance of M1 receptors in the BL, and the expression of M1 receptors seen at both putative PL and putative THAL inputs to the BL (McDonald et al., 2019). Our observed reversal of a small portion of muscarinic inhibition (roughly 11% reversal at PL input and 5% at THAL input) would agree with the anatomical data, but the remainder of muscarinic inhibition remains unexplained. Thus, it remains to be determined what the function of the presynaptic M1 receptors are if not to completely inhibit glutamate release.

We next wanted to determine if M2 mAChRs mediate muscarinic suppression of PL and THAL-evoked fEPSPS. Similar experimental procedures as our M1 experiments were followed, in which stable responses in control ACSF were acquired followed by stable responses in muscarine (10µM). Following muscarinic inhibition, the M2 antagonist AFDX-116 (1µM) was added to the recording ACSF. While AFDX *slightly* reversed a trace amount of muscarinic inhibition, this reversal was not significant at either pathway (Figure 4.7A,B) (PL: n=6, $p>0.05$; THAL: n=6 $P>0.06$). This slight reversal would agree with anatomical data from Muller et al., 2016 & Fajardo-Serrano et al., 2017,

demonstrating the presence of presynaptic M2 receptors. Given that M2 receptors couple to Gi proteins and are largely inhibitory, the slight effects of M2 on fEPSP inhibition would corroborate these anatomical findings, but other muscarinic subtypes must explain the majority of muscarinic receptor-mediated inhibition not explained by either M1 or M2 receptors.

The next set of experiments were aimed at determining if mAChR M3 was responsible for muscarinic inhibition of PL and THAL input to BL. PL and THAL input was again optogenetically evoked and stable baseline fEPSPs were recorded, after which muscarine (10μM) was added to the recording ACSF. Following inhibition of fEPSPs by muscarine, selective M3 antagonist 4-DAMP (1μM) was added to the ACSF. 4-DAMP completely reversed muscarinic inhibition at both pathways to the BL (PL: 104.32 ± 14.67, **p<0.01, n=10, THAL: 105.43 ± 7.26, **p<0.01, n=9) (Figure 4.8A,B). This data agrees with previous studies in other brain regions that have found M3 receptors to inhibit glutamate release by using the M3 antagonist 4DAMP (Hsu et al., 1995; Grillner et al., 1999).

4.3.6 M4 MACHRS PRESENT AT PL, BUT NOT THAL, INPUT TO BL.

While 4-DAMP is commonly used and referred to in the literature as an M3 antagonist, it does show nonselectivity for the other muscarinic subtypes (Doods et al., 1987; Michel et al., 1989). Given the more selective M1 and M2 antagonists did not completely reverse muscarinic inhibition at PL and THAL pathways, we feel confident M1 and M2 receptors are not responsible for strong muscarinic inhibition at these pathways.

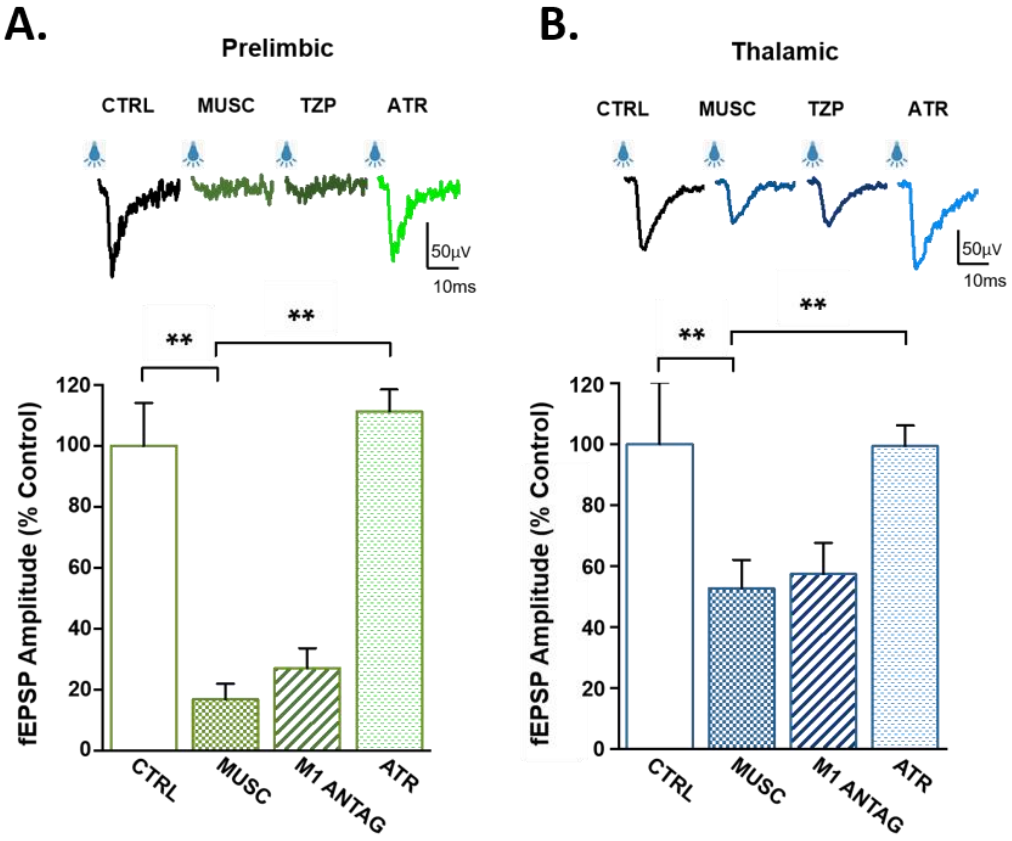


Figure 4.6. Muscarinic receptor activation does not inhibit glutamatergic input at PL or THAL pathways through presynaptic M1 receptors.

A. (Top) Representative traces illustrating glutamatergic inhibition at PL input is inhibited by muscarine (MUSC) compared to control (CTRL), and this inhibition is not reversed when M1 receptors are antagonized by M1 antagonist telenzepine (TZP) but is completely reversed by atropine (ATR). (Bottom) M1 antagonists TZP (1µM) and VU0255035 (5µM) do not significantly reverse muscarinic inhibition (n=9) at PL pathways but this inhibition was completely reversed by atropine (5µM) (111.21 ± 16.07, **p<0.01, n=9) suggesting a muscarinic receptor other than M1 responsible for inhibition at this pathway.

B. (Top**)**Representative traces illustrating glutamatergic inhibition at THAL input is inhibited by muscarine (MUSC) compared to control (CTRL), and this inhibition is not reversed when M1 receptors are antagonized by M1 antagonist telenzepine (TZP) but is completely reversed by atropine (ATR**).** (Bottom) Similar to the PL pathway, at the THAL pathway, M1 antagonist TZP (1µM) did not significantly reverse muscarinic inhibition (n=7), although this inhibition was completely reversed by atropine (5µM) (99.28 ± 6.80, **p<0.01, n=7) suggesting a muscarinic receptor other than M1 responsible for inhibition at this pathway.

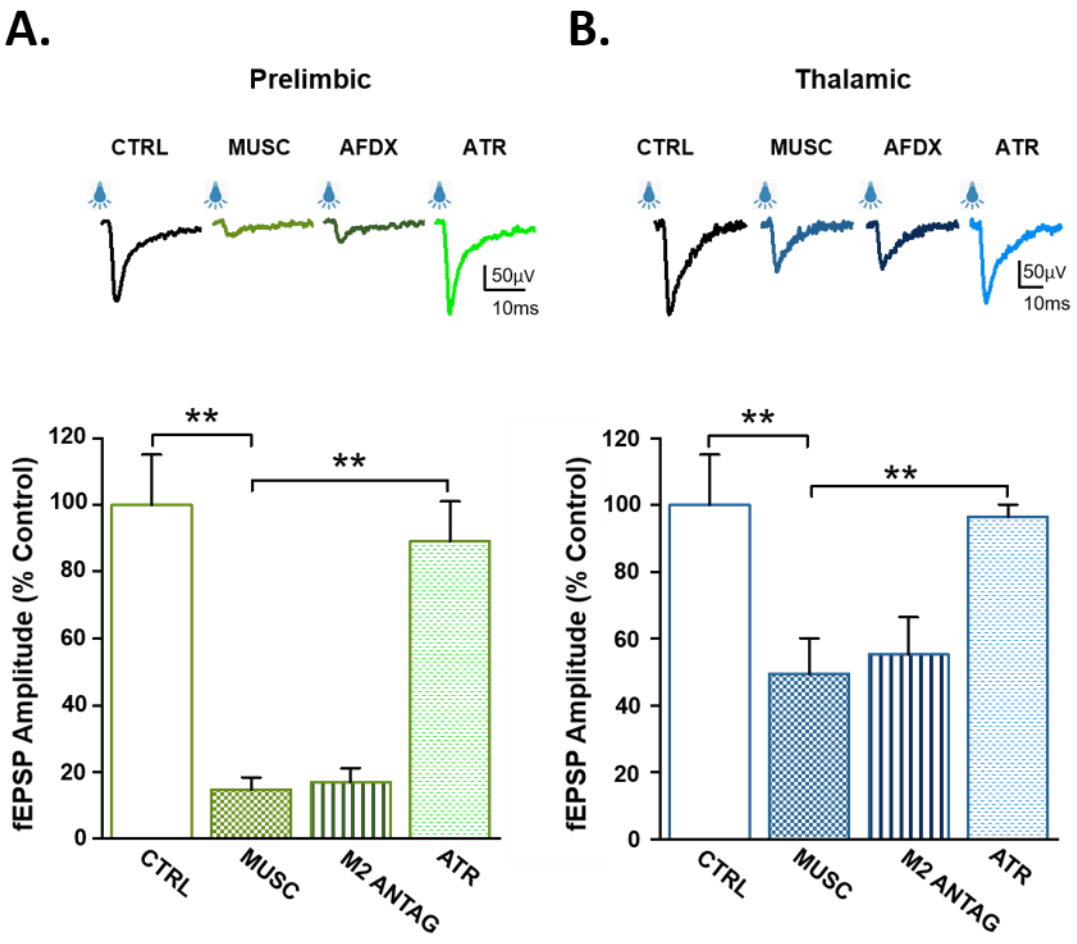


Figure 4.7. Muscarinic receptor activation does not inhibit glutamatergic input at PL or THAL pathways through M2 receptors.

A. (Top) Representative traces illustrating muscarine (MUSC) inhibits fEPSPs at PL input and this inhibition is not reversed by M2 antagonist AFDX-116 (AFDX). (Bottom) M2 antagonist AFDX (1µM) does not significantly reverse muscarinic inhibition (n=6) at PL pathway but this inhibition was completely reversed by atropine (5µM) (89.13 ± 11.88, **p<0.01, n=6) suggesting a muscarinic receptor other than M2 responsible for inhibition at this pathway.

B. (Top) Representative traces illustrating muscarine (MUSC) inhibits fEPSP at THAL input and this inhibition is not reversed by M2 antagonist AFDX-116 (AFDX). (Bottom) Similar to the PL pathway, at the THAL pathway, M2 antagonist AFDX (1µM) did not significantly reverse muscarinic inhibition (n=6), although this inhibition was completely reversed by atropine (5µM) (96.52 ± 3.61, **p<0.01, n=6) suggesting a muscarinic receptor other than M2 responsible for inhibition at this pathway.

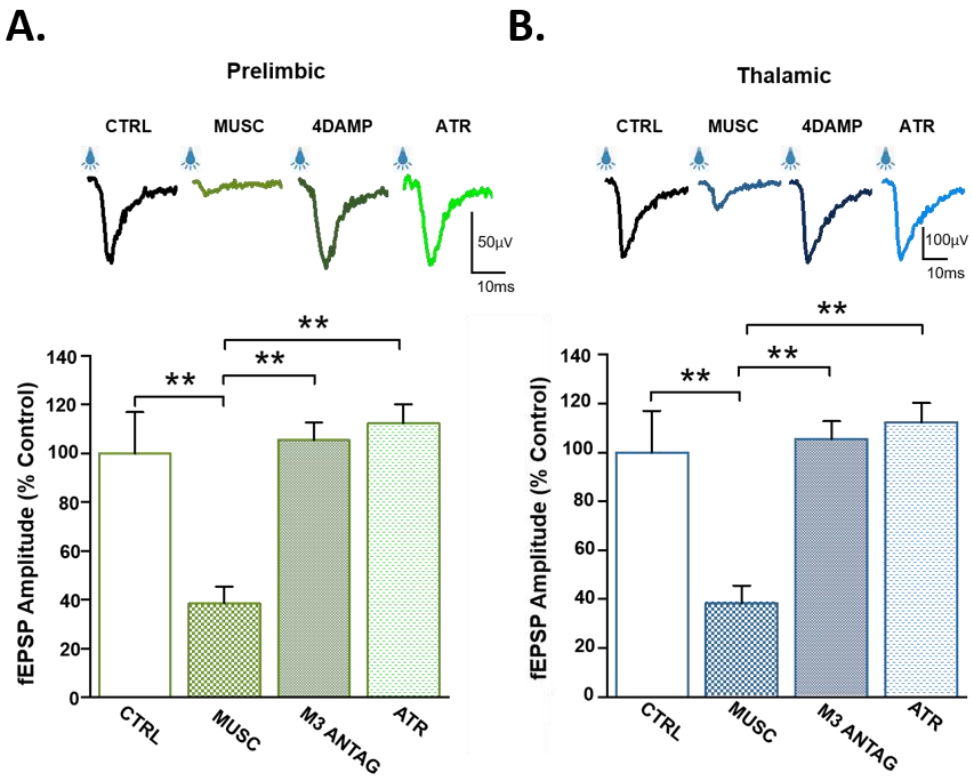


Figure 4.8. Muscarinic receptor activation inhibits PL and THAL input via M3 mediated mechanism.

A. (Top) Representative traces illustrating muscarine (MUSC) inhibits fEPSPs at PL input and this inhibition is completely reversed by M3 antagonist 4DAMP. (Bottom) M3 antagonist 4DAMP (1µM) completely reverses muscarinic inhibition (104.32 ± 14.67, **p<0.01, n=10) at PL pathway, indicated mAChR M3 receptors regulate muscarinic inhibition at this pathway.

B. (Top) Representative traces illustrating muscarine (MUSC) inhibits fEPSP at THAL input and this inhibition is completely reversed by M3 antagonist 4DAMP. (Bottom) Similar to the PL pathway, at the THAL pathway, M3 antagonist 4DAMP (1µM) completely reversed muscarinic inhibition (105.43 ± 7.26, **p<0.01, n=9), indicating M3 receptors mediated muscarinic inhibition at the THAL pathway.

However, M4 can not be ruled out. Due to the lack of availability of specific M4 antagonists and the increasingly common discovery of M4 positive allosteric modulators (PAMs), the latter was used to determine if M4 receptors are responsible for inhibition at PL and / or THAL input to the BL. Allosteric modulators are compounds that bind, as the name suggests, to an allosteric site on the receptor. Positive allosteric modulators will enhance an agonists' effects at that receptor. Here, we used the M4 PAM VU0467154, a positive modulator that, when bound to the allosteric site on M4 receptors, should enhance the functionality of M4 receptors should the agonist also bind. Thus, if M4 receptors are present at PL or THAL input, a low-dose of muscarine or another muscarinic agonist should create a slight effect, and successive application of the M4 PAM would greatly enhance the effect. To test this, a low-dose of oxotremorine (0.3μM) (a muscarinic agonist commonly used in M4 PAM studies) was applied followed by the M4 PAM VU0467154 (3μM). Inhibition by a low dose of oxotremorine at the PL pathway was significantly enhanced after application of the M4 PAM (amplitude significantly decreased from 79.73 ± 2.99 to 46.51 ± 6.70, *$p<0.05$, $n=4$), suggesting M4 receptors are present and inhibit glutamatergic transmission at the PL pathway (Figure 4.9A). However, as these results could be due to an oxotremorine-specific effect by the M4 PAM, experiments at the PL and experiments at THAL were repeated with a low dose of muscarine instead of oxotremorine. These experiments using muscarine instead of oxotremorine found PL glutamatergic fESPS to be similarly inhibited by a low-dose of muscarine (0.3μM), and this inhibition was significantly facilitated by application of M4 PAM VU0467154 (3μM) (amplitude significantly decreased from 63.75 ± 5.51 to 38.33 ± 7.81, *$p<0.05$, $n=5$) at PL pathway, further supporting the presence of M4 receptors at PL pathway (Figure 4.9B).

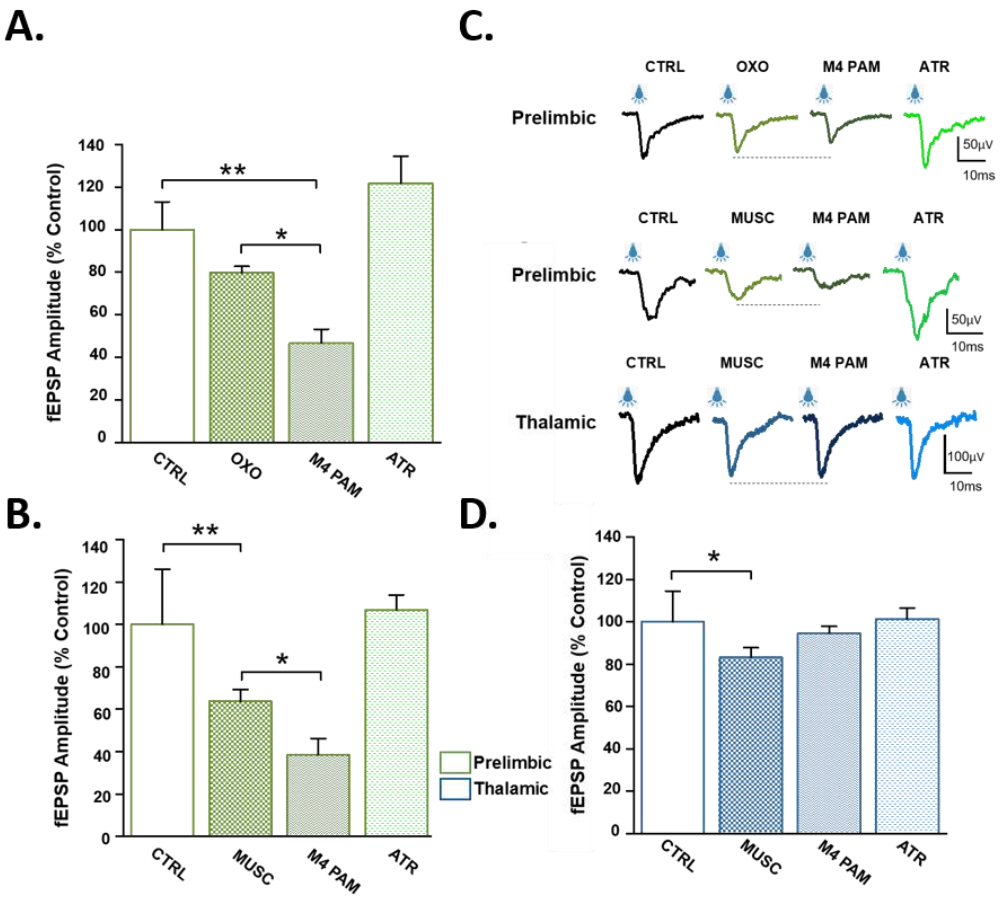


Figure 4.9. M4 mAChRs are present at PL, but not THAL, input to BL.

A. Inhibition by low-dose of oxotremorine (0.03µM) was significantly facilitated by application of M4 PAM VU0467154 (3µM) (amplitude significantly decreased from 79.73 ± 2.99 to 46.51 ± 6.70, *p<0.05, n=4) at PL pathway suggests the presence of functional M4 receptors at PL input.

B. PL input was similarly inhibited by a low-dose of muscarine (0.3µM), and this inhibition was significantly facilitated by application of M4 PAM VU0467154 (3µM) (amplitude significantly decreased from 63.75 ± 5.51 to 38.33 ± 7.81, *p<0.05, n=5) at PL pathway, further supporting the presence of M4 receptors at PL pathway.

C. Representative traces illustrating M4 PAM facilitating inhibition by muscarinic agonists.

D. No presence of M4 receptors at THAL input, as determined by absence of effect of M4 PAM VU0467154 (3µM).

Interestingly, no effect of M4 PAM was seen at the THAL pathways (Figure 4.9D). Taken together, these experiments suggest the presence of M4 receptors at PL input to BL but not at THAL input.

4.4 Discussion

The results from this study provide novel insight into regulation of afferent input to the BL. The regulation of glutamatergic neurotransmission from specific regions to the BL, despite the importance of PL and THAL input in fear learning and the extensive cholinergic receptor presence in the BL, had not yet been examined. Previous studies using electric stimulation to examine regulation of glutamatergic transmission in the BL and LA have been performed, but prior to this current study, there existed no study systematically examining how mAChR subtypes regulate specific afferent projections to the BL (Sugita et al., 1991; Jiang et al., 2016). Results from this study identify mAChRs M3 and M4 could be potential targets for therapeutics aimed at treating and improving symptoms of emotional disorders, including PTSD, anxiety, and the emotional dysregulation seen in patients with Alzheimer's disease.

The results from this study suggest that PL and THAL input to the BL is differentially regulated by mAChRs, such that when ACh levels are elevated in the BL, mAChR activation, via M3 receptors and potentially M4 at PL input, will shift the dynamic to allow more glutamatergic transmission from the THAL than from PL. Thus, signal-to-noise is enhanced to allow the BL to be more receptive to THAL input. These findings are summarized in Figure 4.10 demonstrating optogenetic stimulation of PL and THAL input releases glutamate, but that activation of M3 mAChRs at both pathways inhibits this

glutamatergic transmission. However, suppression is largest at PL, whereas this suppression is only partial at THAL input (fuscia traces represent fEPSPs in muscarine and black in control ACSF).

4.4.1 MACHRS PARTIALLY SPARE THALAMIC INPUT TO THE BL WHILE NEARLY COMPLETELY SUPPRESSING PL INPUT

Results of this study demonstrate that ACh would allow greater neurotransmission from the THAL pathway to the BL than from the PL pathway. However, our results must be considered according to previously published data in the field as well. Because it has been shown that a long-term effect of ACh on cortical input to the BL is a potentiation of responses (Jiang et al., 2016), our data fits into this scheme because we examine acute, short term modulatory effects of ACh on cortical input. It may be possible that acutely, ACh largely suppresses PL input to the BL and spares THAL input, the consequence of which is to allow for rapid quieting of the BL as new information is processed and updated intrinsically, and then, after this transient suppression, plasticity mechanisms dominate to allow for the long term effects seen in previous studies.

Interestingly, in our muscarine experiments at PL and THAL input, we see a slightly larger increase in fEPSP amplitude after muscarine has been applied and atropine antagonizes muscarinic receptors. This increase in amplitude did not reach significance, but on average, antagonizing muscarine with atropine at both the PL and THAL pathways resulted in larger glutamatergic responses. This potential muscarinic-induced plasticity is interesting when we consider that all experiments were done in the presence of NMDA antagonist DAPV. It has been shown in the cortex that muscarine can induce long term

depression in an NMDA-independent manner but whether this occurs in the BL remains an avenue for exploration (McCoy & McMahon 2007). This effect could also be due to tonic ACh being antagonized by atropine and thus it could be interesting to see if there are pathway specific differences in tonic ACh release. When sex was considered, we noticed that in the PL pathway, this enhancement in amplitude after muscarinic application was trending only in males, whereas females showed a mean amplitude in atropine smaller than that in control. While more experiments would be needed in the future to examine this potential sex difference, it highlights the need to explore sex differences in mechanisms underlying the known differences in emotional disorder prevalences in men and women.

4.4.2 CHOLINERGIC MODULATION OF PL AND THAL INPUTS THROUGH M3 RECEPTORS AT PL AND THAL AND M4 RECEPTORS AT PL

Our data supports literature showing muscarinic receptors can suppress inhibition of glutamate presynaptically. Specifically, our data extends findings from CA1 of the lateral amygdala, basal ganglia, nucleus accumbens, hippocampus, neostriatum, and mesencephalon that mAChR M3 receptors can presynaptically inhibit glutamate release (Grillner et al., 1990; Hsu et al., 1995; Shen & Johnson, 2000; Rinaldo & Hansel 2013; de Vin et al., 2015). Our study and these previous studies used a combination of cholinergic antagonists to determine receptor involvement. Due to the lack of antagonists that are particularly selective for subtypes, IC50s need to be taken into consideration when discussing antagonist data and determining subtypes involved in muscarinic inhibition.

Telenzepine, (TZP) an M1 antagonist, has a selectivity for M1 that is 26-fold more selective than M2 and 5-fold more selective than M3 (Doods et al., 1987). We used TZP

at a concentration of 100nm, as its IC50 for M1 receptors in the rodent brain has been shown to be 0.19nM (Baumgold et la., 1992). Thus, our dose of TZP, roughly ten-fold the IC50, should sufficiently antagonist M1 receptors in the BL. Because we saw a slight reversal (although not significant) of M1 receptors, this is most likely due to M1 receptors that are indeed present in the BL as illustrated by anatomical studies (McDonald & Mascagni 2010; Muller et al., 2013; McDonald et al., 2019). Because of this surprising result that M1 receptors were not significantly modulating presynaptic glutamate release but are indeed present in the BL, we performed additional studies on PL input to the BL using a second, more recently developed M1 antagonist, VU0255035 (5µM). This compound displays Ki for M1 receptors that are 45, 62, 84, 168 times more selective than M2, M3, M4 and M5 receptors (Sheffler et al., 2009). These studies confirmed our studies with TZP that antagonizing M1 receptors had no significant effect on muscarinic inhibition.

AFDX-116, the M2 antagonist used in our studies, was shown using autoradiography to have an affinity for M2 receptors that is 25-times more selective for M2 than M3 (Doods et al., 1989). Another study showed AFDX116 exerts an IC50 of 0.36µM for M2 receptors, 4.3µM for M1 receptors, 3.1µM for M3 receptors and 0.79µM for M4 receptors (Buckley et al., 1988). Thus, when determining the presence of M2 receptors using AFDX, the more conservative IC50 values should be taken into consideration. Our AFDX-116 concentration of 1µM, nearly three times the IC50 of AFDX for M2 receptors, should have been sufficient to substantially antagonize M2 receptors. However, because there was no significant effect of AFDX at PL or THAL input, we can rule out M2 receptors at these inputs. Given AFDX116's IC50 of 0.79µM at M4 receptors and our use of AFDX116 at 1µM, there is the potential that we were

antagonizing slightly less than half of M4 receptors if they were there. Our AFDX116 data would suggest that we were either not antagonizing M4 receptors, or they were are not present at either pathway as AFDX at 1μM had no effect on muscarinic inhibition.

4DAMP, while extensively used in the literature as an M3 antagonist, is a relatively nonspecific antagonist for M3 and thus is often used in combination with other antagonists more specific for M1 and M2 (such as TZP and AFDX). 4DAMP displays affinities for both M1 and M3 to similar extents as well as some affinity for M4 receptors (Zubieta & Frey 1993; Moriya et al., 1999). Thus, when used as an M3 antagonist, TZP, VU0255035 or another M1-specific antagonist should be used to first determine M1 effects. In experiments highlighted here, we first used TZP and VU0255 to determine lack of M1 receptors in mediating glutamatergic inhibition. We can therefore conclude that even if our concentration (1μM) of 4DAMP could bind to M1 receptors, they are not present at either the PL or THAL input to BL. However, we could not rule out the potential for 4DAMP antagonizing M4 receptors. Unfortunately, there exists no selective agonist for M4 receptors. However, the advent of several positive allosteric modulators has enabled the investigation of the presence of M4 receptors. Thus, we used the M4 PAM VU-467154 that has been validated across species to potentiate M4 receptors, to determine if M4 receptors were present at either pathway (Byun et al., 2014). We can only conclude the presence of M4 receptors at PL input, as the facilitation of muscarine's effect at PL input by the M4 PAM indicates this subtype is present, but does not indicate that under physiological conditions (ie, not in the presence of a PAM) it would exert an effect.

4.4.2 M3 MUSCARINIC RECEPTORS MODULATE CORTICAL AND THALAMIC INPUT IN THE BL IN A MANNER OPPOSITE TO THAT OF THE CORTEX

ACh shifts the influence of cortical regions from intrinsic signaling to afferent signaling, which is thought to "enhance input relative to feedback" in the cortex (Hasselmo & McGaughy 2004). The value of ACh release in the cortex increasing signal-to-noise filtering to favor afferent input would be, as theorized and summarized elegantly by Hasselmo & McGaughy, to increase receptiveness and attention to incoming sensory stimuli in one's environment while blocking out recurrent obtrusive synaptic transmission (Hasselmo & McGaughy 2004). For example, cholinergic receptors modulate cortical and thalamic inputs to the auditory cortex in a manner consistent with an increase in external signal to noise; muscarinic receptor activation more potently suppressed intrinsic cortical inputs and spared thalamic inputs (Hseih et al., 2000). When viewed from the perspective of projection-type modulation, our experiments would also suggest that ACh functions to spare thalamic input while suppressing cortical input, regardless of whether it is "intrinsic" or "afferent." Similarly, when ACh is elevated in the BL, the signal to noise ratio would favor thalamic input over PL, the potential consequence of which is discussed in Chapter 6.

4.4.2 CHOLINERGIC SUPPRESSION OF PL AND THAL INPUT TO THE BL: IMPLICATIONS FOR EXERCISE

Data demonstrating exercise's influence on the cholinergic system is extensive, with many studies showing that exercise increases ACh release in the brain (Dudar et al., 1979; Tsakaris et al., 2006; Fordyce & Farrar 1991; Hall & Savage 2016). In the BL, the

ACh that is released would bind to the cholinergic receptors shown to be present in this region. We have demonstrated that M3 receptors are activated and suppress PL input but allow THAL input to the BL. If we consider exercise from a historical perspective, as an activity that was evolutionarily necessary for survival (either to flee from a predator, run to catch prey, or run to catch food while avoiding predators), it is rationale to presume that the ability to be physically active and the ability to pay attention to, and update your model of, ones surroundings would similarly evolve in a Darwinian manner. In other words, the value of having developed a neural mechanism whereby ACh is enhanced with physical activity when one is out foraging/surviving/chasing, and this enhanced ACh shifts the BL attention away from cortical input to the BL and towards sensory stimuli in our environment can arguably be rationally understood. The mouse with the amygdala that, when foraging and is confronted with unexpected stimuli, is able to temporarily shut down its BL with ACh in order to pay attention to incoming stimuli and update its model of the world would be better able to appropriately respond to changes in its environment.

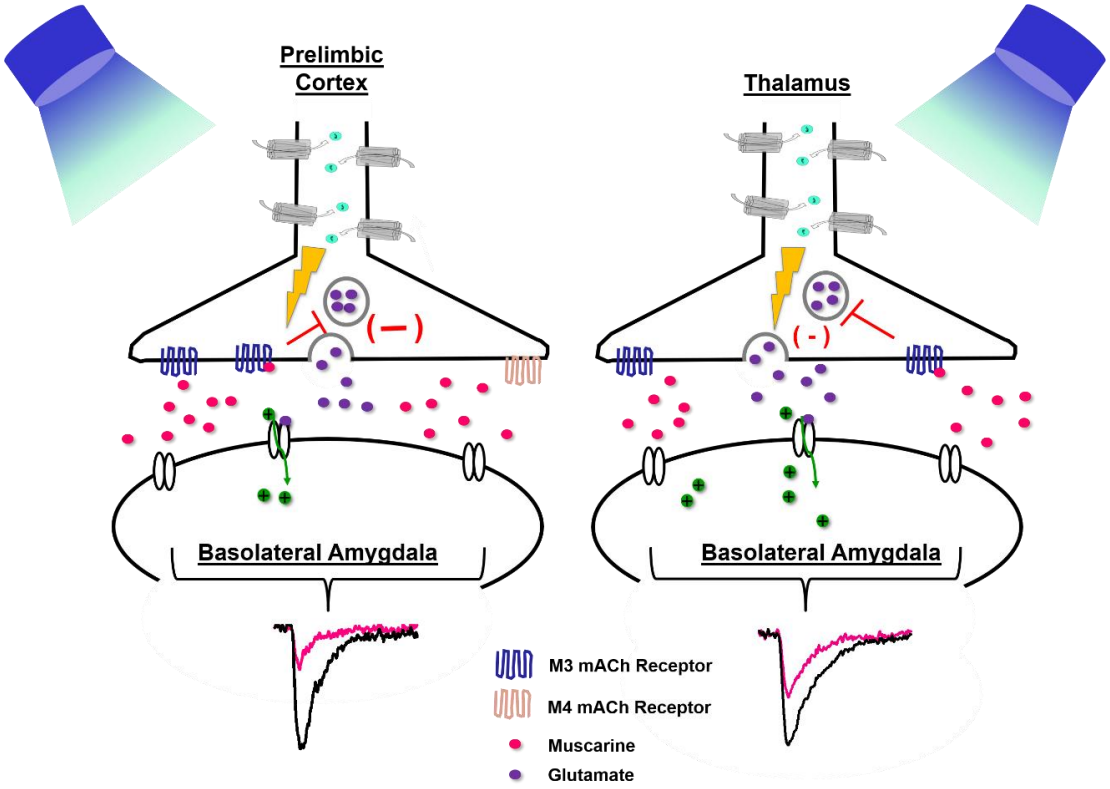


Figure 4.10. Schematic summary of findings described in Chapter 4.
Optogenetic activation of PL and THAL evokes fEPSPS in control (absence of muscarine) black waveforms). Application of muscarine inhibits PL input via M3 and possible M4 mAChRs, while THAL is inhibited through M3 by only 50% (pink waveforms in muscarine).

CHAPTER 5

MECHANISMS OF MUSCARINIC INHIBITION AT PL AND THAL INPUT TO BL

5.1 INTRODUCTION

Fear disorders, including Post Traumatic Stress Disorder (PTSD), general anxiety disorder and phobias affect nearly 20% of Americans (Kessler et al., 2005). One of the common neurological regions involved in these disorders is the amygdala, a region in the temporal lobe critical for emotional learning and memory. Our understanding of the amygdala as a vital center for emotional associations developed out of both human clinical case studies as well as animal models in which the amygdala and its projects were lesioned or damaged (Siebert et al., 2003; Adolphs et al., 1995; Quirk et al., 1995; Rogan et al., 1997; Tovote et al., 2015; Kwon et al., 2014).

Pavlovian conditioning studies, in which a neutral harmless stimulus such as tone (Conditioned Stimulus, or CS) is paired with an aversive stimulus like a shock (Unconditioned Stimulus or US), results in an animal (or even human) forming an emotional association between an otherwise harmless stimulus in our environment (ie a "tone" in many conditioning paradigms) with a fearful stimulus (ie a "shock" in many conditioning paradigms). Once an emotional memory is formed between two stimuli, animals often display fear behaviors allowing scientists to measure their memory of the

CS-US association or their ability to extinguish the memory. By having an observable model of emotional memories, scientists were able to then manipulate aspects of the hypothesized fear circuit in the brain to determine specific mechanisms for emotional memory formation.

Through such conditioning studies, neural regions important for fear acquisition and extinction have been elucidated. The lateral amygdala (LA) is important for the initial acquisition of a tone-shock pairing, as neurons in the LA electrophysiologically respond to both auditory tones and electric shocks, and synapses in the LA undergo plasticity in response to associative learning of a tone-shock presentation (Romanski et al., 1993; Blair et al., 2003).

The basolateral amygdala is also critical for the acquisition and expression of emotional learning and memory, but the exact role of the BL in emotional processing is turning out to be much more complex than previously thought and the exact roles remains largely unknown. Recent studies have suggested the BL functions in processing and updating previously formed associations through studies that have found the BL to discriminate between various noxious stimuli it has encountered and by retaining the memory of a recent conditioned stimulus' saliency (Campese et al., 2019; Sengupta et al., 2018). In the latter study that found the BL to play role in aversive memory by forming a neural representation of the saliency of a recent stimulus, optogenetically inhibited BL neurons during Pavlovian fear conditioning (Sengupta et al., 2018). They found that by inhibiting the BL during the presentative of an aversive stimulus (the US), rats learned and expressed less fear to the CS. Interestingly, they also found that inhibiting BL neurons when the US *should* occur but didn't during extinction training (in other words, when error

prediction was extremely low) enhanced extinction (Sengupta et al., 2018). In studies presented in Chapter 3 and 4, I have shown that ACh briefly inhibits afferent input from PL and THAL to the BL, presumably inhibiting activity in the BL briefly. If one were to extrapolate my findings to this behavioral study (Sengupta et al., 2018), it would be hypothesized that by optogenetically inhibiting the BL briefly, the investigators were in essence mimicking what ACh would do to the BL. Interestingly, ACh is known to be important for learning and memory, so the theory that ACh, by inhibiting the BL through muscarinic receptors, would be causing rats to learn and later express *less* fear to a CS is difficult to understand. In order to fully understand how mAChR activation is affecting the BL at PL and THAL inputs, and thus better understand potential treatment targets for emotional disorders, a more thorough investigation into the mechanisms underlying mAChR suppression of PL and THAL input need to be pursued. Thus, the purpose of this study was to investigate the mechanisms underlying muscarinic inhibition at PL and THAL inputs to the BL. It was hypothesized that given the different amount of inhibition conferred to PL and THAL inputs by mAChRs found in previous studies, that mAChRs at the PL and THAL input would suppress glutamatergic fEPSPS through different mechanisms.

5.2 MATERIALS AND METHODS

To determine mechanisms underlying muscarinic inhibition of PL and THAL input to the BL, field electrophysiology was used in combination with optogenetics. Viral constructs were delivered to the PL and THAL using surgical techniques and coordinates described in Chapter 2.7.

Slices were prepared and incubated following previously describes techniques (Chapter 2.8). Slices were submerged in a recording chamber and gently held in place by a platinum wire. For all experiments in this study except frequency experiments, glutamate release from PL and THAL projections to the basolateral amygdala were stimulated with single or dual light pulses. For frequency experiments, light stimuli was applied as 10-pulse train, with the pulses flashed at 1Hz, 5Hz, 10Hz, 20Hz, 30Hz and 40Hz to release glutamate at various frequencies. Control recording ACSF, unless otherwise described, contained 10μM-100μM picrotoxin or 10μM bicuculline (to block $GABA_A$ receptors), 2μM CGP55845 ($GABA_B$ antagonist) and 50μM L-2-amino-5-phosphonovaleric acid (DAPV) or 10μM MK801 (NMDA antagonist). Experiments examining $GABA_B$ involvement (Chapter 5.3.1) used a control ACSF that consisted of $GABA_A$ antagonists only. When $GABA_B$ receptors were blocked, 2μM CGP55845 was added to the recording medium. Experiments examining muscarinic modulation of NMDA currents used a control ACSF that included 10μM-100μM picrotoxin and 2μM CGP55845N-methyl-D-aspartate (NMDA) to antagonist GABA receptors but did not include D-APV so as not to block NMDA receptors. Glutamatergic components of field potentials were confirmed by inhibiting the response with 25μM of the potent glutamatergic antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) at the conclusion of each experiment.

5.3 RESULTS

5.3.1 mAChRs INHIBIT GLUTAMATERGIC TRANSMISSION AT PL AND THAL SYNAPSES THROUGH $GABA_B$ INDEPENDENT MECHANISMS

Because it is known that $GABA_B$ receptors in the BL inhibit glutamatergic transmission and that activation of mAChRs on postsynaptic interneurons can facilitate the release of GABA that then inhibits glutamate, we first wanted to know if muscarinic M3 receptors inhibit PL or THAL input via a $GABA_B$-dependent mechanism (Pitler & Alger 1992; 1999; Pan et al., 2009). Based on the endogenous ACh data from Chapter 3 demonstrating no effect of GABA receptor action on the cholinergic suppression of glutamate from cortical regions, it was hypothesized that we would also see muscarinic inhibition at the PL and THAL inputs in a manner independent of $GABA_B$ receptor activation. To answer this question, it was first necessary to determine if $GABA_B$ receptors are present at PL and THAL projections to the BL, and if so, do they suppress glutamatergic fEPSPs. To this end, responses were optogenetically evoked from the respective pathways (control fEPSPs shown in black in Figure 5.1A,C top). $GABA_B$ receptors were then activated by bath application of agonist baclofen (2-10μM). Interestingly, both PL and THAL evoked fEPSPs were suppressed to significant extents (PL: 10.67 ± 3.38% of control, $^{**}p<0.01$, n=7; THAL: 28.24 ± 15.66% of control, $^{**}p<0.01$, n=3), demonstrating the presence of $GABA_B$ receptors, that, when activated, inhibit glutamatergic fEPSPs from the PL and THAL input to BL. However, whether mAChRs present on amygdalar interneurons can facilitate the release of GABA to then activate the GABA receptors we found inhibit glutamatergic fEPSPs is unknown. To answer this question, stable evoked fEPSPs were acquired from PL and THAL optogenetic stimulation. CGP55845 ($GABA_B$

antagonist) was left out of the control recording ACSF. After stable baseline responses, muscarine (10μM) was added to the recording medium and responses suppressed as expected. $GABA_B$ receptors were then antagonized to determine if a relief of muscarinic inhibition would result. As hypothesized, antagonizing $GABA_B$ receptors had no effect on muscarinic inhibition, suggesting that muscarinic suppression of glutamatergic fEPSPs at PL ($p>0.05$, $n=7$) and THAL ($p>0.05$, $n=5$) inputs is due to mechanisms that do not involve $GABA_B$ receptors (Figure 5.1B,D).

5.3.2 MUSCARINIC INHIBITION AT THAL, BUT NOT PL, PROJECTIONS IS MEDIATED BY AN ENDOCANNABINOID-DEPENDENT MECHANISM

A potential mechanism whereby mAChR activation inhibits glutamate release is through the facilitation of endocannabinoid (eCB) release. These eCBs, if released from neurons in the BL after mAChR activates mechanisms elevating intracellular calcium influx, could theoretically serve as retrograde messengers and inhibit glutamate from PL and THAL terminals in the BL. This ability for mAChRs to enhance the release of eCBs has been shown in area CA1 of the hippocampus, where mAChR activation enhanced eCB release that then presynaptically reduces glutamate release by activating CB receptors (Kim et al., 2002). In the lateral amygdala, eCBs inhibit both glutamatergic and GABAergic transmitter release through acting on presynaptic CB1 receptors (Azad et al., 2003). Furthermore, experiments in the hippocampus and the cerebellum find G_q-coupled mAChRs, specifically M1 (in hippocampus) and M3 (in hippocampus and cerebellum) mAChRs, to be responsible for inhibition of excitatory glutamatergic transmission, and

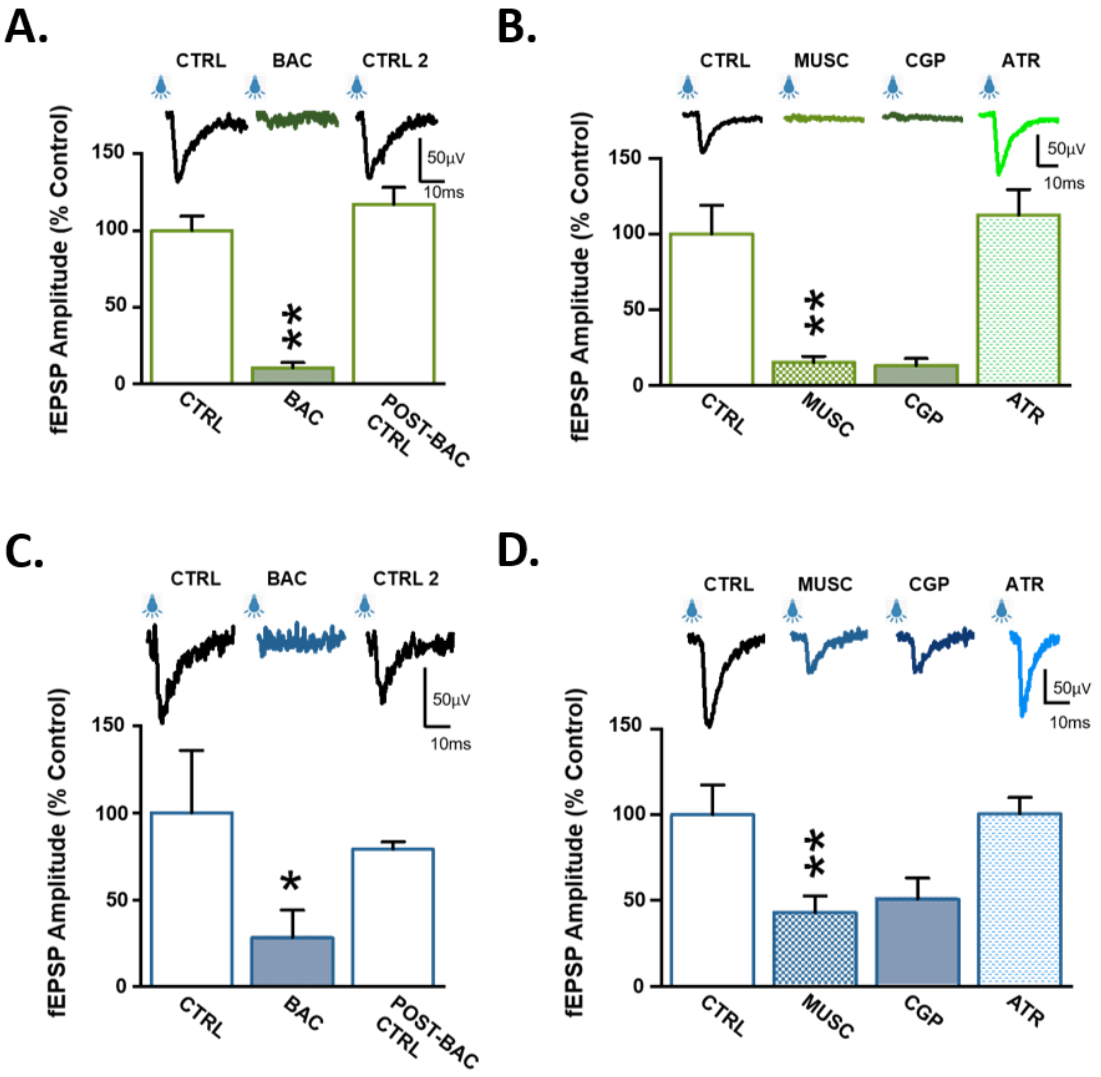


Figure 5.1. Muscarinic inhibition at PL and THAL inputs to BL not mediated by $GABA_B$ receptors.

A. Bath application of $GABA_B$ agonist baclofen (2-10µM) inhibits prelimbic glutamatergic transmission, indicating the presence of $GABA_B$ receptors at PL input to the BL (amplitude reduced from control to 10.67% ± 3.38, **p<0.01, n=7). Top inset: representative traces showing $GABA_B$ activation (green) nearly completely inhibits PL transmission compared to control (black).

B. Bath application of $GABA_B$ antagonist CGP55845 (2µM) had no effect on muscarinic (10µM) inhibition, indicating muscarinic suppression of PL input not mediated by $GABA_B$ receptors (n=7). Top inset: representative races showing muscarine (10µM) (light green) inhibits PL input to the BL, is not reversed by antagonizing $GABA_B$ receptors with CGP55845 (dark green) and is completely reversed by muscarinic antagonist atropine (5µM) (neon green).

C. Similar to the PL input, transmission from the THAL input is inhibited by bath application of $GABA_B$ agonist baclofen (2-10μM), indicating the presence of $GABA_B$ receptors at THAL input to the BL (amplitude reduced from control to 28.24% ± 15.66, **p<0.01, n=3). Top inset: representative traces showing $GABA_B$ activation (blue) nearly completely inhibits THAL transmission compared to control (black).
D. Bath application of $GABA_B$ antagonist CGP55845 (2μM) had no significant effect on muscarinic (10μM) inhibition, indicating muscarinic suppression of THAL input not mediated by $GABA_B$ receptors (n=5). Top inset: representative races showing muscarine (10μM) (light navy blue) inhibits THAL input to the BL, is not reversed by antagonizing $GABA_B$ receptors with CGP55845 (dark navy blue) and is completely reversed by muscarinic antagonist atropine (5μM) (light blue).

that this inhibition possesses functional relevance for preventing plasticity at synapses engaged in this muscarinic facilitation of eCBs (Ohno-Shosaku et al., 2003; Rinaldo & Hansel, 2013). Given the results of our experiments in Chapter 4 showing mAChR M3 receptor activation suppresses glutamate release at both the PL THAL pathways, it is plausible that the mechanism underlying this M3-mediated inhibition is dependent on eCB release. To examine this question, a series of experiments were performed in which CB1 receptor antagonist AM251(1μM) was bath applied following muscarinic inhibition of PL and THAL input. Should muscarine be inhibiting fEPSPs by released eCBS that decrease glutamate release through CB1 receptors, blocking those CB1 receptors should reinstate glutamatergic transmission. Surprisingly, we found antagonism of CB1 receptors to have no effect on muscarinic suppression of fEPSPs at PL input to the BL (p>0.05, n=7), revealing that muscarinic suppression of PL input is through an endocannabinoid-independent mechanism (Figure 5.2A). This was surprising given the presence of cannabinoid receptors at these inputs, as application of CB receptor agonist WIN55,212 (5μM) reduced PL-evoked fEPSPs in a manner reversible by AM251 (19.67 ± 7.37% of

control, **p<0.01, n=6) (Figure 5.2B). This data suggests that even though CB receptors can suppress PL evoked fEPSPs in the BL, muscarinic suppression of PL input does not work through such a mechanism. However, experiments examining THAL projections to the BL found that muscarinic mediation suppression was through a different mechanism. At these inputs, AM251 completely reversed muscarinic inhibition of THAL-evoked fEPSPs (AM251 reversed muscarinic inhibition from 41.50±7.85% of control to 84.48±11.45% of control in the absence and presence of AM251, respectively, *p<0.05, n=7) (Figure 5.2C, D). However, to eliminate the possibility that this effect was simply due to a pharmacological enhancement of glutamatergic transmission by AM251, control experiments were performed in which AM251 was applied and THAL-evoked fEPSPs were measured at the THAL pathway. No significant enhancement of fEPSPs were seen (Figure 5.2E), demonstrating that reversal of muscarinic inhibition was due to a muscarinic-mediated release of eCBs which retrogradely inhibited glutamatergic transmission at the THAL projections.

5.3.3. MACHRs INHIBIT PL INPUT IN A MECHANISM THAT IS DEPENDENT IN PART ON Gi/o-COUPLED PROTEIN

We next wanted to elucidate the mechanism of muscarinic inhibition of PL input, as our prior experiments ruled out $GABA_B$ and CB receptors as mediators in the observed inhibition at this pathway. Our results so far have found M3 receptors and M4 receptors to be present at PL input to the BL. However, whether M3 receptors inhibit glutamate by directly reducing calcium influx presynaptically or by facilitating the release of a

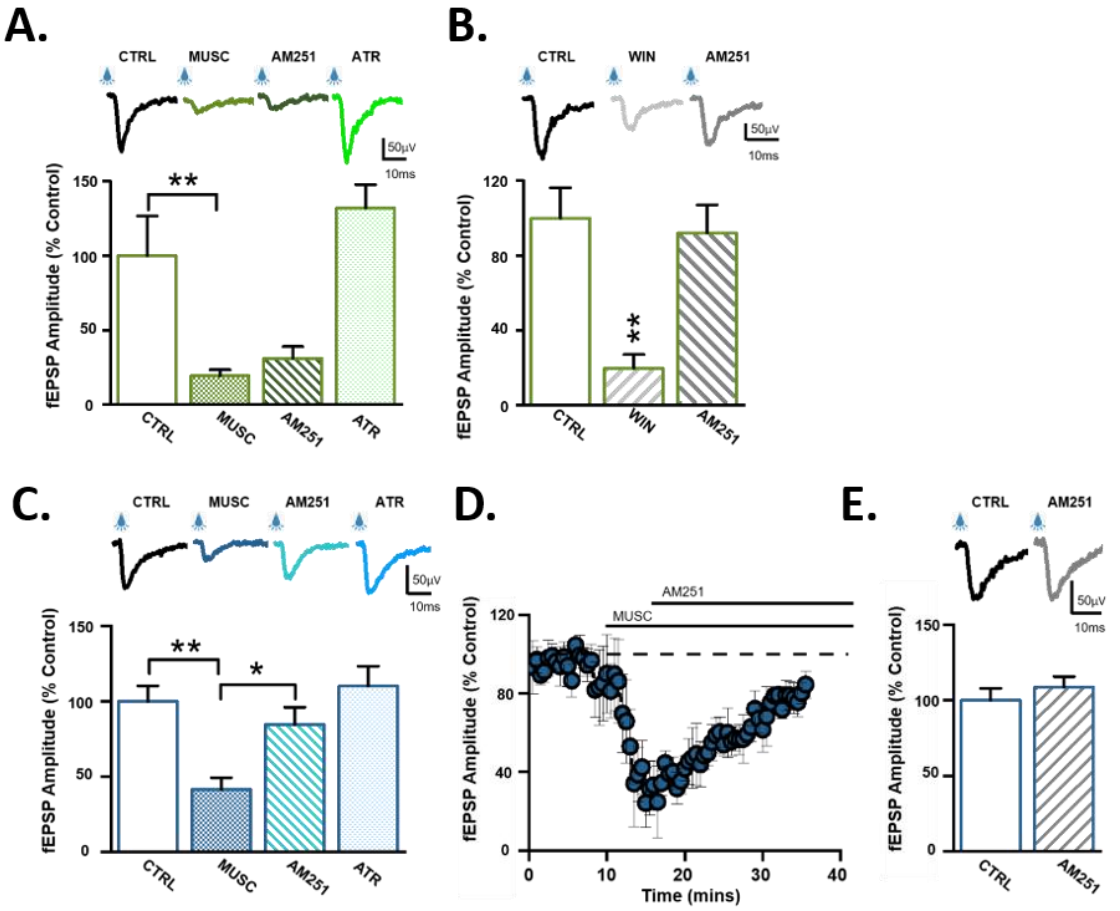


Figure 5.2. Muscarinic inhibition at THAL, but not PL, mediated by an endocannabinoid-dependent mechanism

A. Muscarinic inhibition at PL input is not affected after antagonizing CB1 receptors with AM251 (1µM) (n=7). Top inset: Representative traces illustrating muscarine inhibits fEPSPs (light green) and this is not reversed by AM251 (dark green) but is completely reversed by atropine (neon green).

B. Even through muscarinic inhibition is not through an endobannabinoid-dependent mechanism at the PL input, there are endocannabinoid receptors present at this pathway as indicated by near complete inhibition of fEPSPs by CB agonist WIN 55,212 (5µM) (amplitude reduced from control to 19.67% ± 7.37, **p<0.01, n=6).

C. Application of CB1 antagonist AM251 (1µM) reverses muscarinic inhibition at THAL input (amplitude of response increases from 41.50±7.85 to 84.48±11.45 in the absence and presence of AM251, respectively, *p<0.05, n=7), indicating muscarinic suppression of fEPSPs is through an endocannabinoid-dependent mechanism at this pathway. Top inset: representative traces illustrating muscarine inhibits fEPSPs (light navy blue), AM251 reverses this inhibition (turquoise) and the remainder of the inhibition is reversed by atropine (light blue).

D. Representative experiments demonstrating reversal by AM251 (n=4).
E. Reversal of muscarinic inhibition by AM251 not due to facilitation of the glutamatergic response due to direct action by AM251.

retrograde messenger that can inhibit glutamate is still unknown. Because M3 receptors couple to Gq proteins and M4 receptors to Gi/o proteins, pretreating slices with an alkylating agent that inhibits Gi/o proteins should block the effects of muscarine at PL input if the effect is entirely M4 modulating a Ca2+ channel via Gi/o protein and have no effect if M3 is inhibiting transmitter release through a completely Gi-independent mechanism (Fryer 1992; Shapiro et al., 1994). To test if mAChR suppression of PL input is through a Gi/o protein-dependent mechanism, baclofen (2µM), an agonist of $GABA_{B,}$ was chosen as a positive control as it is dependent on Gi/o coupled mechanism and was shown in previous experiments to inhibit PL input. After baclofen suppressed PL input, it was washed out and muscarine (10µM) was bath applied. After stable, suppressed responses in muscarine, muscarine was washed out and a Gi/o inhibitor, n-ethylmaleimide (NEM) (50µM) was bath applied to slices for a minimum of 15 minutes. Following preincubation with NEM, muscarine (10µM) was again applied to slices and amplitude of the response after NEM treatment was compared with the amplitude of responses before NEM treatment. Baclofen was also applied following NEM treatment as a positive control to determine if NEM was appropriately inhibiting Gi/o receptors. Preincubation of slices with NEM was sufficient to inhibit Gi/o proteins, as effects by baclofen were significantly inhibited by NEM (Figure 5.3A). However, NEM only partially blocked muscarinic inhibition, suggestive that muscarinic inhibition of PL pathway is partially due to a Gi/o protein-dependent mechanism (pre-NEM amplitude in muscarine was 26.12 ± 7.15 % of

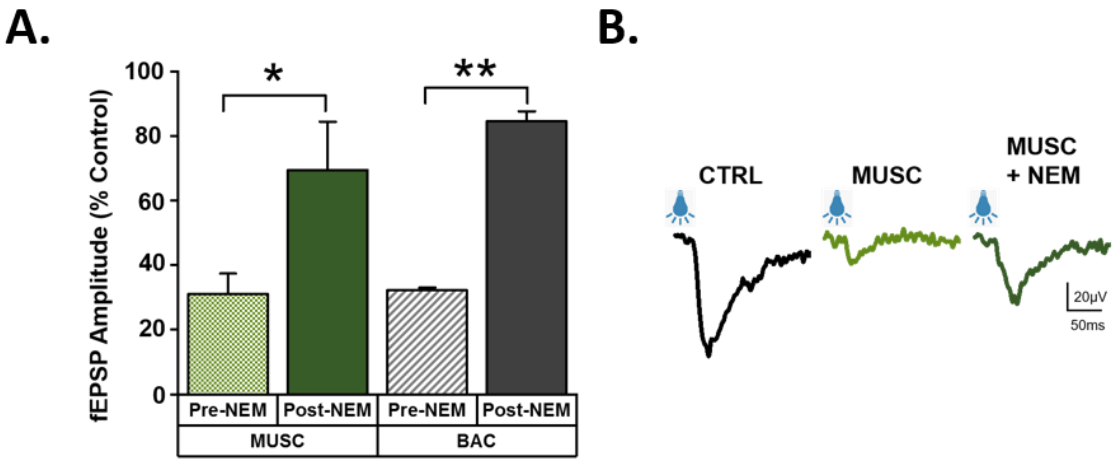


Figure 5.3. Muscarinic inhibition of PL input dependent in part on GI coupled mechanism.

A. Muscarinic inhibition at PL input to BL is partially dependent on a Gi-coupled mechanism, as preincubation with Gi inhibitor N-ethylmaleimide (50 μM) partially inhibited the response (pre-NEM amplitude compared to control was 26.12% ± 7.15 and post-NEM amplitude compared to control was 68.97% ± 15.25, *$p<0.05$, $n=6$).

B. Representative traces illustrating muscarinic inhibition in the absence of Gi inhibitor (light green) largely suppresses the fEPSP but when the Gi is inhibited this suppression is partially blocked (dark green).

control and post-NEM amplitude was 68.97 ± 15.25% of control, *p<0.05, n=6) (Figure 5.3A, B). Representative traces illustrate inhibition by muscarine (light green) and reversal of muscarinic inhibition by pretreatment with NEM (dark green). It remains unknown, however, as to what is mediating the remaining 30% of muscarinic inhibition at PL input.

5.3.4 MACHR AT PL INPUT TO BL FACILITATES NMDA CURRENT

It is known in the hippocampus, striatum, lateral amygdala and other brain regions that mAChRs, specifically M1 receptors, can enhance the NMDA current in postsynaptic neurons through activation by muscarinic agonists and endogenous ACh (Markram & Segal 1990; Marino et al., 1998; Aramakis et al., 1999; Weisskopf et al., 1999; Buchanan et al., 2010; Fernandez de Sevilla & Buno 2010). Given the BL's importance in associative learning, the density of ACh projected to the BL, and NMDA receptors' crucial role in learning and plasticity, we wanted to know if mAChRs similarly enhance NMDA responses in the BL at PL-BL synapses. To investigate this, slices were prepared from animals expressing opsins in the PL. Control ACSF containing 0µM Mg^{2+}, picrotoxin (10µM), CGP55845 (2µM) and CNQX (10µM) was utilized to block $GABA_A$, $GABA_B$, AMPA and Kainate receptors, respectively. As previous experiments in this study (see Chapter 4) found M3 receptor activation to inhibit glutamate release, the release of which is necessary to obtain and detect NMDA responses, M3 receptors were blocked by also adding 4DAMP (1µM) to the recording medium. Thus, evoked responses measured in this ACSF was termed "control" and reflects the isolated excitatory NMDA field potentials ($fEPSP_{NMDA}$) (Figure 5.4A,B). Once NMDA potentials were measured in control, muscarine (10µM) as added to the recording medium. In agreement with what has been

observed in previous studies, muscarinic receptor activation greatly facilitated the NMDA responses (Figure 5.4A,B). Summarized experiments showing muscarinic enhancement of the NMDA response is shown in Figure 5.4A. NMDA responses were confirmed by subsequent application of NMDA receptor antagonist D-APV (50μM) at the conclusion of the experiment. Representative traces from PL input is shown in Figure 5.4B. The isolated NMDA components in control are shown in grey. Application of muscarine at PL (green) greatly increased the NMDA responses. These responses were confirmed to due to NMDA receptors as application of APV (black traces) abolished responses in the PL pathway (Figure 5.4B). This data demonstrates that mAChR in the BL, when activated, can enhance the NMDA response following PL stimulation. Future experiments will be necessary to determine the functionality of this enhancement of NMDA responses.

5.3.5 MUSCARINIC INHIBITION OF PL, BUT NOT THAL, TRANSMISSION FAILS AT GAMMA FREQUENCY

Because ACh levels fluctuate with different behavioral and internal states, we wanted to know how mAChR activation regulates PL and THAL inputs during behaviorally relevant oscillatory patterns of these afferent regions. Higher frequency oscillations (20-70Hz), or gamma frequency oscillations, occur during wakefulness, during acquisition of emotional associative learning, and slow-wave sleep and allow for long-range synchrony between brain regions (Singer and Gray 1995; Popescu et al., 2009; Headley and Pare 2013). Given ACh's importance and the BL's importance for acquisition and expression of fear learning and gamma's presence during acquisition of associative learning, we wanted to know if cholinergic modulation changes or is consistent during

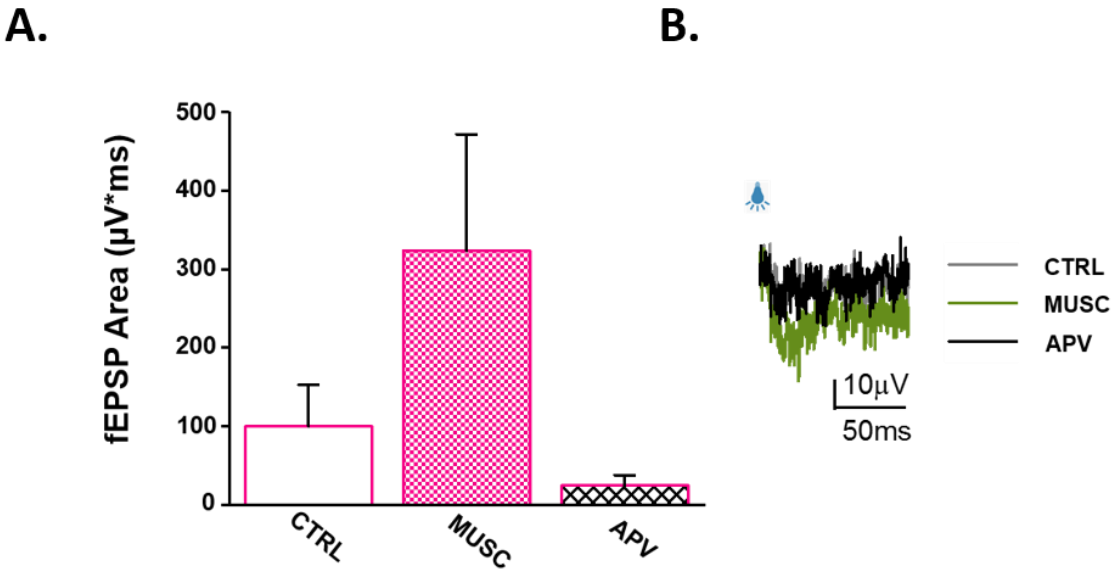


Figure 5.4. Muscarinic receptor activation facilitates NMDA current at PL synapses in BL.

A. Summary bar graph of experiments demonstrating application of muscarine increased the NMDA current (pink bar) and this current was completely blocked by application of NMDA antagonist APV (black bar).

B. Representative traces illustrating muscarinic facilitation of NMDA current at PL. Black (control) represents isolated NMDA current prior to muscarine application (green).

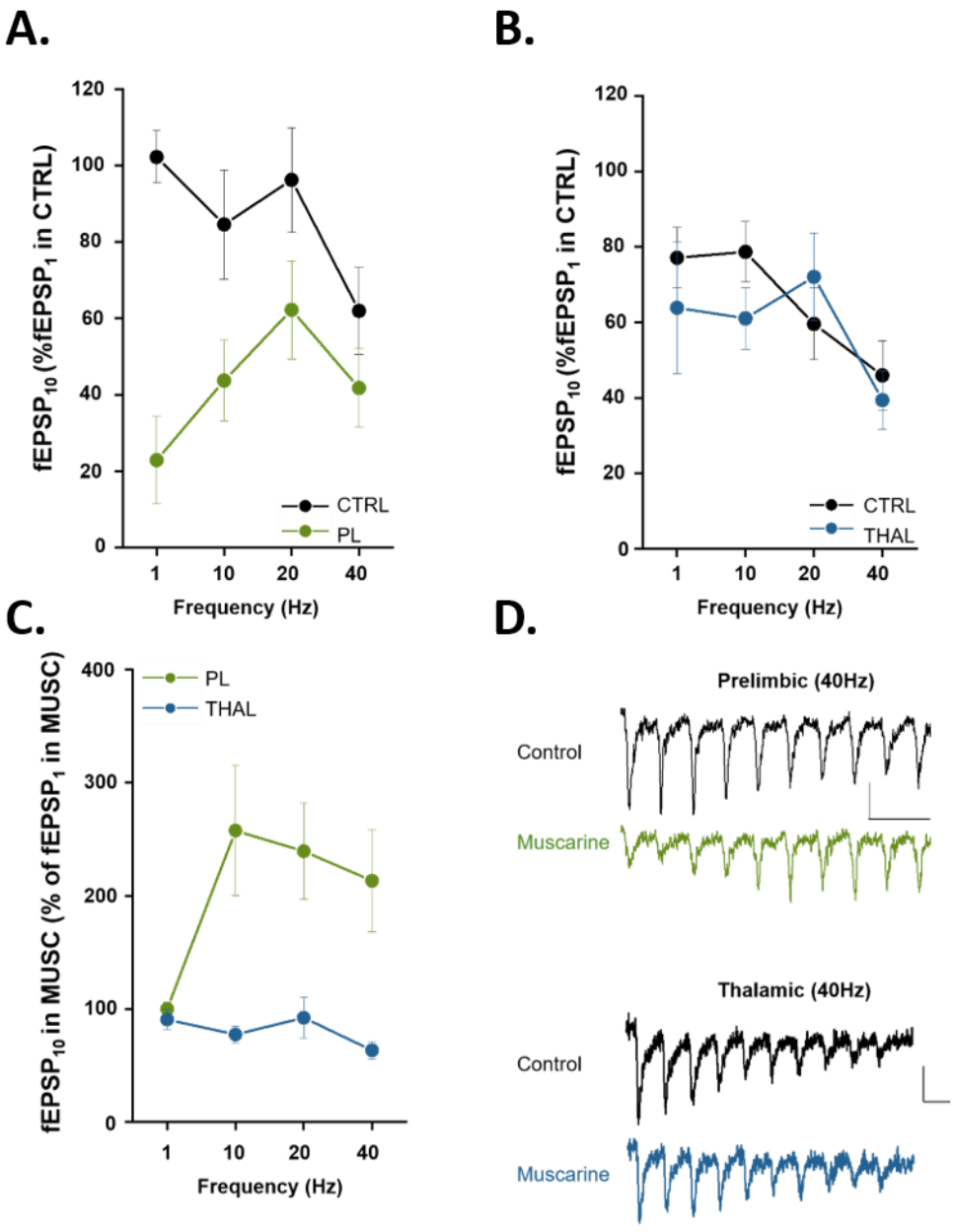


Figure 5.5. Muscarinic inhibition of PL, but not THAL, input fails at gamma frequency

A. Averaged data showing amplitude of the 10th fEPSP at PL pathway in muscarine (green) compared to control. In muscarine, the last fEPSP facilitates at 40Hz whereas in control it depresses at 40Hz compared to control.

B. Averaged data showing amplitude of the 10th fEPSP at THAL pathway in muscarine (blue) compared to control At the THAL pathway, responses continued to depresses whether muscarine present or not.

C. Amplitude of the 10th fEPSP in muscarine at each pathway as a % of the first in mucarine
D. Representative traces illustrating consistent muscarinic inhibition in THAL and relieved muscarinic inhibition in PL.

different frequency bands. Animals were injected in either the PL or THAL, and slices prepared as in previous experiments. To examine how input from the PL and THAL is transmitted to the BL during different frequencies, 1Hz, 10Hz, 20Hz and 40Hz trains consisting of 10 pulses were applied to the PL or to the THAL input and the resulting fEPSPS in the BL measured (representative gamma frequency traces shown in Figure 5.4D top & bottom). As expected at the lowest frequency (1Hz), each of the ten fEPSPs were consistent in amplitude, meaning that when the PL and THAL are firing at slow rates in the absence of ACh, successive action potentials will evoke transmitter release that reliably is received by the BL (Figure 5.5, A,B). However, when muscarine (10μM) was applied and PL stimulated at 1Hz, each of the pulses was consistently inhibited by roughly 80%, and the last pulse was no different than the first pulse (Figure 5.5, AB). However, as frequency increased from 1Hz to 10Hz, 20Hz and 40Hz trains, expected run-down of the fEPSP amplitude was seen in control. However, at the PL input, when muscarine was present (green), the amount of muscarinic inhibition across the ten fEPSPs was relieved, such that the last pulse of the 10-pulse train resulted in an fEPSP that was larger than the first pulse in 10Hz, 20Hz and 30Hz experiments (Figure 5.5A). Interestingly, muscarinic inhibition of THAL input behaved quite differently at increasing frequencies of stimulation of the THAL input. At low frequencies in control (1Hz), we saw consistent sized fEPSPs across the train. Application of muscarine at 1Hz stimulation of THAL input also resulted in consistently inhibited fEPSPs (Figure 5.4B). However, at higher frequencies, this

muscarinic inhibition (blue) was not relieved, and in fact appeared to still run down similar to control experiments. Representative traces from high-frequency experiments (gamma frequency of 40Hz) displayed in Figure 5.5D (top and bottom). In the top figure we can see the run-down of glutamatergic transmitter release at 40Hz in control (black waveforms) but in the presence of muscarinic inhibition, we see a relief of inhibition across the train (green waveforms). In the bottom figure, we see the THAL pathway also displaying classic run down of transmitter release in control (black waveforms) but no relief of inhibition in the presence of muscarine (blue waveforms).

5.4 DISCUSSION

In these studies we have demonstrated a differential cannabinoid-dependent regulation of glutamate release by mAChRs whereby mAChRs facilitate the release of CBs that then inhibit glutamate from the THAL, but not PL, input to BL. These different mechanisms of muscarinic suppression were reflected in different frequency-dependent inhibition of the PL and THAL input by mACHRs. When the PL and THAL were stimulated at low frequency (1 Hz), inhibition by muscarine at both the PL and THAL inputs remained consistently strong across each pulse of the ten pulse train. However, when the PL and THAL were stimulated at high gamma frequency (40Hz), muscarinic inhibition at the THAL pathway remained strong throughout the ten pulses of the train but muscarinic inhibition at the PL input was relieved. Intriguingly, when we further examined potential postsynaptic mechanisms of inhibition at PL input to the BL, we found that mAChRs have the capability to regulate NMDA currents. Taken together, this suggests

that even though the PL input may be inhibited at low frequencies, the glutamatergic transmission that is relieved at higher frequencies may be enough to facilitation NMDA currents through postsynaptic mAChRs. Overall, these studies suggest distinct mechanisms of muscarinic inhibition that confer different short-term plasticity events at PL and THAL inputs, and potential long-term plasticity at PL inputs.

5.4.1 MACHRS INHIBIT THAL, BUT NOT PL, INPUT THROUGH CB1 RECEPTORS.

Our results show that in the BL, a similar inhibition of glutamate release by endocannabinoids occurs like that shown in the LA (Azad et al., 2003). However, in our experiments, we found that mAChRs mediate this cannabinoid-mediated inhibition of glutamate, a mechanism that was not explored in the LA. Since our study showed muscarinic suppression of PL input is partially dependent on Gi/o-coupled mechanisms and THAL input is completely dependent on muscarinic mediated cannabinoid actions, discovery mechanisms distinct to the PL or THAL that do not involve Gi/o proteins would be beneficial to develop drugs targeting one pathway or the other. Because endocannabinoids, through actions on their CB receptors, can affect both voltage dependent and voltage independent K+ channels as well as inhibit presynaptic voltage-dependent calcium channels, future studies would be needed to determine the mechanisms of this cannabinoid-dependent muscarinic suppression at the THAL pathway (Deadwyler et al., 1995; Mackie et al., 1995; Mcallister et al., 1999; Hoffman & Lupica, 2000; Schweizer 2000).

Additionally, while we examined muscarinic suppression of glutamatergic transmission at each pathway in the BL, it would be interesting to see if muscarinic

modulation of cannabinoid release at THAL inputs also affects GABA transmission in the BL to exert a disinhibition of BL. In the LA, cannabinoids inhibit *both* glutamatergic and GABAergic transmission but their actions affect glutamatergic transmission more to cause an overall decrease of excitation in the LA (Azad et al., 2003). Whether this is occurring in the BL would be most interesting to determine if the BL, specifically after enhanced cholinergic tone and THAL input to BL, is largely inhibited or excited after muscarinic receptors facilitate cannabinoid release. Our data, just focusing on THAL input to pyramidal neurons and endocannabinoid mediated inhibition of glutamate, would suggest that in the event of increased ACh levels, eCB mediated suppression of glutamate would cause an overall decrease in BL excitability following THAL stimulation. Thus, to examine the larger picture, interneurons would need to be recorded from to determine if muscarinic facilitation of eCBs also disinhibits the BL to offset the inhibitory effects of suppressed glutamate.

The presence of muscarinic-mediated cannabinoid signaling at THAL input raises many questions for future investigations. For example, it has been shown in the dorsal cochlear nucleus that when postsynaptic M1 or M3 mAChRs are activated and increase eCB signaling at the same time as NMDA receptors, LTD occurs in an eCB-dependent manner (Zhao & Tzounopoulos 2011). Given our findings that M3 receptors enhance eCB signaling at the THAL pathway, it would be interesting to see if a similar plastic effect occurs in the BL and determine if a long-term physiological consequence, if any, arises from our observed mechanism of muscarinic mediated eCB release.

The finding that there are indeed CB receptors at PL input, but mAChRs don't inhibit them is in and of itself extremely interesting from a therapeutic perspective. Since

M3 receptors are present peripherally and thus drugs that act on M3 receptors are not first choices for emotional disorders given their widespread side effects (ie dry mouth, nausea, constipation, etc.), other targets that function in their place could be further investigated. Since we found application of WIN inhibits PL input to a similar extent as muscarine, this could possibly be an avenue of future investigation.

5.4.2. mAChRs INHIBIT PL GLUTAMATERGIC TRANSMISSION IN A MECHANISM THAT IS PARTIALLY MEDIATED BY A GI/O COUPLED PROTEIN

In efforts to determine the mechanism of muscarinic inhibition of PL input to the BL, an inhibitor of the Gi protein, n-ethylmaleimide (NEM), was used as this agent inhibits pertussis-sensitive G-proteins (Shapiro et al., 1994). Since NEM is an unstable compound, a positive control was chosen to include in experiments to ensure that NEM was actually blocking Gi proteins. We used baclofen, a $GABA_B$ agonist, as the control because of $GABA_B$'s known intracellular mechanisms that involve coupling to a Gi/o protein and regulating adenylyl cyclase activity, GIRK channels and voltage gated calcium channels. Thus, if NEM inhibited the effects of baclofen, it would be functioning appropriately. Experiments in which NEM failed to inhibit baclofen were excluded.

A possible explanation for NEM's inhibition of muscarinic responses could be due to NEM inhibiting the Gi/o protein to which M4 receptors are known to couple. If this mechanism accounts for NEM's suppression of muscarinic inhibition, then most of muscarinic's effects at the PL input would be due to presynaptic M4 receptors that, upon activation by muscarine, the Gi/o$_{\beta\gamma}$ subunit dissociates and can interact with voltage gated calcium channels (Brown 2010).

However, another potential explanation for a partial NEM-effect would be if the M3 mAChRs are located on postsynaptic amygdalar neurons that then release a retrograde messenger that also works through a Gi/o coupled mechanism to inhibit transmitter release. Such retrograde messengers that can bind to receptors that are able to couple to Gi/o proteins include opioids, somatostatin, adenosine, etc. (Heinke et al., 2011; Yudin & Rohacs 2018). It is therefore of interest to examine the potential for mAChRs to act on postsynaptic M3 receptors that may facilitate the release of these retrograde messengers.

5.4.3 PL INPUT MAY INDUCE POTENTIATION AT SYNAPSES IN THE BL DURING GAMMA FREQEUENCY STIMULATION

We also demonstrate, for the first time in the BL, that muscarinic activation can enhance NMDA currents in postsynaptic neurons following PL stimulation. These findings agree with studies finding a similar muscarinic receptor activated enhancement of the NMDA current in other brain regions (Markram & Segal 1990; Segal 1992; Calabresi et al., 1998). In the hippocampus and striatum, M1 receptors mediate this NMDA current enhancement (Calabresi et al., 1998; Dennis et al., 2016). It is therefore reasonable to hypothesize that postsynaptic M1 receptors in the BL would also be responsible for this enhancement of NMDA currents, as recent anatomical studies found they are present in the BL at some putative cortical and thalamic synapses but not all, raising the possibility that they distinctly regulate certain projections but not all to the BL (Muller et al. 2013; McDonald et al., 2019).

This data, taken together with our frequency data, raises the possibility of an interesting modulatory mechanism whereby PL input, inhibited by mAChRs, would be

relieved at gamma frequency. The glutamatergic transmission that does overcome muscarinic inhibition at gamma frequency bind to NMDA receptors, and, if M1 receptors are also present the NMDA current would be enhanced, presumable through a SK channel-dependent mechanism as has been known to occur at Schaffer collaterals in the hippocampus (Buchanen et al.,2016). Experiments to test this hypothesis would involve repeating the frequency experiments in the absence of NMDA antagonists APV, and determining if PL input is facilitated when the PL is firing at 40Hz in the presence of muscarine or released ACh compared to control.

CHAPTER 6

GENERAL DISCUSSION & SIGNIFICANCE

6.1 FINDINGS OF THE STUDY

1. Endogenous ACh, when released in a theta-burst pattern to mimic the endogenous firing rate of basal forebrain cholinergic neurons, inhibits afferent glutamatergic input to the BL. This extent of inhibition was similar regardless of the amount of blue light pulses applied, possibly due to rapid actions of AChEsterase at synapses but also possibly due to ACh rundown. Pharmacologically inhibiting muscarinic, but not nicotinic, ACh receptors reversed this cholinergic inhibition, demonstrating the role of muscarinic receptors in mediating this inhibition of afferent input. Furthermore, this released ACh did not suppress afferent input through GABA or NMDA receptors, as blocking GABA and NMDA receptors caused no change in cholinergic inhibition. Notably, the cholinergic suppression of glutamatergic input was acute and temporary; Maximum inhibition by endogenous ACh occurs between 50and 250 ms after release of ACh, with inhibition diminishing after 500 ms. Exploratory experiments in which acetylcholinesterase inhibitor physostigmine was applied resulted in a drastic increase in cholinergic suppression, possibly due to an increase of extrasynaptic ACh.

2. Activation of mAChRs confers different amounts of inhibition of PL and THAL input to the BL whereby nearly all of PL glutamatergic transmission is suppressed but about half

of THAL input breaks through to the BL. Furthermore, no difference between males and females was seen. This inhibition by muscarinic receptors is ultimately due to mechanisms that retain neurotransmitters in the presynaptic terminal and prevent their release.

3. M3 and M4 mAChRs were responses for muscarinic suppression at PL while M3 mAChRs were responsible for muscarinic suppression at THAL input. However, the reversal of muscarinic inhibition by 4DAMP alone could not determine receptor involvement, so a combination of antagonists were used and their selectivities for the different subtypes taken into account.

4. M3 mediated inhibition was through a GABA-independent mechanism at both PL and THAL input, but dependent on the release of endocannabinoids at THAL, but not PL, input as blocking CB1 receptors eliminated the effect of muscarine.

4. Though not significant due to a smaller sample size, muscarinic receptors facilitated the NMDA current at PL input to the BL. This is likely due to postsynaptic mAChRs on BL neurons. Which receptors would mediate this NMDA current, however, remains unknown.

5. mAChRs display frequency-dependent gating of glutamatergic inhibition. When the PL and THAL are firing slowly, more THAL glutamatergic transmission will occur over PL, and the BL will be tuned to attend to THAL input. However, when the PL and THAL are firing at higher gamma frequencies, the THAL input will remain suppressed whereas

muscarinic inhibition of PL will be removed; in this context, the BL would be tuned to attend to PL input.

Results from this study provide significant contributions to the field of cholinergic modulation of neural regions. Our studies are the first to provide an understanding of how mAChRs regulate glutamatergic transmission from specific regions to the BL. We offer novel findings that mAChRs differentially regulate excitatory transmission from the PL and THAL, key regions in emotional processing and emotional memory. The pharmacological data showing M3 receptors inhibit PL and THAL input through eCB-independent and dependent mechanisms, respectively, and the presence of M4 receptors at PL input could be investigated as possible pharmaceutical targets to pair with behavioral therapies to treat emotional disorders involving the amygdala.

Emotional disorders that involve the amygdala impact millions of Americans a year, oftentimes drastically reducing the quality of life (Kessler et al., 2005). Unfortunately, the current drug treatments for emotional disorders often have unpleasant side effects that reduce compliance. There therefore is a need to identify targets and behavioral interventions for more effective treatments for emotional disorders including PTSD, anxiety, depression, etc. Our studies utilizing optogenetics to release endogenous ACh demonstrate the ability for endogenous ACh to regulate amygdalar function. These findings could serve as motivation for future behavioral and clinical studies to investigate treatment interventions that would increase ACh levels in the brain. Together with the pharmacological data, behaviors could enhance cholinergic tone in the BL, and pharmacological treatments could manipulate the receptors to which that ACh could bind

to achieve a desired clinical outcome that may treat or alleviate symptoms of emotional disorders.

6.2 CHOLINERGIC INHIBITION OF PL AND THAL INPUTS: IMPLICATION FOR BEHAVIOR.

Why projections from the PL would be nearly completely inhibited but the projections from the THAL would be partially spared is an intriguing behavioral question. In attempting to assign functional relevance to the inhibition of PL input, it is necessary to ask what neurons PL input contacts in the BL. It is known that PL inputs contact both inhibitory interneurons and excitatory neurons in the BL, but that the excitation of BL seems to predominate (Brinley-Reed et al., 1995; Rosenkranz & Grace, 2001; Lihtik et al., 2005; Hubner et al., 2014). Taking this into account, cholinergic inhibition of PL input to the BL would then cause an overall inhibitory effect in the BL. This is intriguing because there are interconnected loops between the PL and BL in which PL neurons activate BL neurons that feedback to inhibit the PL (McGarry & Carter, 2016). In other words, the BL sends projections to the PL that shut down PL activity; excitation by the PL, then, to the BL, would be a "go trigger" that induces excitation of BL neurons that feedback to inhibit the PL. In other words, if the PL induces excitatory fEPSPs in the BL, it likely that it is inhibiting itself via feedback inhibition coming from the BL. If we take this feedback inhibitory model into a situation in which ACh is elevated in the amygdala, we now have PL activity largely suppressed. Thus, ACh prevents the PL from exciting the BL, which in turn prevents the PL from being inhibited by the BL due to feedback inhibition.

Therefore, during enhanced cholinergic tone in the BL, the BL is suppressed, and the PL is active.

This theoretical model of cholinergic regulation of PL-BL coupling is intriguing given imaging studies in human showing lower activity in the prefrontal cortex when viewing aversive images in patients with PTSD (Shin et al., 2004). To take our findings into this translational realm, one possible explanation for what is seen in humans with PTSD could be a downregulation or disruption in M3 receptors in the BL that fail to appropriately inhibit PL input to the BL, and in turn, results in increased amygdalar activity and increased feedback inhibition of the PL. Indeed, if we view PTSD symptoms as manifestations of a disruption in shifts between arousal states, keeping in mind the role of ACh in inhibiting slow oscillations in the cortex to regulate arousal states, then a potential underlying cause for dysregulated fear behaviors could be a disruption ACh's ability to inhibit PL input to the BL that then dysregulates feedback inhibition to the PL.

Indeed, behavioral data showing synchrony between PFC and BL predicts freezing ("fear") behavior would support this hypothesis (Karalis et al., 2014). Our data suggests that afferent input to the BL would be temporarily suppressed (for only 500ms-1s based on our data from Chapter 3) but not intrinsic recurrent activity, while the THAL input would be partially spared. It may be that for this brief, 1s interval, in which PL input would be cholinergically inhibited, incoming information will be processed in the BL intrinsically as the animal receives sensory input about its environment and information about unexpected stimuli and error prediction from the THAL. Then, after the transient period of suppression, this inhibition will be relieved so that the BL can, if appropriate given the environment, resume inhibition of the PL and display an appropriate fear behavior.

However, the temporal synchrony of PL and basal forebrain firing should also be considered when determining consequences of cholinergic tone in the BL. Given our data showing released ACh inhibits PL input to the BL and literature demonstrating the basal forebrain to fire at theta-frequency (Lee et al., 2015), it is interesting to speculate on the consequences of PL and basal forebrain firing patterns that would synchronously arrive in the BL. If the PL is also firing at theta frequency *and* doing so in synch with the basal forebrain, then it could be possible that PL input to the BL would be completely suppressed for the entirety of the synchronous oscillatory events of both regions. Every time the PL input arrives at the BL, it would theoretically be inhibited by ACh released at the same time. Thus, a behavioral state in which there is synchronous oscillation between the two regions would ensure inhibition of the PL.

6.3 CHOLINERGIC INHIBITION OF THE BASOLATERAL AMYGDALA: IMPLICATIONS FOR EXERCISE AS A POTENTIAL TREATMENT FOR EMOTIONAL DISORDERS

A hyperexcited amygdala is often a clinical observance in individuals with anxiety and PTSD (Ressler 2010). Unfortunately, many of the medications prescribed for anxiety, PTSD, depression and other disorders involving the amygdala tend to have unfavorable side effects that can result in noncompliance with treatment. Fortunately, exercise and physical activity offers many health benefits, mental health benefits. In a recent study using data from the World Health Survey collected across 47 countries and adjusted by sociodemographics, depression and geographic location, individuals who were more active had lower odds for anxiety than those who were more sedentary (Stubbs et al., 2017).

Interestingly, both acute and chronic exercise influences the cholinergic system in many ways, both in manners that would elevate cholinergic functioning. Acute bouts of exercise, including moderate walking and moderately intense running both increase acetylcholine levels in the brain (Uchida et al., 2006; Dudar et al., 1979). Cholinergic tone is also increased following exercise as a result of reduced activity of acetylcholinesterase, which would keep ACh in the synapses longer; in essence it would mimic our experiments using physostigmine in Chapter 5 (Sunanda et al., 2000). Chronic exercise training not only increased cholinergic neurons in a diseased group that experiences a loss of cholinergic neurons, but is also increased ACh levels in the brain when both health controls and diseased groups performed an attentionally-demanding task (Hall and Savage 2016). In other words, the groups that exercised saw increased ACh, the neurotransmitter important for attention and learning, during the task in which they most needed it.

Given the clinical observations of increased amygdala activity in anxiety, these studies showing enhanced cholinergic tone in the brain with exercise, and our study showing enhanced ACh inhibits the BL, it is then easily comprehended how exercise may be an efficacious intervention for anxiety and emotional disorders. Another disorder for which exercise may alleviate symptoms via amygdalar modulation is in Alzheimer's disease. One of the symptoms often seen in Alzheimer's disease is emotional dysregulation. The cholinergic hypothesis of Alzheimer's disease would suggest that these emotional symptoms are due to a decline in cholinergic functioning. Indeed, exercise has been shown to reduce depression and improve mood in a randomized control trial (Williams & Tappen 2008) and improve cognition in the elderly. However, future studies

are needed to specifically look at the relationship between exercise and emotional regulation in this population.

Taken together, our data showing the modulatory mechanisms of cholinergic inhibition of the BL provides novel insight into how amygdalar function is regulation by ACh. Combining these findings with translational studies to examine pharmacological manipulation of these receptors in various behaviors states and disorders (anxiety, PTSD, Alzheimer's disease, etc.) would hold tremendous potential to discover therapies improving emotional health.

6.4 FUTURE DIRECTIONS

1. Experiments in this first aim of this study found and examined muscarinic regulation of afferent input to the BL. Our electric stimulation and optogenetic protocol may have eliminated the ability to detect any nicotinic regulation of afferent input to the BL because of the quickly desensitizing nature of this receptor subtype. Thus it would be necessary to pursue experiments with potentially less light released ACh and/or less inter stimulus interval between ACh and recordings in order to detect nicotinic modulatory actions at these inputs. Such studies would contribute to a thorough understanding of cholinergic modulation of the BL by both ionotropic and metabotropic receptor types.

2. There are gender differences in human prevalence of emotional disorders, but we did not encounter any differences in muscarinic regulation in our experiments. However, we combined both males and females after no difference in muscarinic inhibition was found.

It may be there while muscarinic inhibition may be the same between genders, there could be different mechanisms underlying this inhibition.

3. The findings that physostigmine may confer an enhancement of cholinergic functioning is most interesting. Pursuing studies to look at how inhibiting ACHE, by increasing AChE in the synapse, could increase the inhibitory effect of ACh could be used in combination with drugs targeting specific muscarinic subtypes to maximize pharmacological effects. This would be especially important if there are differences in M4 receptors given the existence of PAMs for this subtype that could be administered together with physostigmine to potentiate muscarinic receptor functioning at specific synapses.

4. NMDA experiments were, with the exception of one exploratory experiment, carried out at PL input to the BL. Because the PL conveys glutamatergic transmission important for fear acquisition and expression and the THAL conveys glutamatergic transmission that conveys information thought to represent unexpectedness and information pertinent to an individual's state, the ability for muscarinic receptors to enhance plasticity at one of the inputs over the other would be intriguing to know. Especially given the difference in *presynaptic* muscarinic inhibition, it would be striking if THAL inputs also potentiated with muscarinic activation. In this situation, ACh would be inhibiting PL input the most, while allowed about 50% of THAL to come through. If both PL and THAL were slowly firing, it wouldn't matter if the PL input also had the ability to potentiate NMDA receptors because nearly all the glutamatergic transmission would be suppressed anyway. However,

at the THAL input 50% transmission may be enough to facilitation NDMA current and engage mechanisms of plasticity.

5. Another interesting avenue would be to piece apart the difference in frequency gating of these receptors. I would hypothesize that this difference in short term plasticity at 40Hz between PL and THAL is due to the different mechanisms: endocannabinoids being released and retrogradely traveling to inhibit neurotransmitter release versus possible direct inhibition of voltage gated calcium channels by mAChR G protein subunits at PL input.

6. Lastly, an exciting area to explore is taking this circuitry data into a behavioral realm and determining if individuals may have differences in their cholinergic systems. If so, it would be important for future human clinical trials to determine what impacts those individual differences may have for behavior. One area of which this study leads itself very well is to exercise a set of animals and determine if there are individual differences in increased ACh in response to exercise. If so, could this difference affect the ability for acquire or extinguish fear memories? If there is a difference, is the mechanistic difference at the levels of ACh release in response to exercise or as differences in long-term adaptation to exercise, or is the difference at the level of receptors? It may be possible that individuals will display different cholinergic receptors at baseline, and exercise may differentially regulate receptor expression, etc. Similarly, there may be baseline differences that are innate to individuals and will not be changed by exercise. These differences are also important to explore for drug development and the creation of future translational studies.

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