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MUSCARINIC MODULATION OF BASOLATERAL AMYGDALA

Lei Liu
University of South Carolina - Columbia

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by

Lei Liu

Bachelor of Medicine
Jiangsu University,2004

Master of Science
Shanxi Medical University, 2007

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Accepted by:

David Mott, Co-Major Professor

Alexander McDonald, Co-Major Professor

Marlene Wilson, Chairman, Examining Committee

Richard Goodwin, Committee Member

Roger Newman-Norlund, Committee Member

Lacy Ford, Vice Provost and Dean of Graduate Studies

DEDICATION

I would like to dedicate this manuscript to my wife, Jennifer, my parents, Zhuan Liu and Faling Wang, my parents-in-law, Dennis and Anita Faglier, and our dogs and cats. Without their love, caring, patience, understanding, support, encouragement I would not be where I am today.

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ABSTRACT

The basolateral amygdala (BL) receives a dense cholinergic innervation from the basal forebrain. Despite the importance of muscarinic acetylcholine receptors (mAChRs) in fear learning, consolidation, and extinction, there have been no studies that have systematically investigated the functional role of mAChRs in regulating emotional processing in the BL. To address this critical knowledge gap we combined brain slice whole-cell recording, optogenetics, and immunohistochemistry to determine how muscarine, acting on mAChRs, regulates neuronal oscillations, synaptic transmission and plasticity in the BL.

Neurons in the BL oscillate rhythmically during emotional processing, which are thought to be important to integrate sensory inputs, allow binding of information from different brain areas and facilitate synaptic plasticity in target downstream structures. We found that muscarine induced theta frequency rhythmic inhibitory postsynaptic potentials (IPSPs) in BL pyramidal neuron (PN). These IPSPs synchronized PN firing at theta frequencies. Recordings from neurochemically-identified interneurons revealed that muscarine selectively depolarized parvalbumin (PV)-containing, fast firing, but not PV, regular firing or somatostatin (SOM)-containing interneurons. This depolarization was mediated by M3 mAChRs. Dual cell recordings from connected interneuron-PN pair

indicated that action potentials in fast firing, but not regular firing interneurons were strongly correlated with large IPSCs in BL PNs. Furthermore, selective blockade of M3, but not M1 mAChRs suppressed the rhythmic IPSCs in BL PNs. These findings suggest that muscarine induces rhythmic IPSCs in PNs by selectively depolarizing PV, fast firing interneurons through M3 mAChRs. Furthermore, we found that rhythmic IPSCs were highly synchronized between PNs throughout the BL.

The BL receives extensive glutamatergic inputs from multiple brain regions and recurrent collaterals as well. They are important for fear learning and extinction, which are tightly regulated by local GABAergic inhibition. We found that mAChRs activation suppressed external glutamatergic inputs in a frequency dependent and pathway specific manner but kept recurrent glutamatergic transmission intact. In addition, muscarine disinhibited BL PNs by attenuating feedforward and GABAergic inhibition. In agreement with these observations, long term potentiation (LTP) induction was facilitated in the BL by mAChRs activation.

Taken together, we provided mechanisms for cholinergic induction of theta oscillations and facilitation of LTP in the BL.

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±	Plus/minus
α	Alpha
β	Beta
Ω	Ohm
μ	Micro
A	Amp
Hz	Hertz
M	Molar
G_q	Excitatory G protein coupled receptor
G_i	Inhibitory G protein coupled receptor

List of Abbreviations

ACh ..... Acetylcholine

BF ..... Burst Firing

BL ..... Basolateral

CB ..... Calbindin

CCK ..... Cholecystokinin

CEA ..... Central Medial

CR ..... Calretinin

CS ..... Conditioned Stimulus

FF ..... Fast Firing

IL ..... Infralimbic

ITC ..... Intercalated Nucleus

PTSD ..... Post-traumatic Stress Disorder

mAChR ..... Muscarinic Acetylcholine Receptor

US ..... Unconditioned Stimulus

VTA ..... Ventral Tegmental Area

LA ..... Lateral Nucleus

LTP ..... Long Term Potentiation

LTD ..... Long Term Depression

mPFC ..... Medial Prefrontal Cortex

NPY ..... Neuropeptide Y

PL ..... Prelimbic

PV ..... Parvalbumin

RF..... Regular Firing

SF ..... Stutter Firing

SOM.....Somatostatin

VIP .....Vasoactive Intestinal Peptid

CHAPTER 1

GENERAL INTRODUCTION

1.1 SIGNIFICANCE

Anxiety disorders, including posttraumatic stress disorder (PTSD), are the most prevalent mental disorders in the US, which affect affecting 40 million American adults each year. Amygdala is a brain region thought to be the center of emotion (LeDoux, 2000). Dysfunction of amygdala causes emotional disturbances. Basolateral nucleus of amygdala (BL) receives the densest cholinergic innervations from the basal forebrain (Svendsen and Bird, 1985, Hellendall et al., 1986, Emre et al., 1993, Azouz and Gray, 2000), indicating cholinergic signaling plays a important role in regulating BL functions. In line with this idea clinical evidence found that Alzheimer's patients are more likely to have PTSD (Childress et al., 2013). Moreover one of the most common commobilities of Alzheimer's disease is emotional disturbances such as anxiety (Mori et al., 1999, Gauthier et al., 2002). One of the hallmarks of Alzheimer's is disrupted cholinergic system (Mesulam, 2013a). mAChRs-mediated mechanisms in the BL are primary mediators of the neuromodulation involved in memory consolidation of emotionally arousing experiences by the amygdala (McGaugh, 2004), and have also been implicated in the consolidation and extinction of contextual fear conditioning memory (Vazdarjanova and McGaugh, 1999, Boccia et al., 2009). These findings clearly suggest that therapeutic

modulation of mAChRs-mediated mechanisms in the BL nucleus could be important for treating a number of major neuropsychiatric diseases involving impairments in emotional learning, including anxiety disorders, drug addiction, and Alzheimer's disease (Salinas et al., 1997, McIntyre et al., 2002). For example, there is a 40% reduction in the cholinergic innervation of the BL nucleus in Alzheimer's disease (Emre et al., 1993), and impairments of emotional event memory in Alzheimer's patients are correlated with the extent of amygdalar involvement (Mori et al., 1999). In fact, AChE inhibitors (AChEIs), such as donepezil, the current gold standards for symptomatic treatment of Alzheimer's disease, is effective for improving mood and reducing anxiety in AD patients (Gauthier et al., 2002). However, there has been no study examined its effects in the amygdala or physiological mechanisms by which it may affect anxiety. Despite the remarkably dense cholinergic innervation of BL, and its critical importance for learning and memory, surprisingly little is known about cholinergic modulation of BL circuits. Most previous studies have investigated cholinergic modulation of potassium channels in pyramidal projection neurons (Washburn and Moises, 1992a, b, Womble and Moises, 1992, 1993, Yajeya et al., 1997, Yajeya et al., 1999, Power and Sah, 2008)

It is the first step towards developing a comprehensive understanding of the muscarinic cholinergic modulation of BL circuits, which should lead to the development of novel pharmacological treatments for diseases involving the amygdala, including anxiety disorders, drug addiction, and Alzheimer's disease.

1.2 AMYGDALA FUNCTION

The amygdala is brain structure which is located deep in the temporal lobe. The amygdala function was first revealed from lesion studies in both animals and humans. Monkeys with amygdala lesions exhibited a loss of fear and anger as well as other phenotypes including increased exploration, hypersexualtity, hyperrorality, and etc. (Weiskrantz, 1956, Zola-Morgan et al., 1991). The phenotype produced by amygdala lesions has also been seen in many other animal species (Goddard, 1964). Clinical cases showed that patients had focal bilateral amygdala lesions due to Urbach-Wiethe disease had emotional deficits, especially not being able to exhibit fear-related behaviors, which was called Kluver-Bucy syndrome (Feinstein et al., 2011). With the development of optogenetics in the last decade, which provides temporal, spatial, and genetic precise manipulation of neuronal activities, investigators have further demonstrated the role of the amygdala in emotion and emotion-related mental disorders. For example, optogenetic activation and inhibition of central amygdala increased and reduced, respectively, anxiety in mice (Tye et al., 2011). All of these studies pinpoint that the amygdala is an essential brain area that is responsible for generation of emotion and producing adaptable behaviors to salient external cues or stimuli.

The progress of our understanding of the amygdala function was hampered at the early stage of the field due to the abstract complexity of emotion itself. The difficulty of measuring emotion made it extremely uneasy to study the amygdala function. Pavlovian fear conditioning is a simple task in which a neutral conditioned stimulus (CS), such as a

tone, is paired with an unconditioned stimulus (US), such as a foot shock. By repeating such pairing for several times, animals can learn to associate the CS to the US. Thus CS is able to produce the fearful behaviors. The fearful behaviors including freezing and startle response which can be directly measured (LeDoux, 2000). Later when CS is repeatedly presented alone animals learn to dissociate CS from US. CS no longer produces fear, a phenomenon called fear extinction. Fear extinction is not simply erasure of previously learned fear memory trace but rather a new learning process (Rescorla, 2001, Bouton et al., 2006, Myers and Davis, 2007). Fear extinction is context dependent, meaning that fear memory emerges once the CS is presented in a different context rather than the one where fear extinction was performed (Maren et al., 2013). Fear extinction memory is very labile in that fear memory can spontaneously recover over time and can show reinstatement when exposed to aversive stimuli (Myers and Davis, 2007). These simple behavioral tasks are clinically relevant. Fear conditioning is a good model for studies of the genesis of anxiety disorders (Davis, 1992, Rosen and Schulkin, 1998, Davis and Whalen, 2001). PTSD is considered as a deficit of fear extinction. Studies of fear conditioning and extinction clearly implicated the central role of the amygdala and its afferent and efferent projections in fear processing (Adolphs et al., 1998, LeDoux, 2000).

In addition to generating emotional behaviors, the amygdala is important to modulate emotional memory. When memory traces are tagged with salient stimuli and contexts, they are always remembered faster and persist much longer than neutral ones. In the perspective of evolution, emotional memories are the most valuable ones for animals to remember for the sake of survival. The amygdala is well positioned to play a pivotal role in the emotional memory modulation. The amygdala makes reciprocal

connections with many cortical and subcortical brain regions, acting as a hub for the communications between brain structures (Sah et al., 2003). For example, the amygdala is reciprocally connected with neuromodulatory systems, such as basal forebrain cholinergic system (Carlsen et al., 1985, Zaborszky et al., 1997, Zaborszky et al., 1999) and ventral tegmental area (VTA) dopaminergic system (Lee et al., 2011) both of which are important in memory modulation (Rosenkranz and Grace, 2002, Bissiere et al., 2003, Power et al., 2003a, Power et al., 2003b, Boccia et al., 2009). In lines with this, many studies have showed the major contribution of the amygdala to this process. fMRI studies showed that the amygdala was lit up during emotional encoding (Cahill et al., 1996, Nili et al., 2010). Pharmacological manipulations showed that the amygdala activities during and short after emotional learning were necessary for the facilitation of emotional memory (Packard et al., 1994). The amygdala plays a role not only in fear-related behaviors but also in reward-related behaviors (Everitt et al., 1999, Di Ciano and Everitt, 2004). Projections from the amygdala to nucleus accumbens mediate reward reinforcement behaviors (Stuber et al., 2011). This suggests that the amygdala is also involved in drug addiction. It has been further found that projections from the amygdala to prelimbic PFC and the NA are part of the critical drug-seeking circuits (McFarland and Kalivas, 2001, McFarland et al., 2004, LaLumiere and Kalivas, 2008). Furthermore it has been proposed that the amygdala encodes the representation of state value (Morrison and Salzman, 2010).

Taken together, the amygdala function can be described as responding external salient stimuli, generating appropriate behavioral responses, and formation of long lasting emotional memory.

1.3 AMYGDALA ANATOMY

The amygdala comprises multiple nuclei based on the cell types and afferent and efferent connections they make. Amygdala nuclei can be divided into four groups. These are cortex-like group, which includes lateral (LA), basolateral (BL), and basomedial nucleus; superficial cortex-like group: which is composed of anterior cortical nucleus, bed nucleus of the accessory olfactory tract, nucleus of the lateral olfactory tract, periamygdaloid cortex, and posterior cortical nucleus; striatum-like central medial group, which includes medial and central nuclei; and intercalated cell nucleus (McDonald, 1998, Sah et al., 2003). In many literatures LA and BL are often combined and treated as one nucleus named as BLA.

1.3.1 Afferent and Efferent Connections to the amygdala

Each amygdala nucleus has different inputs from multiple brain regions (McDonald, 1998). BLA is the major nucleus to receive external inputs (McDonald, 1998, Sah et al., 2003, Pape and Pare, 2010). The afferent inputs to the BLA can be grouped into the ones from cortical and thalamic brain areas and the ones from the neuromodulatory systems. Cortical and thalamic inputs carry different modalities of sensory information to the amygdala, including visual, auditory, olfactory, somatosensory, gustatory and visceral modality (Mcdonald and Mascagni, 1996, Mcdonald et al., 1996, McDonald, 1998, Sah et al., 2003). These projections are glutamatergic and are from layer V pyramidal cells of specific sensory cortex (Ottersen et al., 1986, Amaral and Insausti, 1992). The cortical and thalamic inputs form axonal bundles and enter into the BLA via external capsule and internal capsule, respectively

(McDonald, 1998, Sah et al., 2003). These glutamatergic inputs are thought to carry CS information which is critical in fear-related associative learning such as fear conditioning (LeDoux, 2000, Maren and Quirk, 2004). BLA also receives polymodal information from prefrontal cortex and hippocampus (Maren and Fanselow, 1995, McDonald and Mascagni, 1997, Marek et al., 2013). PFC projections mainly target the BL nucleus (Mcdonald et al., 1996), which is thought to be important in fear extinction (Marek et al., 2013) (Milad and Quirk, 2002, Herry et al., 2010). Hippocampus projections to the BL nucleus carry context information which is important for contextual fear conditioning (Goosens and Maren, 2001, Maren et al., 2013).

Central medial nucleus is the major output station of the amygdala (Sah et al., 2003). BLA relays the sensory information to the central medial nucleus. It then projects to hypothalamus and brain stem responsible for generation autonomic changes and appropriate behaviors (Sah et al., 2003). Originally the central medial nucleus is considered as a passive relay station of BLA activity to the downstream fear circuits. However, evidence from recent studies suggests that plasticity and modulations also happen at CEA (Ciocchi et al., 2010, Haubensak et al., 2010, Li et al., 2013, Penzo et al., 2014). One study used optogenetics to activate BLA pyramidal cells the same time when a mouse was given a tone (CS) so that long term potentiation (LTP) of that particular CS pathway was established (Johansen et al., 2010). Thereafter the animals were able to show freezing when the same tone was played alone. However, the fear effect which was measured by freezing time was much lower than the one seen in the mice traditionally fear conditioned (Johansen et al., 2010). These results suggest that although LTP at

synapses in BLA is important for fear conditioning plasticity occurred elsewhere, possibly including CEA, is also involved in fear learning.

BLA projects not only to CEA but also to brain structures outside of the amygdala, including mPFC, hippocampus, striatum, and nucleus accumbens. (Tye and Janak, 2007, Herry et al., 2008, Popescu et al., 2009, Stuber et al., 2011, Felix-Ortiz et al., 2013, Jennings et al., 2013, Felix-Ortiz and Tye, 2014, Senn et al., 2014, Stamatakis et al., 2014). This indicates that BLA and CEA can act as both parallel and serial circuits. The projections to the striatum are involved in habit learning circuitry (Popescu et al., 2009, Stamatakis et al., 2014). BLA projections to nucleus accumbens are important in reward-related behaviors such as drug addiction (Tye and Janak, 2007, Stuber et al., 2011, Jennings et al., 2013). As mentioned earlier BLA receives inputs from mPFC and the hippocampus. It also projects back to these two limbic structures (Felix-Ortiz et al., 2013, Felix-Ortiz and Tye, 2014, Senn et al., 2014). The exact roles of these reciprocal connected circuits are not clear. Some studies showed that BLA projection to the hippocampus plays a role in generation of anxiety (Felix-Ortiz et al., 2013, Felix-Ortiz and Tye, 2014). Other studies have suggested that there are two populations of pyramidal cells differentially involved in fear learning and fear extinction (Herry et al., 2008, Tye et al., 2011, Senn et al., 2014). One population of BLA pyramidal cells called fear neurons receive inputs from hippocampus, whereas the other population of BLA pyramidal cells called extinction neurons receive inputs from mPFC (Herry et al., 2008). Furthermore fear neurons project to prelimbic (PL) subdivision of mPFC while extinction neurons send axons to infralimbic (IL) subdivision (Senn et al., 2014). Fear neurons are activated during fear learning while extinction neurons are recruited during fear extinction (Herry

et al., 2008, Senn et al., 2014). Selective manipulations of these PL and IL projection pathways specifically impaired fear learning and fear extinction, respectively (Senn et al., 2014). These results suggest that specific behaviors may be encoded in particular neuronal circuits between remote brain structures. The amygdala is well suited to be the central hub for the emotional circuits.

Although the afferent and efferent projections mentioned above are glutamatergic, some other projections do use other neurotransmitter or neuromodulators to communicate. For example, BLA receives cholinergic, dopaminergic, serotonergic, noradrenergic, and etc. modulations from the neuromodulatory systems (Brinley-Reed and McDonald, 1999, Muller et al., 2007b, Pinard et al., 2008, Muller et al., 2009, 2011, Zhang et al., 2013). One study showed subpopulations of somatostatin (SOM) interneurons in BLA send GABAergic projections to the basal forebrain (McDonald et al., 2012), possibly providing feedback inhibition back to cholinergic neurons in the basal forebrain. The amygdala can be modulated brain state dependently by different neuromodulators. Meanwhile the amygdala is able to affect the neuromodulatory systems to broadly regulate brain functions in many brain areas such as consolidation of long term emotional memory remotely stored in multiple brain structures. These afferent and efferent connections indicate that there is extensive local computation of information coming into the amygdala before it sends out to generate behavioral outcomes.

How the amygdala integrates and computes incoming information is a critical question to pursue. Accumulated evidence points to many emotional mental disorders such as anxiety disorders, PTSD, etc. are caused by abnormal or inappropriate information computation by the amygdala such that the abnormal brain is not able to

flexibly lead to appropriate behaviors when being in constantly changing environmental situations (Martin et al., 2009, Mahan and Ressler, 2012, Parsons and Ressler, 2013, Duvarci and Pare, 2014). For example, in PTSD patients, the amygdala is no longer able to process safe environmental signals and inhibit fear responses (Mahan and Ressler, 2012, Parsons and Ressler, 2013). The comprehensive knowledge of mechanisms of which the amygdala computes is still lacking. However, researchers have started to shed light on this big question. Glutamatergic and GABAergic transmissions which are the basic elements in the amygdala neuronal circuits have been dissected by many studies. They play a critical role in fear conditioning and extinction.

1.4 GLUTAMATERGIC AND GABAERGIC TRANSMISSION IN FEAR CONDITIONING AND EXTINCTION

1.4.1 Fear conditioning

Pavolovian fear conditioning and fear extinction have been one of the most powerful behavioral models for studying associative learning and fear memory formation and storage in the amygdala (Fanselow and LeDoux, 1999, LeDoux, 2000, Fanselow and Poulos, 2005). Excitatory glutamatergic transmission has been the major focus for study of fear conditioning and extinction. Synaptic plasticity, LTP, of the sensory glutamatergic inputs from thalamus and cortex (CS) to the BLA has been thought to be the mechanism of fear conditioning (Chapman et al., 1990, Miserendino et al., 1990, Sigurdsson et al., 2007, Sah et al., 2008, Mahan and Ressler, 2012, Nabavi et al., 2014). CS and US fibers converge onto BLA. US inputs depolarize BLA neurons while CS-BLA pathway is activated in the same time window. Synapses at that particular CS-BLA pathway are

potentiated. Therefore, when the same CS is presented alone, it will be able to increase firing of those BLA projection neurons. This N-methyl-D-aspartate receptor (NMDAR) dependent LTP is similar as seen in other brain regions such as the hippocampus (Sah et al., 2008, Johansen et al., 2011). NMDARs at the dendritic spines of BLA projection neurons (PN) are blocked by Mg^{2+} at resting membrane potential. US depolarizing BLA projection neurons removes Mg^{2+} blockade of NMDARs. At the same time glutamate released from the CS glutamatergic terminals binds to NMDARs at the same synapses to open the channel. Opening of Ca^{2+} permeable NMDARs allows Ca^{2+} entry to the compartmented spines which further actives Ca2+/calmodulin-dependent protein kinase II (CaMKII). Activated CaMKII phosphorylates AMPARs to increase their conductance and increases insertion of more AMPARs to the membrane surface at the same spines (Johansen et al., 2011). In this way synaptic potentiation is pathway specific. In other words, only the CS inputs paired with US not others are potentiated after fear conditioning training. Blockade and occlusion experiments support the idea that NMDARs dependent LTP at sensory inputs to the BLA projection neurons underlies acquisition and storage of associative fear learning (LeDoux, 2000, Maren and Quirk, 2004, Johansen et al., 2011).

While excitatory glutamatergic circuits have been extensively studied in fear conditioning as has been shown above, little is known about the involvement and modification of GABAergic inhibitory circuits in fear learning and memory. Evidence from recent studies indicates that modification of local inhibitory circuits in the amygdala play an important role in fear conditioning (Bissiere et al., 2003, Ehrlich et al., 2009, Trouche et al., 2013, Courtin et al., 2014). Pharmacological increase of GABA release in

the amygdala impairs fear memory acquisition and expression (Davis, 1979, Sanger and Joly, 1985, Harris and Westbrook, 1995, 1999, 2001). In contrast, decrease of GABA release in the amygdala facilitates fear learning and fear memory retrieval (Guarraci et al., 1999, Tang et al., 2007). In line with this, in vitro electrophysiology studies showed that blockade of GABAa and GABAb receptors in the BLA facilitates LTP inductions at thalamic pathways (Bissiere et al., 2003, Shaban et al., 2006, Shin et al., 2006, Tully et al., 2007, Pan et al., 2009). GABAergic feedforward inhibition tightly controls BLA pyramidal cells activity (Li et al., 1996, Lang and Pare, 1997, Szinyei et al., 2000, Szinyei et al., 2007). This feedforward inhibition gating LTP induction can be modulated by multiple neuromodulators. For example, dopamine, noradrenaline, and opioids suppress feedforward inhibition, and thus facilitate LTP induction (Bissiere et al., 2003, Loretan et al., 2004, Shaban et al., 2006, Tully et al., 2007). Whereas others, including serotonin, increase feedforward inhibition, thereby suppress LTP induction (Stutzmann and LeDoux, 1999). Mechanisms of modulation of GABAergic inhibition include presynaptic regulation of GABA release and affecting local interneuron excitability by direct depolarizing or hyperpolarizing interneuron or changing presynaptic glutamate release to interneurons (Bissiere et al., 2003, Loretan et al., 2004, Kroner et al., 2005). In vivo electrophysiology and behavioral studies further showed the requirement of neuromodulatory inputs to amygdala for fear conditioning (Rosenkranz and Grace, 2002, Kroner et al., 2005). This inhibition gating LTP induction indicates the flexibility of the brain. The amygdala is instructed by neuromodulatory system to decide when is the right time for learning to happen.

1.4.2 Fear extinction

Fear extinction is a process of formation of inhibitory memory suppressing fear memory retrieval (Milad and Quirk, 2002, Myers and Davis, 2007, Herry et al., 2010). Fear memory is encoded by fear neurons in the BLA, whereas extinction memory recruits BLA extinction neurons (Han et al., 2007, Herry et al., 2008, Han et al., 2009, Josselyn, 2010, Senn et al., 2014). During high fear state fear neurons are active but extinction neurons are inhibited (Herry et al., 2008, Senn et al., 2014). In contrast, during low fear state it is the opposite. The exact roles of fear and extinction neurons remain elusive. It seems that fear neurons and extinction neurons inhibit each other. This inhibition is possibly through local interneurons. It is likely that during fear conditioning LTP is inducted at CS-fear neurons pathway synapses while during fear extinction LTP happens at CS-extinction neurons pathway synapses. In this way, after fear conditioning fear neurons are more likely excited by CS inputs, thereby lead to fear-related outputs. In contrast, after fear extinction, CS activation of a group of extinction neurons drives some local interneurons which project to fear neurons, thereby provide inhibition to them. It is likely that fear memory and extinction memory traces coexist within the BLA but compete with each other for retrieval. Under different circumstances it may favor expression of a particular memory trace and suppress the other. One can imagine that anything affects the balance of the competition between fear and extinction memory traces would cause emotional disturbances. In agreement with this idea studies showed that blockade of synaptic plasticity in the BLA interfered fear extinction (Falls et al., 1992, Lu et al., 2001, Lin et al., 2003, Herry et al., 2006, Sotres-Bayon et al., 2007, Sotres-Bayon et al., 2009). Increase of GABAergic transmission also impairs fear

extinction learning(Hart et al., 2009). These findings indicate that fear extinction acquisition also requires NMDARs dependent synaptic plasticity at BLA pyramidal cells or interneurons. Inhibitory circuits in the BLA are also under modification by fear extinction training. For example, decrease of GABAergic transmission in the BLA impairs fear extinction memory retrieval (Harris and Westbrook, 1998b, a). A recent study found that the number of axonal terminals from a subset of PV interneurons to cell bodies of fear neurons but not extinction neurons significantly increased after fear extinction training (Trouche et al., 2013). This indicates that after fear extinction fear neurons recruited in the fear memory would receive more inhibition compared to other neurons. The mechanism of this modification is not known. In fact, inhibitory interneurons are the major targets of multiple neuromodulatory systems (Asan, 1998, Cassell et al., 1999, Guarraci et al., 1999, Fuxe et al., 2003, Muller et al., 2007b, Pinard et al., 2008, Muller et al., 2011). Different neuromodulators which are released under different behavioral states may be able to regulate fear learning and extinction through local interneurons.

1.4.3 Intercalated nucleus

Another group of inhibitory neurons in the intercalated nucleus (ITC) have also been shown to play a role in fear extinction (Likhtik et al., 2008). These inhibitory neurons act as an interface between the BLA and the CEA. Glutamatergic inputs from BLA pyramidal cells and mPFC can activate ITC neurons which lead to inhibition of its projection targets in the CEA and thus suppress fear responses (Pare and Smith, 1998, Likhtik et al., 2008). mITCs receive projections from the IL area of the mPFC (Mcdonald et al., 1996, Vertes, 2004, 2006). The activity of mPFC is increased after fear extinction

and is required for the expression of extinction (Milad and Quirk, 2002, Anglada-Figueroa and Quirk, 2005, Burgos-Robles et al., 2007, Sotres-Bayon et al., 2008). One study showed that ablation of mITCs impaired fear extinction memory acquisition, expression and retrieval (Likhtik et al., 2008). This suggests that ITC is involved in fear extinction circuits.

1.5 Morphology and Physiology of the BLA

The BLA contains two main cell types: pyramidal cells and interneurons (McDonald, 1992a, b, Pare and Smith, 1998). They are anatomically and physiologically different (Table 3.1, Figure 3.1, Figure 3.5).

1.5.1 Pyramidal cells in the BLA

Pyramidal cells make up about 80% of the BLA neuronal population. These neurons resemble the pyramidal cells in the cortex and hippocampus in that they are large, spiny, pyramidal-like projection neurons that use glutamate as a neurotransmitter (Hall, 1972, McDonald, 1982b, a, Carlsen, 1988, Carlsen and Heimer, 1988, McDonald, 1992a). Unlike in the cortex and hippocampus, BLA pyramidal cells do not form parallel apical dendrites to have organized laminar structures. Instead the directions of their apical dendrites are randomly laid out to form a salt and pepper like structure (McDonald, 1992a, Washburn and Moises, 1992a, Rainnie et al., 1993, Pare et al., 1995, Faber et al., 2001). Their axons branch out several axonal collaterals within the BLA before projecting to their efferent targets (McDonald, 1982b, Smith and Pare, 1994). These collaterals innervate neighboring pyramidal cells and interneurons to provide feedback inhibition. BLA pyramidal cells receive thalamic and cortical inputs which form

asymmetrical glutamatergic synapses containing both AMPA and NMDA receptors (Farb and LeDoux, 1997, 1999). NMDA receptors contain two types of subunits, NR1 and NR2. The NR2 subunit has four subtypes: NR2A, NR2B, NR2C, and NR2D (Traynelis et al., 2010). In most synapses in the brain NMDARs are composed by NR1 and NR2A or NR2B subunits (Traynelis et al., 2010). In the cortex and hippocampus, it has been shown that NR2A and NR2B subunits undergo a developmental switch (Monyer et al., 1994). NMDA receptors contain NR2B subunits until about a week after birth. After then NMDARs subunits switch from NR2B to NR2A (Monyer et al., 1994). However, in the amygdala one studied showed that selective NR2B blocker impaired fear conditioning acquisition in adult animals, suggesting that NR2B containing NMDARs exist in the BLA of adult animals (Rodrigues et al., 2001).

Kainate receptors, belonging to glutamate receptors family, have also been shown to be present in some glutamatergic synapses in the BL (Li and Rogawski, 1998). Similar to AMPARs, kainate receptors are involved in basal glutamatergic neurotransmission (Lerma et al., 2001, Lerma, 2003). The major differences are native kainate receptors coupled with accessory protein NETO hardly show any desensitization characteristics seen in AMPARs (Straub and Tomita, 2012, Fisher and Mott, 2013). Moreover, kainate receptor-mediated currents have much slower kinetics, which is thought to be ideal for EPSP temporal summation to integrate glutamatergic inputs (Fisher and Mott, 2012, 2013). The exact role of kainate receptors in the BLA is not known.

The anatomical, physiological, and glutamatergic synaptic properties among BLA pyramidal cells are very similar. Therefore, BLA pyramidal cells have been typically seen as a homogenous group of neurons. However, accumulating findings suggest that

BL pyramidal cells are functionally heterogeneous. For example, in a reversal reinforcement learning task, a population of BL pyramidal cells responds to aversive learning while another population of BL pyramidal cells is recruited in appetitive memory trace (Stalnaker et al., 2007). In addition, the hypothesis that there are two populations of BL pyramidal cells, fear and extinction neurons, has been recently proposed by researchers (Herry et al., 2008). In the cortex and hippocampus, it has been suggested that pyramidal cells can be functionally grouped by whether receiving common excitatory and/or inhibitory inputs (Lee et al., 2014). It would be interesting to examine whether BLA pyramidal cells innervated by a same PV interneuron or a group of interconnected PV interneurons form a functional ensemble. Different BLA ensembles may project to the same targets to perform a same function. Since PV interneurons can powerfully control pyramidal cells output (Cobb et al., 1995, Woodruff and Sah, 2007a), according to this hypothesized circuitry model one can imagine that different ensembles can be easily maneuvered by simply controlling a single or a few PV interneurons.

1.5.2 Interneurons in the BLA

Interneurons make up 20% of the neuronal population (McDonald, 1985a, b, McDonald and Augustine, 1993, Sah et al., 2003). They are spine-sparse non-pyramidal neurons and use GABA as inhibitory neurotransmitter (Hall, 1972, McDonald, 1982b, Millhouse and DeOlmos, 1983, McDonald, 1985a, Carlsen and Heimer, 1988). Their axonal innervations typically are limited locally within the BLA but with some exception that subpopulations of SOM interneurons in the BL project to the basal forebrain (McDonald et al., 2012).

Like in the cortex and hippocampus, interneurons in the BLA are heterogeneous based on their morphology, electrophysiology, and neurochemistry. Based on firing patterns, BLA interneurons can be divided into four groups: fast firing, regular firing, burst firing, and stutter firing interneurons (Rainnie et al., 2006, Woodruff and Sah, 2007b). Fast firing and stutter firing interneurons fire action potentials at high frequency and show little adaptation, while regular and burst firing interneurons fire at low frequency and show a lot of adaptation (Rainnie et al., 2006, Woodruff and Sah, 2007b). BLA interneurons can also be differentiated by their expression of neurochemical markers. There are two non-overlapping groups of interneurons: one group express calbindin (CB), the other express calretinin (CR) (Kemppainen and Pitkanen, 2000, McDonald and Mascagni, 2001a). Both CB and CR interneurons can be subdivided based on their co-expression with other calcium binding proteins and/or neuropeptides. Some CR interneurons co-express VIP and/or CCK with some overlap (McDonald and Mascagni, 2002, Mascagni and McDonald, 2003). Interneurons express PV, SOM, or CCK individually without overlapping (McDonald and Mascagni, 2001a, 2002, Mascagni and McDonald, 2003, Davila et al., 2008). These interneurons often are CB+ (McDonald and Mascagni, 2001a, 2002, Mascagni and McDonald, 2003, Davila et al., 2008). The diversity of these neurochemical markers expressions in the BLA interneurons are functionally relevant, which correlate with their postsynaptic projection targets. PV and CCK interneurons often project to somas of BLA pyramidal cells and form basket-like synapses, suggesting that they are basket cells (McDonald et al., 2005, Muller et al., 2006). Basket cells are important for controlling outputs of pyramidal cells and synchronization (Freund and Buzsaki, 1996). Some other PV interneurons project to

proximal and/or distal dendrites (Muller et al., 2006). There are some PV interneurons forming axo-axonic synapses with pyramidal cells, which are named chandelier cells (Muller et al., 2006, Rainnie et al., 2006). On the other hand, majority of SOM interneurons project to distal dendrites of BLA pyramidal cells, which are thought to play a role in regulation inputs to the pyramidal cells and gating induction of synaptic plasticity (Muller et al., 2007a). VIP interneurons are specialized to project to CB+ interneurons, which provide a mechanism of disinhibition in the BLA (Muller et al., 2003, Pi et al., 2013). Other groups of interneurons also synapse onto other interneurons but are restricted to the ones that belong to a same group (Woodruff and Sah, 2007b).

The electrophysiological properties of interneurons were mainly studied in PV interneurons. One study in mice with PV interneurons were tagged by GFP showed that PV interneurons can be any of those four firing patterns (Woodruff and Sah, 2007b). However, PV interneurons with same firing patterns are more likely interconnected by gap junctions and chemical synapses (Muller et al., 2005, Woodruff and Sah, 2007b), suggesting that same types of PV interneurons may act as functional groups. Unlike PV interneurons, CCK interneurons have broader action potentials at low frequency with adaption (Jasnow et al., 2009, Sosulina et al., 2010). Different from other types of interneurons, CCK interneurons express CB1 receptors, which can be found in their somas, dendrites, and presynaptic terminals as well(McDonald and Mascagni, 2001b). Therefore, PV and CCK basket cells have different firing patterns and express different modulatory receptors, suggesting that they are different types of basket cells which may undergo differential modulation. More importantly different types of interneurons are recruited during different brain states. For example, during fear conditioning SOM

interneurons are inhibited by excitation of PV interneurons by CS stimuli (Wolff et al., 2014). As mentioned above, SOM interneurons provide dendritic inhibition on BLA pyramidal cells. Inhibition SOM interneurons would allow CS information to come in and facilitate synaptic plasticity at the BLA pyramidal cells dendrites. Therefore, this disinhibitory effects by selectively recruiting PV interneurons would promote CS-US association. In contrast, during US stimulation, such as foot shock, both PV and SOM interneurons activities are reduced (Wolff et al., 2014). Release of the perisomatic inhibition break by silencing PV interneurons would increase outputs of BLA pyramidal cells to boost postsynaptic US-related responses. One study found that BL interneurons responses under different circumstances were cell type specific (Bienvenu et al., 2012). In vivo SOM but not PV basket or chandelier cells firings were phase locked with hippocampal theta oscillation (Bienvenu et al., 2012). It was further found that PV axo-axonic cells but not PV basket cells or other CB+ interneurons respond to aversive stimuli (Bienvenu et al., 2012). Taken together, these results suggest that specific types of BLA interneurons play a defined role in the BLA microcircuits. There are two major roles of interneurons having been extensively studied. One is inhibitory gating of synaptic plasticity. The other is perisomatic inhibition regulates neuronal synchronization and oscillation.

1.6 INHIBITORY GATING OF SYNAPTIC PLASTICITY

In the BLA, the pyramidal cell activity is tightly controlled by powerful feedforward inhibition (Ehrlich et al., 2009). Therefore in the BLA induction of LTP fails without blockade of GABAa receptors (Bissiere et al., 2003). One study found that dopamine was able to gate LTP induction by reduction of feedforward inhibition

(Bissiere et al., 2003). Dopamine inhibited the monosynaptic IPSC of pyramidal cells through a presynaptic mechanism, meanwhile increased inhibition onto interneurons by exciting a subgroup of interneurons (Bissiere et al., 2003, Chu et al., 2012). The feedforward interneurons gating LTP induction are thought to be SOM interneurons (Bissiere et al., 2003). These dendritic projection SOM interneurons are able to provide strong inhibition at specific compartmental spines with little effects on other compartments. Thereby this characteristic provides possibilities of pathway specific gating of LTP induction.

1.7 PERISOMATIC INHIBITION AND NEURONAL OSCILLATION

Neuronal oscillation is rhythmic, synchronized neuronal activity of a large number of neurons, which can be observed by EEG and LFP recordings (Buzsaki et al., 2012, Buzsaki and Wang, 2012, Buzsaki and Watson, 2012). Several frequency bands of neuronal oscillations have been discovered. These are alpha (8-13 Hz), delta (1-4 Hz), theta (4-8 Hz), beta (13-30 Hz) and gamma (30-70 Hz) frequency band (Buzsaki et al., 2013, He, 2014). Different frequencies of oscillations are correlated with specific brain states and behaviors. Alpha oscillations are seen during relaxed wakefulness (Jensen et al., 2014, Sigala et al., 2014). Delta oscillations are found during non-REM sleep (Mascetti et al., 2011). Gamma oscillations are thought to play a key role in cognitive processing (Buzsaki and Wang, 2012, Khazipov et al., 2013, Tsubo et al., 2013). Theta oscillations in the hippocampus are found in EEG or LFP recordings during exploratory behaviors (Buzsaki, 2002, 2005). It has been proposed to serve as a reference for hippocampal place cells coding of physical positions (Buzsaki and Draguhn, 2004). Neuronal oscillation is caused by synchronization of a group of neurons firing, the

function of which involves feature binding and facilitation of synaptic plasticity. (Pare and Collins, 2000, Buzsáki and Draguhn, 2004, Pape et al., 2005, Bauer et al., 2007b, Cardin et al., 2009b, Sohal et al., 2009, Lesting et al., 2011). It has been hypothesized that synchronization of neuronal firing may be a way used by spatially distributed neurons to respond to a same stimulus so that neuronal ensembles could bind different features of a object or concept together (Ward, 2003). When individual neuronal activities are synchronized to a same phase, the amplitude of electrical current from each neuron can be added up rather than averaged out to produce magnified neuronal activities within the neuronal ensemble. Neuronal oscillations also facilitate LTP at the pathways from the synchronized brain structures. For example, the induction of LTP would only be facilitated when the input come at the peak not the trough of the neuronal oscillation phase. In other words, only the inputs from the brain structure of which the neuronal activity is synchronized with the oscillation generating brain structure can be strengthened, which further promotes feature binding. Neuronal oscillations are also found in pathological conditions. For instance, it is featured with large scale, high amplitude neuronal oscillations during epileptic seizures (Isomura et al., 2008). The functional neuronal assemblies are no longer segregated, which may explain why the consciousness disappears during seizures.

Although neuronal oscillations were mostly studied in the cortex and hippocampus, they have also been reported in the amygdala. Rhythmic LFP at theta frequency were found in the BLA during emotional arousal (Pare and Collins, 2000). During fear memory retrieval and consolidation theta frequency synchrony between hippocampus and BLA is increased (Seidenbecher et al., 2003, Narayanan et al., 2007).

Moreover, this theta oscillation originates from synchronized firing of BLA neurons and then influences hippocampal CA1 pyramidal cells activity. During fear extinction and extinction memory retrieval, infralimbic mPFC neuronal firing is phase-locked to BLA and hippocampus theta oscillation (Sangha et al., 2009). These findings indicate that generation of synchronized neuronal oscillations in the BLA and other brain structures may represent the interactions between BLA and related brain areas and play a critical role in fear related learning and behaviors. How do firings of individual projection neurons become synchronized under certain conditions? Studies in the cortex and hippocampus suggest that interneurons providing perisomatic inhibition can provide narrow time window for allowing pyramidal cells to fire, and thereby they fire synchronously (Csicsvari et al., 1999, Pike et al., 2000, Freund, 2003, Whittington and Traub, 2003, Hájos et al., 2004, Traub et al., 2004, Bartos et al., 2007, Freund and Katona, 2007, Mann and Paulsen, 2007, Woodruff and Sah, 2007a, Gulyás et al., 2010, Ryan et al., 2012). It has also been shown that inhibition provided by PV interneurons was able to phase reset innervated pyramidal cells (Woodruff and Sah, 2007a, Courtin et al., 2014). Synchronized firing caused by depolarization rebound after hyperpolarization can be seen in a group of pyramidal cells receiving same inhibition (Cobb et al., 1995, Woodruff and Sah, 2007a). Selective subtypes of interneurons are sensitive to neuromodulators (Kawaguchi, 1997), which could explain why some neuromodulators, such as ACh, can robustly induce neuronal oscillations in vitro and in vivo (Alonso et al., 1996, Klink and Alonso, 1997, Fisahn et al., 1998, Chapman and Lacaille, 1999, Fellous and Sejnowski, 2000, Fisahn et al., 2002, Steriade, 2004, Zhang et al., 2010, Nagode et al., 2011).

1.8 CHOLINERGIC MODULATION

1.8.1 Acetylcholine

ACh is a neurotransmitter found in neuromuscular and neuronal synapses. In the neuromuscular synapses it acts as a fast neurotransmitter that induces muscular contraction. In contrast, in the CNS neuronal synapses it is described as slow modulatory effects. Acetyl-coenzyme A (acetyl-CoA) and choline are the precursors of ACh synthesis (Ferguson et al., 2003, Brandon et al., 2004). Acetyl-CoA is mainly generated from glucose metabolism, while choline is from membrane-bound phosphatidylcholine, dietary choline, and choline reuptake. ACh is formed by a catalytic reaction by choline acetyltransferase (ChAT), by which Acetyl group of Acetyl-CoA is transferred to choline. ChAT is synthesized in the soma and transported along the axons down to the terminals, which makes ACh synthesis in the axonal terminals be possible. Once ACh is synthesized, it is loaded into synaptic storage vesicles by vesicular acetylcholine transporter (VAChT), and then is ready to be released into the synaptic cleft (Ferguson et al., 2003, Brandon et al., 2004). ACh release is mediated by presynaptic Ca2+ entry caused by depolarization of presynptic terminals. Once ACh is released into the synaptic cleft, it can be quickly hydrolyzed by acetylcholinesterase (AChE) into choline. Choline is then reuptaken into presynaptic terminals by choline transporters for reuse. This is the rate-limiting step of ACh synthesis. Unlike ChAT or VAChT, AChEs are found in both cholinergic neurons and cholinoreceptive neurons. Therefore ChAT and VAChT can be used to identify cholinergic neurons and their axons as well, whereas AChE can be used for determining the places of cholinergic neurotransmission.

1.8.2 ACh Receptors

Nicotinic receptors and muscarinic receptors are the two types of ACh receptors found in the CNS. They are named after their selective agonists: nicotine and muscarine. Nicotinic receptors are ligand-gated ionotropic receptors with a pentameric structure, which mediate fast cholinergic actions in the CNS (Albuquerque et al., 2009, Papke, 2014). There are 12 nicotinic subunits have been found in the CNS, ranging from α2 to α10 and β2 to β4. α4β2 nicotinic receptors and α7 receptors are the most common ones found in the CNS (Albuquerque et al., 2009, Papke, 2014). ACh binding to these nicotinic receptors produces a fast onset but short duration effects. Opening of the channels are permeable to cations, including Na^{+}, K^{+}, and Ca^{2+}. Once activated, nAChRs undergo rapid desensitization (Albuquerque et al., 2009). nAChRs are present in both pre and post-synaptic sites to regulate presynaptic transmitter release and directly depolarize postsynaptic membrane potentials (Sahin et al., 1992).

1.8.3 Muscarinic Receptors

mAChRs are metabotropic G-protein coupled receptors with typical seven transmembrane spanning domains structure, mediating slow, modulatory effects in the CNS (Vaidehi et al., 2014). Like other G-protein coupled receptors transduction of mAChRs signals involves activation of G-proteins. G-protein consist three subunits: α, β, and γ subunits. When α subunit binds with GDP, they form an inactive trimer. Activation of G-protein causes the replacement of GTP to GDP, which causes G-protein subunits dissociate into two parts: α-GTP and β γ dimer. Both of them interact with downstream effectors. After phosphorylation of target proteins α-GTP is hydrolyzed into α-GDP. This

promotes the subunits re-association to become back to the inactive trimer (Vaidehi et al., 2014).

There are five subtypes of mAChRs existing in the CNS: M1, M2, M3, M4, and M5 receptors. Of which M1, M3 and M5 receptors are Gq coupled receptors, while M2 and M4 receptors are Gi coupled receptors (Hulme et al., 2003, Brown, 2010). Activation of G_s coupled M1, M3, or M5 receptors causes activation and dissociation of α subunit of G-proteins, which binds and activates phospholipase C (PLC). PLC further catalyzes the cleavage of membrane-bound phosphatidylinositol biphosphate (PIP2) into diacylglycerol (DAG) and inositol triphosphate (IP3) acting as the second messengers. Protein kinase C (PKC) activated by DAG phosphorylates various downstream target proteins. IP3 binds to and activates IP3 receptors located on the membrane of the smooth endoplasmic reticulum, leading to Ca2+ release to cytosol. The consequences of elevated cytosolic Ca2+ concentration include Ca2+ dependent enzymes and kinases such as CaMK (Brown, 2010). In addition to the effects of α subunit of G-proteins, the dissociated Gβγ dimer also regulates various ion channels, such as G-protein-regulated inwardly rectifying K+ channels (GIRKs), P/Q- and N-type voltage-gated Ca2+ channels. This effect is much faster (usually within seconds) than traditional metabotropic effects (Hulme et al., 2003, Brown, 2010).

Activation of Gi coupled M2 or M4 receptors leads to the inhibition of adenylyl cyclase (AC), an enzyme produces cAMP as a second messenger from ATP. Inhibition of AC causes reduced activity of cAMP dependent protein kinase A (PKA). This reduces phosphorylation of PKA-target protein, thus leads to decreased function of these proteins. Therefore generally speaking M1, M3, and M5 Gq coupled receptors have excitatory

effects whereas M2 and M4 Gi coupled receptors produce inhibitory effects. Although various G-protein coupled metabotropic receptors share same signaling pathways, they may produce different effects. It was proposed that G-protein coupled receptors, second messengers, and downstream effectors may form functional clusters restricted in small microdomains (Delmas et al., 2004). Moreover the difference of spatial distributions of various metabotropic receptors also makes each type of receptors have unique functions in neural networks.

1.8.4 The Basal Forebrain

Besides the presence of cholinergic interneurons in some brain regions, such as basal ganglia and striatum, the majority of cholinergic projection neurons are found in the basal forebrain (Zaborszky et al., 1999). The basal forebrain is a brain structure located in the medial and ventral part of the forebrain. It is a highly complex structure consisting of multiple substructures, including medial septum, diagonal band complex, ventral pallidum, substantia innominata, extended amygdala and peripallidal regions. Different basal forebrain nuclei innervate different brain areas (Zaborszky et al., 1999). For example, cholinergic innervations of the hippocampus are mainly from medial septum, while basolateral amygdala receive heavily cholinergic projections from ventral pallidum and substantia innominata (Zaborszky et al., 1993). This structure is involved in many brain functions, such as learning and memory, cortical activation, and attention (Arnold et al., 2002, Parikh et al., 2007). Dysfunction of the basal forebrain has been implicated in mental disorders including Alzheimer's disease (Mesulam, 2013b).

The cellular composition of the basal forebrain includes cholinergic projection neurons, GABAergic projection neurons, and local interneurons expressing different calcium binding protein and other neurochemicals (Gritti et al., 1997, Zaborszky et al., 1999, Duque et al., 2000, Zaborszky and Duque, 2000, 2003, Hur and Zaborszky, 2005, Jones, 2008). Interestingly, cholinergic neurons only make up about 20% of the total neuronal population in the basal forebrain (Zaborszky and Duque, 2000). The basal forebrain receives top-down modulation by projections from prefrontal, piriform, and insular cortices (Mesulam and Mufson, 1984, Zaborszky et al., 1997). It also receives inputs from other neuromodulatory systems, including adrenergic, dopaminergic, noradrenergic, and serotonergic systems (Zaborszky et al., 1993, Gaykema and Zaborszky, 1996, 1997, Hajszan and Zaborszky, 2002). In addition, amygdala (Grove, 1988, McDonald, 1991, Petrovich et al., 1996) and hypothalamus (Cullinan and Zaborszky, 1991) send outputs to the basal forebrain as well. The prefrontal projections mainly target on non-cholinergic neurons (Zaborszky et al., 1997), while inputs from amygdala, hypothalamus, and neuromodulatory systems synapse on both cholinergic and non-cholinergic neurons (Zaborszky et al., 1999).

1.8.5 Functions of Cholinergic Modulations

Behavioral studies on animals with lesions of the cholinergic neurons in the basal forebrain indicated that ACh plays a critical role in regulation of attention (Robbins et al., 1989, Dunnett et al., 1991, Muir et al., 1992, Roberts et al., 1992, Voytko et al., 1994, Chiba et al., 1995, Turchi and Sarter, 1997, McGaughy and Sarter, 1998, Baxter et al., 1999, Newman and McGaughy, 2008). For example, cortical cholinergic deafferentation impaired cue detection rate in rats performing an attention task, while response accuracy

in blank trials was not affected (McGaughy et al., 1996). Microdialysis studies also showed increased cortical ACh release during attention task performing (Arnold et al., 2002). These results suggest that cholinergic system is active at the moment of cue detection during attention tasks. Moreover in vivo recordings found that neurons in the basal forebrain increased firing during cues presentation which predict salient stimuli (Rigdon and Pirch, 1986). The basal forebrain neurons also respond to aversive stimuli (Richardson and DeLong, 1991). The responses were with rapid onset and short duration (within hundreds of milliseconds) (Richardson and DeLong, 1991), suggesting that in these cases phasic rather than volume cholinergic transmission mediated the behaviors.

1.8.6 The effects of ACh on pyramidal cells and interneurons

The effect on electrophysiological properties of pyramidal cells and interneurons has been extensively studied in the cortex and hippocampus. ACh depolarizes pyramidal cells through activation of mAChRs (Madison et al., 1987). This is mediated by increase of input resistance by blocking potassium channels (Madison et al., 1987). In addition, mAChRs activation blocks M-current and slow afterhyperpolarization to make pyramidal cells more receptive to incoming inputs and prolong firing by reducing spike adaptation (Weight and Votava, 1970, Constanti and Sim, 1987, Madison et al., 1987). ACh enhances persistent spiking of pyramidal cells in prefrontal cortex (Haj-Dahmane and Andrade, 1996, 1997, 1998), entorhinal cortex (Klink and Alonso, 1997, Egorov et al., 2002, Fransen et al., 2006, Tahvildari et al., 2007). This persistent firing is thought to be responsible to temporal information retention and is necessary for trace conditioning learning.

The effects of ACh on interneurons depend on the interneuron subtype. In the cortex it has been found that SOM and regular firing interneurons not PV or fast firing interneurons were depolarized by mAChRs agonist (Kawaguchi, 1997). Moreover regular firing not fast firing interneurons are engaged in nAChRs activation (Xiang et al., 1998). In the cortex SOM interneurons providing dendritic inhibition receive strong glutamatergic inputs from neighboring pyramidal cells (Silberberg and Markram, 2007) and weak inputs from thalamic inputs (Cruikshank et al., 2010), suggesting that they are feedback inhibitory interneurons. Activation of SOM interneurons would facilitate feedback inhibition to neighboring pyramidal cells. This may explain how ACh sharpens receptive fields of cortical pyramidal cells.

1.8.7 The effects of ACh on Glutamatergic and GABAergic transmission

In the cortex, mAChRs activation suppresses recurrent glutamatergic synaptic transmission between neighboring pyramidal cells through a presynaptic mechanism (Hasselmo and Bower, 1992, Hasselmo and Schnell, 1994, Hasselmo and Cekic, 1996, Kimura and Baughman, 1997, Kimura, 2000). This would reduce the size of distributed network of excitation within the cortex and enhance the responses to external inputs. In the other hand, ACh activates presynaptic nAChRs located on the thalamocortical terminals and lead to facilitate glutamate release from that pathway (Metherate and Ashe, 1993, Hsieh et al., 2000). This is thought to be important for increase of signal/noise ratio, sharpening receptive fields and improving information flow from other brain areas to the cortex (Giocomo and Hasselmo, 2007). In the cortex and hippocampus, ACh, through M2 receptor activation, inhibits GABA release from PV and CCK interneurons (Freund, 2003). It has been shown that ACh has an important role in the induction of

theta oscillations (Bland and Colom, 1993). PV and CCK perisomatic projection interneurons have been shown to be involved in the generation of Gamma oscillation (Freund, 2003, Hájos et al., 2004, Gulyás et al., 2010). Therefore, it is paradoxical that ACh inhibits GABA release from PV and CCK interneuron. However, Lawrence (Lawrence, 2008)proposed that by reducing GABA release ACh reserves GABA vesicles in the terminals against vesicles depletion for transmission at high frequency, such as gamma. In this case, ACh acts like a high pass filter in the circuit.

1.8.8 Cholinergic innervations of the amygdala

Although as mentioned above basal forebrain cholinergic neurons project to most of the brain regions, the innervations of different areas do not show equal density. It has been shown cholinergic innervations become denser in paralimbic areas than cortical areas (Mesulam, 2004). The density further increases in the core limbic region including the hippocampus and amygdala (Ben-Ari et al., 1977). Within the amygdala BL is the nucleus where mainly receives cholinergic inputs. In fact, in all mammals, compared to any other part of the brain the BL receives the densest cholinergic innervation indicated by the expression levels of ChAT, VAChT and AChE (Girgis, 1980, Svendsen and Bird, 1985, Hellendall et al., 1986, Emre et al., 1993). As different portions of the basal forebrain are connected to different forebrain regions, BL cholinergic input comes from the ventral pallidum and substantia innominata (Zaborszky et al., 1999). mAChR-mediated mechanisms in the BL nucleus are primary mediators of the neuromodulation involved in memory consolidation of emotionally arousing experiences by the amygdala (McGaugh, 2004), and have also been implicated in the consolidation and extinction of contextual fear memory (Vazdarjanova and McGaugh, 1999, Boccia et al., 2009), reward

devaluation learning(Salinas et al., 1997), amphetamine-motivated conditioned place preference learning (McIntyre et al., 2002) and conditioned cue reinstatement of cocaine seeking (See, 2005). These findings suggest that cholinergic modulation in the BL could potentially be one of therapeutic targets for treating a number of neuropsychiatric disorders. For example, it has been shown that there is significant reduction of the cholinergic innervation of the BL in Alzheimer's disease (Emre et al., 1993). Indeed, one of the co-morbidities is mood disturbances and impairment of emotional memory (Mori et al., 1999). AChE inhibitors, the most common drugs for treatment of Alzheimer's disease, also improve mood and reduce anxiety in Alzheimer's patients (Gauthier et al., 2002). Cholinergic inputs target both pyramidal cells and interneurons in the BL (Muller et al., 2011). The majority of cholinergic terminals synapse onto distal dendrites and spines of BL pyramidal cells (Muller et al., 2011). This is consistent with findings from electrophysiological studies that cholinergic transmission increases the excitability of BL pyramidal cells through activation both muscarinic receptors (Washburn and Moises, 1992b) and nicotinic receptors (Klein and Yakel, 2006). About less than 10% of the cholinergic terminals in the BL synapse onto PV interneurons (Muller et al., 2011). Although only a very few percentage of cholinergic terminals project to PV interneurons, considering PV interneurons only make up about 6% of the total neuronal population in the BL this indicates that same as pyramidal cells PV interneurons are also heavily innervated by cholinergic inputs. Indeed, it also has been shown BL interneurons were depolarized by both muscarinic (Washburn and Moises, 1992a, Yajeya et al., 1997) and nicotinic agonists (Zhu et al., 2005). In the hippocampus, PV interneurons are interconnected by gap junctions and chemical synapses (Freund and Buzsaki, 1996). It

has been shown that activation of a network of PV interneurons plays a key role in the generation of neuronal oscillations in the hippocampus (Freund and Buzsaki, 1996, Freund, 2003). Similarly, In the BL PV interneurons form a network via connections by gap junctions (Muller et al., 2005, Woodruff and Sah, 2007b). PV interneurons providing robust perisomatic inhibition may be able to synchronize neighboring pyramidal cells firing (Rainnie et al., 2006, Woodruff and Sah, 2007b, a). Therefore, cholinergic innervation of BL PV interneurons may be involved in generation of neuronal oscillations in the BL during emotional related behaviors.

As mentioned before, cholinergic neurons only make up about 20% of the basal forebrain neuronal population. However, only about 20% of the axons are from non-cholinergic neurons in the basal forebrain (Carlsen et al., 1985, Zaborszky et al., 1986).Therefore the BL receives much stronger cholinergic modulation than other types from the basal forebrain.

1.8.9 mAChRs subtypes Expressions in the BL

Early studies using receptor binding autoradiographic technique demonstrated that in rodents and primates, BL contains M1 and M2 receptors (Cortes and Palacios, 1986, Mash and Potter, 1986, Spencer et al., 1986, Cortes et al., 1987, Mash et al., 1988), and putative M3 and M4 receptors (Smith et al., 1991). In situ hybridization studies further suggested that M1 receptor is the most dominant mAChRs subtype in the BL (Buckley et al., 1988). Studies using antibodies double labeling M1 receptors and pyramidal cells or interneurons revealed that in the BL M1 receptors are highly expressed in pyramidal cells exclusively (McDonald and Mascagni, 2010). Electron microscopy studies further found

that about 60% of the spines of pyramidal cells express M1 receptors (Muller et al., 2013). Majority of the glutamatergic terminals synapse onto those M1R+ spines also express M1 receptors (Muller et al., 2013). In other words, M1 receptors are present on both pre and post synaptic membranes of many glutamatergic synapses on BL pyramidal cells. This seems odd that ACh would have two opposite effects on the same synapses. On one hand, ACh excites postsynaptic spines through postsynaptic M1 receptors. On the other hand, ACh would inhibit glutamate release through presynaptic M1 receptors and thereby decreases postsynaptic spines excitation. It would be tempting to speculate that ACh may preserve glutamate in the terminal for high frequency transmission by reducing transmitter release probability. Therefore, cholinergic transmission could suppress spontaneous, low frequency glutamatergic transmission but facilitate high frequency ones, such as theta or gamma frequency transmission from hippocampus and cortex. Along with the M1Rs-mediated postsynaptic excitation, ACh could promote LTP induction at those spines. In the electron microscopic study, the authors also found that some GABAergic terminals express M1Rs too (Muller et al., 2013). These GABAergic axons are from both the basal forebrain GABAergic neurons and local BL interneurons (Muller et al., 2013). This suggests that cholinergic transmission can counteract the GABAergic modulation originated from the basal forebrain. By inhibiting GABAergic transmission, ACh can further promote LTP induction in BL pyramidal cells. In contrast to M1Rs localization, M2 receptors are mostly found in SOM and NPY interneurons in the BL (McDonald and Mascagni, 2011). Information on other mAChRs subtypes localization in the BL has not yet been studied. All these anatomical and other behavioral findings on the mAChRs in the BL suggest that they may play a critical role in emotional

memory formation and consolidation. However, to understand the defined function of ACh transmission in the BL and the underlying mechanism requires further studies combining multiple techniques.

1.9 SIGNIFICANCE REVISITED

As discussed above, mAChRs play an important role in the physiology and pathophysiology of emotional memory (LeDoux, 2000, Sah et al., 2003). However, there have been no studies that have systematically examined the mechanisms by which mAChRs regulate fear memory formation and extinction. To understand this significant knowledge gap first step we need to approach is to define the effects of activation of mAChRs on different types of neurons in the BL and what it affects on the information inputs to the BL. Understanding activation of distinct mAChRs differentially modulates what subpopulations of neurons in the basolateral amygdala (BL) will help us comprehend how the amygdala processes and sends information and how cholinergic transmission regulates it. Knowing distinct glutamatergic and GABAergic inputs to these neurons will tell us how cholinergic transmission would filter and modulate incoming information and regulate communications between brain structures and amygdala. These studies are important because BL neurons play an essential role in the cellular processes that underlie emotional memory (Goddard, 1964, LeDoux, 2000). BL neurons receive the densest cholinergic innervation among all targets of the basal forebrain (Girgis, 1980, Svendsen and Bird, 1985, Hellendall et al., 1986, Emre et al., 1993), suggesting that ACh must play a critical role in regulating amygdala function. In line with it, numbers of behavioral studies have shown blockade of mAChRs in the BL impairs fear memory consolidation (McGaugh, 2004), whereas activation of them enhances fear memory

formation, consolidation and extinction (McGaugh, 2004). Moreover, Alzheimer's disease, anxiety disorders and schizophrenia, which are commonly associated with emotional disturbances, are thought to result, at least in part, from abnormal cholinergic transmission. As there have not been effective treatments for these diseases, cholinergic transmission and associated mAChRs could potentially be novel therapeutic targets.

The project was aimed to understand what activation of mAChRs does to neurons inside the BL and to incoming glutamatergic projections to the BL, and thus shed light on how cholinergic transmission regulates information processing and flow in and out the amygdala.

1.10 HYPOTHESIS AND SPECIFIC AIMS

Our overarching hypothesis was that mAChRs differentially modulate distinct BL interneuronal subpopulations and inputs, resulting in alterations in synaptic transmission, plasticity and network oscillatory activity. We proposed to address this hypothesis through the following specific aims:

Specific Aim 1: To define muscarinic modulation of BL interneurons. While previous studies have investigated muscarinic agonists on BL PNs, the effects of muscarinic agonists on BL interneurons are unknown. We hypothesized that muscarine selectively depolarizes a BL interneuronal subpopulation.

Specific Aim 2: To examine functional effects of BL interneurons activation by muscarine. Previous studies have shown activation of mAChRs induces neuronal oscillations in the hippocampus and cortex via interneurons activation. However, it is unknown whether this is the case in the amygdala. We hypothesized that muscarinic

activation of a distinct interneuronal subpopulation in the BL generates neuronal oscillations.

Specific Aim 3: To determine presynaptic muscarinic modulation of glutamatergic and GABAergic transmission. Muscarinic modulation of glutamatergic and GABAergic transmission has been extensively studied in the cortex and hippocampus. This modulation is thought be important for increasing signal noise ratio and facilitating synaptic plasticity. However, these are not known in the amygdala. We hypothesized that mAChRs in the BL produce frequency-dependent and pathway-specific modulation of synaptic transmission to the BL PNs.

CHAPTER 2

GENERAL METHODS

2.1 ANIMALS

All animal care and use procedures were performed in accordance with the National Institutes of Health guidelines for care and use of laboratory animals and approved by the institutional Animal Care and Use Committee at the University of South Carolina. Male Sprague Dawley rats (14 - 28 days old) were used in all the experiments.

2.2 PREPARATION OF BRAIN SLICES

Under deep isoflurane anesthesia, male Sprague Dawley rats were decapitated, and brains were removed and immersed in ice-cooled oxygenated ACSF (artificial cerebrospinal fluid). Coronal brain slices, 300 mm thick, were cut using a vibratome (VT1000S; Leica, Nussloch, Germany). Brain slices were incubated in warmed (32–34°C), bubbled ACSF containing (in mM) 120 NaCl, 3.3 KCl, 1.0 NaH_2PO_4, 25 $NaHCO_3$, 10 glucose, 0.5 $CaCl_2$, and 5 $MgCl_2$ and bubbled with a 95% O_2/5% CO_2 gas mixture at pH 7.4. Osmolarity was 301–308 mOsm.

2.3 WHOLE-CELL RECORDING

Our whole-cell recording techniques have been described previously (Mott et al., 1997, Mott et al., 2008). Slices were individually transferred to a recording

chamber maintained at 32–34°C and continuously perfused with oxygenated ACSF, pH 7.4, containing 1.5mM $CaCl_2$ and 1.5mM $MgCl_2$. Whole-cell recordings were obtained using borosilicate glass electrodes (6-8 MΩ) filled with an internal solution containing (in mM) 130 K-gluconate, 5 KCl, 10 HEPES, 0.5 EGTA, 2 $MgCl_2$, 2 MgATP, and 0.3 NaGTP, pH 7.3. Biocytin (Sigma, St. Louis, MO) 0.2–0.4% was added for later visualization of the neuron morphology. Whole-cell patch-clamp recordings were made from the anterior subdivision of the BL, and that the nucleus could be easily identified in the slice based on its position between the external capsule (located lateral to the BL) and the intermediate capsule (located medial to the BL). Both capsules could be easily identified with infrared-differential interference contrast optics. Putative interneurons in the BL could be visually identified. In this study the criteria that we used to identify the candidate interneurons are that their somatic diameters were less than 15um. They were further confirmed by measuring their electrophysiological properties, for example, they usually had relatively high input resistance, and by their morphologies via performing post hoc immunohistochemistry. Voltage-clamp recordings were made at a holding potential of -70 mV. Series resistance was 10-25 MΩ, and recordings in which series resistance changed significantly were discarded. Responses were recorded using a Multiclamp 700B amplifier and filtered at 1 kHz. Responses were digitized by a Digidata 1440A analog-to-digital (A-D) board (Molecular Devices, Sunnyvale, CA) in a Windows-based computer using pClamp 10 software.

2.4 PUFF APPLICATION

For some experiments muscarine was applied by puff application to increase the speed of drug application. Muscarine (50 μM) was made in ACSF with similar OSM as

the bath solution which was filled into a glass pipette. The pipette was made by the same puller used to make recording electrode pipette. The size of the pipette tips was made to have 1-2 MΩ if filled with recording internal solution. The pipette was mounted on a pipette holder which was connected to picopump with an air tank through which the pressure and during of application can be adjusted.

2.5 OPTOGENETICS

2.5.1 Virus injection

Male Sprague Dawley rats (around 30 days old) were anesthetized with isoflurane. A small hole was drilled at the appropriate coordinates for prelimbic mPFC and midline thalamus dependent upon each experiment (prelimbic mPFC, 3.5 mm anterior and 0.5 lateral to the bregma; midline thalamus, 2.0 mm posterior and 0.0 lateral to the bregma). Following this, 2 µL AAV-CAMKII-hChR2(H134R)-EYFP (UNC vector core) was delivered through an injection pipette. Electrophysiology experiments were carried out after 6-8 weeks after injection.

2.5.2 Activation of ChR2

Slice illumination was carried out using a blue LED bulb (470 nm) placed directly between the condenser and the recording chamber. The intensity of illumination is adjustable by the controller (ThorLabs Inc, Newton, New Jersey).

2.6 IMMUNOHISTOCHEMICAL ANALYSIS

There are two major cell classes in the BLC, glutamatergic pyramidal projection neurons and GABAergic interneurons. Although these cells do not exhibit a laminar

organization, their morphology, synaptology, electrophysiology, and pharmacology are remarkably similar to their counterparts in the cerebral cortex(Carlsen and Heimer, 1988, McDonald, 1992b, Washburn and Moises, 1992a, Rainnie et al., 1993, Paré et al., 2003). Dual-labeling immunohistochemical studies suggest that the BL contains at least four distinct subpopulations of GABAergic interneurons that can be distinguished on the basis of their content of calcium-binding proteins and peptides. These subpopulations are: (1) parvalbumin+/calbindin+ neurons, (2) somatostatin+/calbindin+ neurons, (3) large multipolar cholecystokinin+ neurons that are often calbindin+, and (4) small bipolar and bitufted interneurons that exhibit extensive colocalization of calretinin, cholecystokinin, and vasoactive intestinal peptide (Kemppainen and Pitkanen, 2000, McDonald and Betette, 2001, McDonald and Mascagni, 2001a, 2002, Mascagni and McDonald, 2003, 2009). In the present study we performed triple-labeling immunofluoresence on slices/sections containing neurons that were filled with biocytin during recording to determine their phenotype. Antibodies to PV, SOM and CR were used to identify the three most numerous interneuronal subpopulations (Mascagni and McDonald, 2003).

Slices were fixed overnight in 4% paraformaldehyde in 0.1 M phosphate buffer at 4 degrees C. Approximately half of the slices were resectioned at 75 μm on a vibratome, and the other half were processed for immunohistochemistry without resectioning. All antibodies were diluted in phosphate-buffered saline (PBS; pH 7.4) containing 0.5% Triton X-100 and 1% normal goat serum. Sections/slices were first incubated in a primary antibody cocktail containing mouse anti-parvalbumin (PV; 1:5,000; Swant, Bellinzona, Switzerland) and rabbit anti-somatostatin-28 antibodies (SOM; 1:2000, Peninsula Laboratories, San Carlos, CA) overnight at 4°C. Sections were then rinsed in 3

changes of PBS (10 min each), and then incubated in a secondary antibody cocktail of Alexa-488-conjugated goat anti-mouse IgG (1:400; Invitrogen, Eugene OR) and Alexa-633-conjugated goat anti-rabbit IgG (1:400; Invitrogen) for 3 hours at room temperature, followed by incubation in Alexa-546-conjugated streptavidin (1:6,000; Invitrogen) for 3 hours for biocytin visualization. Secondary antibodies were highly cross-adsorbed by the manufacturer to ensure specificity for primary antibodies raised in particular species. Sections were then rinsed in 3 changes of PBS (10 min each), mounted on glass slides using Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and examined with a Zeiss LSM 510 Meta confocal microscope. Triple labeling fluorescence of Alexa-488, Alexa-546, and Alexa-633 dyes was analyzed using filter configurations for sequential excitation/imaging via 488 nm, 543 nm, and 633 channels, respectively. Biocytin-filled neurons that were not immunostained for PV or SOM in the first round of staining were subjected to a second round of immunofluorescence staining using a rabbit anti-calretinin antibody (CR; 1:1000; Chemicon).

Biocytin-filled neurons in the BL were considered interneurons if they exhibited a non-pyramidal morphology and had dendrites that were aspiny or spine-sparse (McDonald, 1982b, McDonald and Betette, 2001, McDonald and Mascagni, 2001a, 2002, Mascagni and McDonald, 2003). In most cases a single digital photomicrograph was taken through the cell bodies of these neurons at an optical section thickness of 10 µm. However, in some cases a z-series was compiled using images taken at an optical section thickness of 1 µm in order to better visualize neuronal morphology. Confocal digital images were adjusted for brightness and contrast in Photoshop 6.0. A total of 25 slices/sections containing BL pyramidal neurons that were filled with biocytin during

electrophysiological recording were processed for biocytin/PV/SOM triple labeling along with slices/sections that contained interneurons. Pyramidal cells were easily recognized on the basis of their distinctive morphology. They exhibited large pyramidal or piriform somata, thick spiny apical dendrites, and thinner spiny basal dendrites. As expected, none of these 25 pyramidal cells exhibited immunoreactivity for interneuronal markers, indicating method specificity and a lack of "cross-talk" between the 543 nm channel used to image biocytin and the 488 nm and 633 channels used to image the interneuronal markers.

The antibodies used to selectively label distinct interneuronal subpopulations in the BL in this study have been shown to be specific for their respective immunogens. Each produced the characteristic pattern of marker immunostaining seen in previous studies of the rat BL (Kemppainen and Pitkanen, 2000, McDonald and Betette, 2001, McDonald and Mascagni, 2001a, 2002, Mascagni and McDonald, 2003, 2009). The mouse monoclonal PV antibody utilized in this study (Swant #235) is one of the most widely used PV antisera in studies of the central nervous system. The immunogen used to generate the antibody was carp-II PV. The specificity of this antibody has been well documented (Celio et al., 1988). The polyclonal antibody to somatostatin (# T-4547; Peninsula Laboratories) was raised in rabbit against somatostatin-28. Studies conducted by the manufacturer indicate that it recognizes somatostatin-28 but does not react with various other neuropeptides including substance P, CCK, or VIP. The rabbit polyclonal antibody to calretinin (# AB5054, Chemicon) was raised against recombinant rat calretinin. Western blot studies conducted by the manufacturer indicate that it is specific

for calretinin and recognizes both calcium-bound and calcium-unbound conformations of this protein.

2.7 STATISTICAL ANALYSIS

Analysis was performed using pClamp 10 (Molecular Devices) and Origin (MicroCal, Northampton, MA) software packages. Statistical comparisons were performed using the independent/paired samples t test or one-way ANOVA with post hoc tests. Non-parametric analysis (Chi-Square Fisher's test) was used to compare the response to muscarine between interneuron types. Values are given as mean ± SE. Significant main effects were defined by $p<0.05$.

CHAPTER 3

DIFFERENTIAL MODULATION OF BL INTERNEURONS BY MUSCARINE

3.1 INTRODUCTION

The BL is critical for the generation of emotional behavior and formation of emotional memory (Sah et al., 2003, Pape and Pare, 2010). Understanding the neuronal mechanisms of emotional information processing in the BL requires knowledge of the anatomy and physiology of its constituent neurons. Inhibitory interneurons in the BL can be divided into subpopulations defined by distinct electrophysiological properties or neurochemical markers (Sah et al., 2003, Spampanato et al., 2011). In the BL amygdala, PV interneurons project to perisomatic and dendritic domains of pyramidal cells (Muller et al., 2006). They also project to other types of interneurons including themselves to form axo-dendritic or axo-axonal connections (Muller et al., 2005, Woodruff and Sah, 2007b). SOM+ interneurons preferentially project to distal dendrites and spines of pyramidal cells (Muller et al., 2007a). Therefore, different neuromarkers containing interneurons have different functions in the neuronal network. PV+ interneurons tightly control pyramidal cells fire action potentials through perisomatic inhibition thus are critical for regulating output of the pyramidal cells, while SOM+ interneurons are important for regulating synaptic plasticity and information input to the pyramidal cells.

However, few studies have examined whether the electrophysiological and neurochemical properties of these interneurons subgroups coincide (Rainnie et al., 2006, Woodruff and Sah, 2007b, Sosulina et al., 2010). Thus, one goal of this study was to determine whether electrophysiological properties of interneurons in the BL correlate with their neurochemical content.

The BL receives dense cholinergic innervation from basal forebrain (Muller et al., 2011). Cholinergic modulation of amygdala is important for memory consolidation (Power et al., 2003b). Deprivation of cholinergic modulation from amygdala impairs emotional learning, contextual fear conditioning, and other amygdala functions (Power et al., 2003b). Cholinergic projections modulate emotional responses through actions on both excitatory and inhibitory circuits in the BL. While the effect of ACh on pyramidal cells has been described (Washburn and Moises, 1992b), little is known about cholinergic modulation of interneuron function. Anatomical studies showed that muscarinic receptors were differentially expressed by BL neurons (McDonald and Mascagni, 2010, 2011). For example, M1 receptors were found exclusively in pyramidal cells in the BL (McDonald and Mascagni, 2010). M2 receptors were found in the neurons which contained glutamatic acid decarboxylase (GAD), SOM, and neuropeptide Y (NPY), but not PV, calretinin (CR), or cholecystokinin (CCK) (McDonald and Mascagni, 2011). Previous studies in frontal cortex have indicated that regular firing interneurons containing SOM are more sensitive to mAChR activation than PV fast firing interneurons (Kawaguchi, 1997). Therefore, a second goal of this study was to determine whether mAChRs activation differentially modulates distinct interneuron subpopulations in BL. We determined whether BL interneurons show a similar sensitivity to mAChR activation.

3.2 MATERIALS AND METHODS

3.2.1 Brain slices preparation

Coronal (300 µM thick) amygdala slices were prepared from 18 -28 days old male Sprague Dawley rats.

3.2.2 Electrophysiological characterization

Transient (750 ms) hyperpolarizing current steps of increasing amplitude (50–250pA) were used to determine the input resistance (Rm). Membrane input resistance was calculated from the peak voltage deflection obtained in response to the first -100pA step in each series of current steps. Transient (750 ms) depolarizing current steps of increasing amplitude (range 20–120pA) were used to determine the firing properties of interneurons. The duration of the action potential at half-amplitude (action potential half-width) was measured at the point halfway between the potential at which the action potential began to rise and its peak. The rising time of the action potential was measured from the point at 10% to the point at 90% of the action potential amplitude. The firing frequency adaptation was expressed as the ratio of the time interval of the last three spikes of the train to the one of the first three spikes and termed the adaptation ratio, which, if less than unity, indicates spike frequency adaptation occurred during the spike train. The amplitude of the fast afterhyperpolarization (fAHP) after an action potential was determined by measuring the peak downward deflection of the fAHP from the membrane potential at the point immediately before the action potential. The amplitude of the slow AHP (sAHP) after trains of 8–10 action potentials was measured as the peak downward deflection from the resting membrane potential immediately after the train.

Muscarinic current was isolated by addition of CNQX (50 μM), D-APV (50 μM), bicuculline (20 μM) and TTX (1 μM) to the bath. In order to investigate which subtype of muscarinic receptors mediate the responses, we also bath applied selective M1 receptor antagonist (Telenzepine, 100 nM) (Sigma, St. Louis, MO) or selective M3 receptor antagonist (4-DAMP, 1 μM) (Ascent) with muscarine (10 μM) (Sigma, St. Louis, MO). In order to validate telenzepine we used, whole-cell recordings were also made from prefrontal cortex layer V pyramidal cells.

3.3 RESULTS

3.3.1 Electrophysiological properties of BL interneurons

In the present study, we recorded 133 interneurons as well as pyramidal cells exclusively from BL. Consistent with previous studies (Rainnie et al., 2006, Woodruff and Sah, 2007b) four different action potential firing patterns of interneurons have been identified, which were bursting firing pattern (BF) (4/133), regular firing pattern (RF) (59/133), fast firing pattern (FF) (55/133), and stutter firing pattern (SF) (14/133) (Figure 3.1A). As has been described previously (Rainnie et al., 2006), we also observed that different types of interneurons had distinct electrophysiological properties. We injected inward current to depolarize cells to fire action potentials (Fig. 3.1A). BF interneurons fired a burst of action potentials at the beginning then quickly settled into regular firing patterns. RF interneurons fired action potentials regularly and at low firing frequency similar to pyramidal cells. FF interneurons fired at much higher frequency than regular firing interneurons. For SF interneurons, they fire action potentials similar to fast firing interneurons but stutteringly. When we injected hyperpolarizing current to the cells

(Figure 3.1B), we found that BF and RF interneurons but not FF or SF showed depolarizing sags in response to the injected transient depolarizing current , which were similar to pyramidal cells. This agreed with previous studies (Rainnie et al., 2006). Electrophysiological parameters of these recorded BL interneurons were summarized in (Table 3.1). In general, BF and RF showed similar electrophysiological properties, while FF and SF are alike. The resting membrane potentials of all types of interneurons were similar to pyramidal cells ($p>0.05$). All interneurons had much higher input resistances than pyramidal cells ($p<0.05$). Compared to BF and RF interneurons, FF and SF had lower input resistance, shorter action potential half width and rise time, much larger fast AHP and less action potential adaptation.

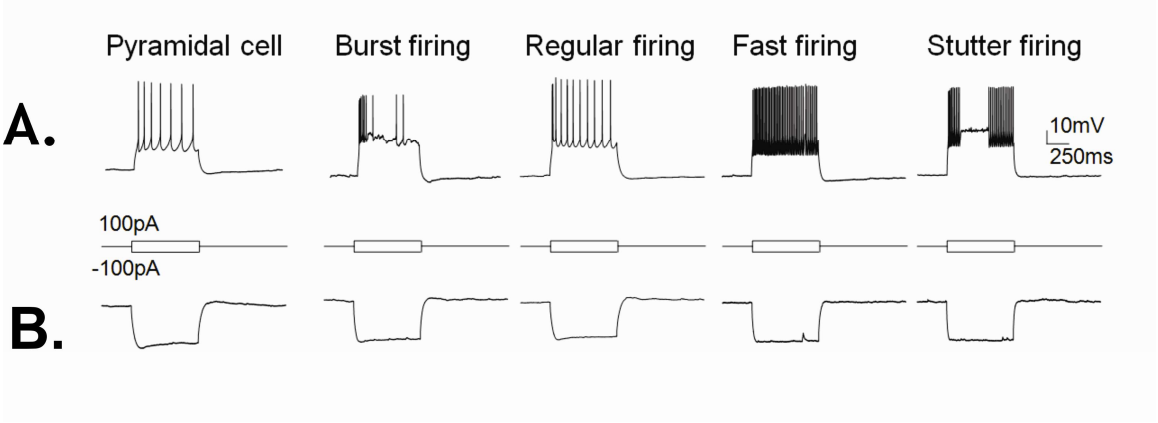


Figure 3.1 Action potential firing patterns of pyramidal cells and interneurons.

A. Based on voltage responses to depolarizing current step interneurons in BL can be divided into 4 groups, burst firing, regular firing, fast firing, and stutter firing interneurons. Different groups of BL interneurons also show different

B. Voltage responses to hyperpolarizing current step.

Table 3.1 Electrophysiological properties of pyramidal cells and interneurons

Firing Pattern	Vm (mV)	Rm (MΩ)	A.P. Halfwidth (msec)	A.P. rise time (msec)	Fast AHP (mV)	Slow AHP (mV)	Adaptation Ratio
Pyramidal Cells	-64 ± 4	137 ± 1	1.1 ± 0.1	0.46 ± 0.04	6 ± 1	1.4 ± 0.2	8.0 ± 4.7
BF (4/133)	-65 ± 3	344 ± 40	1.2 ± 0.2	0.55 ± 0.03	4 ± 2	5 ± 1	6.2 ± 1.5
RF (59/133)	-63 ± 1	369 ± 26	1.5 ± 0.1	0.56 ± 0.05	4 ± 1	2.2 ± 0.5	3.2 ± 0.5
FF (55/133)	-63 ± 2	224 ± 14 *	0.7 ± 0.1 *	0.36 ± 0.02 *	15 ± 1 *	1.8 ± 0.6 *	1.5 ± 0.1 *
SF (14/133)	-62 ± 3	215 ± 31 *	0.6 ± 0.1 *	0.31 ± 0.03 *	16 ± 2 *	1.7 ± 0.3 *	1.2 ± 0.1 *

This table exhibits the mean values and SEMs for the resting membrane potential (Vm), input resistance (Rm), action potential half width (A.P. Halfwidth), action potential rise time (A.P. rise time), fast afterhyperpolarization (Fast AHP), slow afterhyperpolarization (Slow AHP), and spike frequency adaptation (Adaptation Ratio) for four groups of BL interneurons with different firing patterns. Note that there is no significant difference of all parameters either between burst firing (BF) (4 cells) and regular firing (RF) (59 cells) interneurons or between fast firing (FF) (55 cells) and stutter firing (SF) (14 cells) interneurons ($p>0.05$). However all parameters except Vm of BF and RF are significantly different from FF and SF ($p<0.05$). All values are expressed as means ± SEMs.

3.3.2 Interneurons show differential responses to muscarine

In the hippocampus and neocortex, mAChRs agonists selectively modulate subpopulations of interneurons (Kawaguchi, 1997, Lawrence, 2008). For example, in neocortex, RF but not FF interneurons are depolarized by mAChRs agonists (Kawaguchi, 1997). Since BL amygdala and cortex are cytoarchitecturally similar (McDonald, 1982b, 1984), we examine whether it is also the case in the BL. Neurons were recorded in voltage clamp mode held at -70mV. Muscarinic currents were isolated by addition of CNQX (50 μM), D-APV (50 μM), bicuculline (20 μM) and TTX (1 μM) to the bath to block neurotransmission. Application of muscarine induced inward currents in a portion of recorded neurons (Figure 3.2A). Among all of the recorded interneurons, we found that 3 out 4 BF firing interneurons had muscarinic currents, 9 out 43 RF interneurons had muscarinic currents, 35 out 44 FF interneurons had muscarinic currents, and 5 out 8 SF interneurons had muscarinic currents (Figure 3.2B). Although a few percentage of RF interneurons had muscarinic currents, the amplitude was significant smaller than the ones in FF interneurons ($p<0.05$). The finding that FF interneurons rather than RF interneurons tend to have muscarinic currents is opposite from what have been found in neocortex, indicating that ACh modulates the amygdala differently from the cortex and hippocampus.

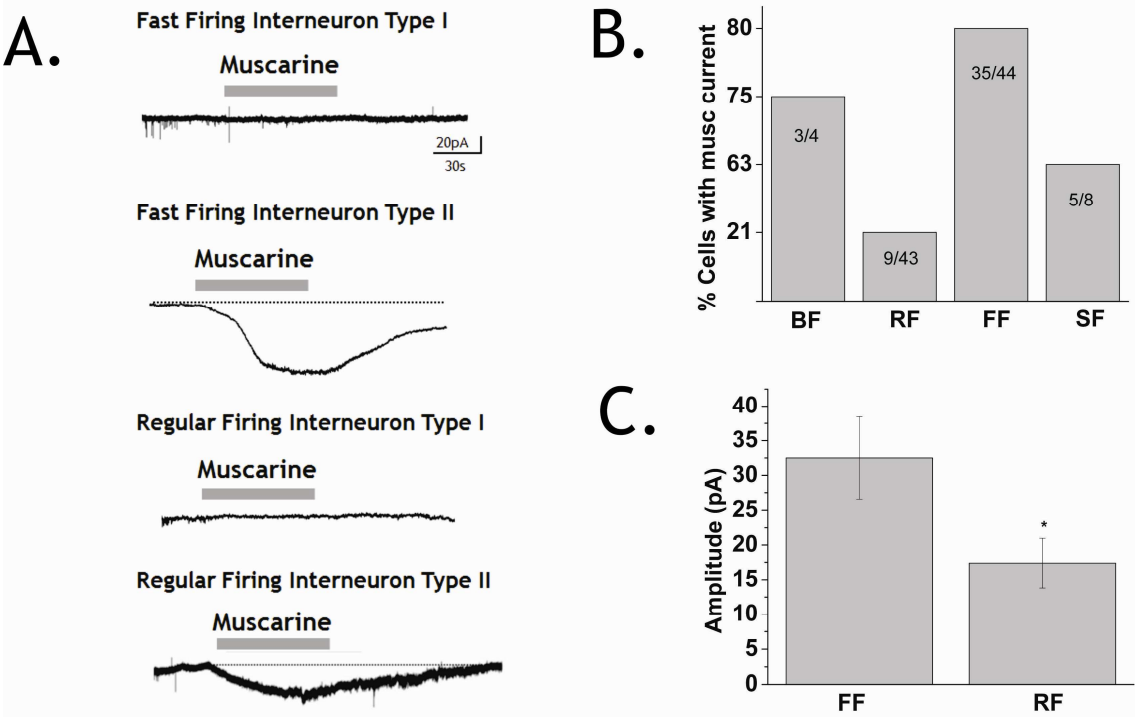


Figure 3.2 Muscarinic responses of BLA interneurons

Muscarinic current was isolated by addition of CNQX (50 μM), D-APV (50 μM), bicuculline (20 μM) and TTX (1 μM) to the bath. All cells were held at -70mV under voltage clamp.

A. Top to bottom: the first trace: one example of a fast firing interneuron which does not have muscarinic response (FF interneuron type I). The second trace: downward reflection represents muscarine (10 μM) mediated inward current in a FF interneuron and went back to baseline after muscarine was washed out (FF interneuron type II). The third trace: one example of a regular firing interneuron which does not have muscarinic current (RF interneuron type I). The fourth trace: One example of a regular firing interneuron which has muscarinic current (RF interneuron type II).

B. The bar graph shows the percentage of interneurons from each electrophysiological subpopulation that exhibit a muscarinic current. 3 out 4 BF have muscarinic current. 9 out 43 RF have muscarinic current. 35 out 44 FF have muscarinic current. 5 out 8 SS have muscarinic current.

C. The bar graph shows in cells with a muscarinic current the current amplitude is significantly smaller in regular firing (n=8) than in fast firing cells (n=18, $P < 0.05$).

3.3.3 Distinct muscarinic responses of FF and RF interneurons

We further examined muscarinic modulation of BL interneurons firing. The least depolarizing current step (750 ms) was injected to each recorded cell, which was just large enough to make the cell fire a train of spikes (Figure 3.3A). Membrane potentials were maintained at -60 mV by injecting current to the cells. Firing frequency, action potential adaptation, slow AHP, and action potential half width were examined before and after bath application of muscarine (10 μM; Figure 3.3B,C,D). Since the majority of interneurons we found in BL were RF and FF interneurons, in this study we focused on these two types. We further divided them into two categories determined by whether they had muscarinic currents or not. FF type I and RF type I interneurons did not have muscarinic currents, while FF type II and RF type II interneurons did. We found that muscarine (10 μM) did not affect firing frequency, action potential adaptation, slow AHP and action potential half width in FF type I (n=5) and RF type I interneurons (n=5) ($p>0.05$). Muscarine (10 μM) did not alter firing frequency, action potential adaptation, and action potential half width but significantly decreased slow AHP in FF type II interneurons (n=8) ($p<0.05$) (Figure. 3.3B,C,D). In contrast, muscarine (10 μM) significantly increased firing frequency and decreased action potential adaptation and slow AHP (n=5) ($p<0.05$) but did not change action potential half width in RF type II interneurons (n=5) ($p>0.05$) (Figure. 3.3B,C,D).

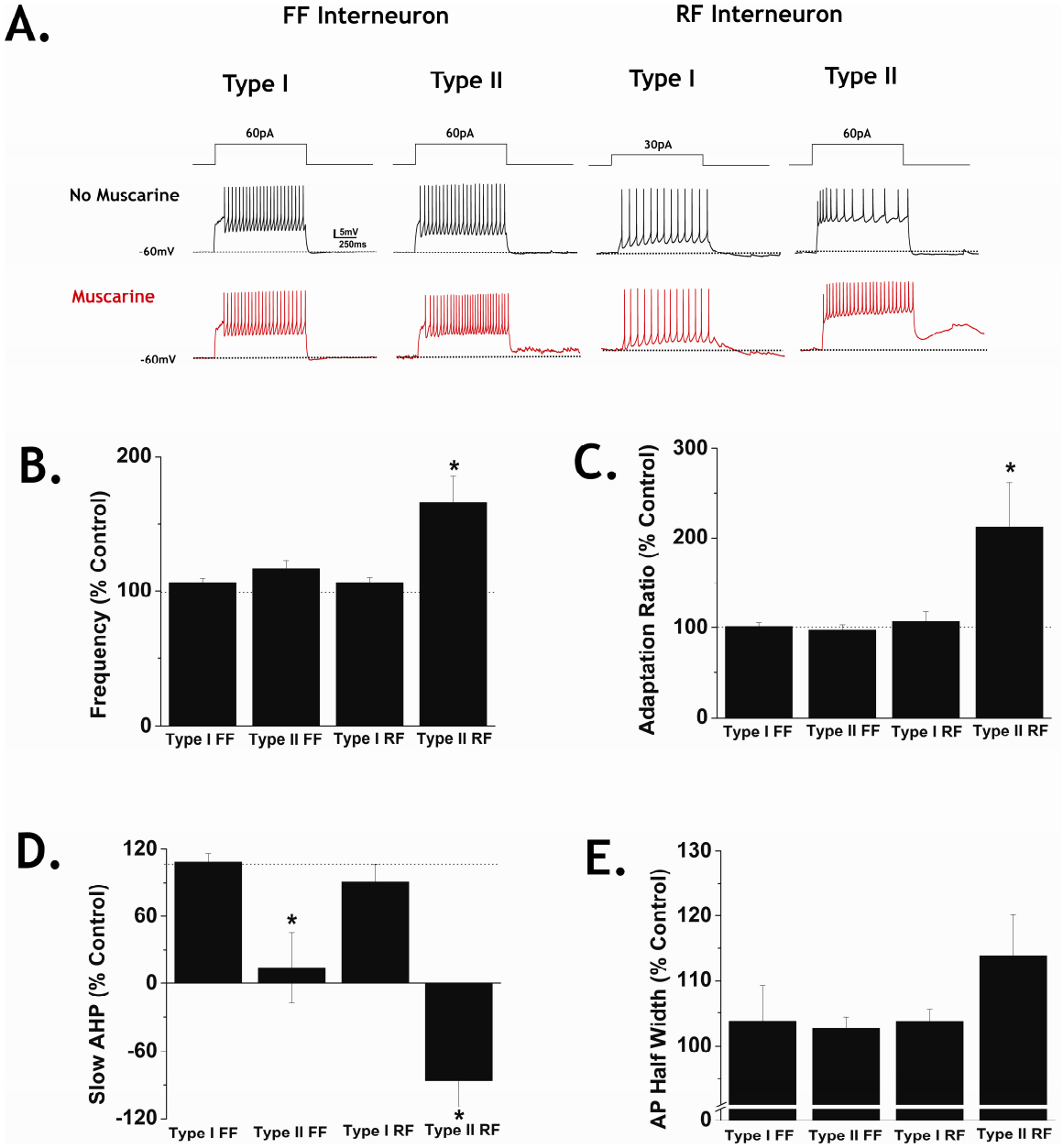

A.
FF Interneuron
RF Interneuron
Type I
Type II
Type I
Type II
60pA
60pA
30pA
60pA
No Muscarine
5mV
250ms
-60mV
Muscarine
-60mV
B.
Frequency (% Control)
200
100
0
*
Type I FF
Type II FF
Type I RF
Type II RF
C.
Adaptation Ratio (% Control)
300
200
100
0
*
Type I FF
Type II FF
Type I RF
Type II RF
D.
Slow AHP (% Control)
120
60
0
-60
-120
*
*
Type I FF
Type II FF
Type I RF
Type II RF
E.
AP Half Width (% Control)
130
120
110
100
0
Type I FF
Type II FF
Type I RF
Type II RF

Figure 3.3 Fast and regular firing interneurons exhibit distinct muscarinic response

A. Sample waveforms showing the effect of muscarine (10 μM) on fast firing and regular firing cells. Fast and regular firing cells are separated into those in which muscarine had no effect (Type I) and those in which it did (Type II).

B. Bath application of muscarine significantly increased firing frequency in type II RF interneurons (n=5), but not in type I FF (n=5), type II FF (n=8), type I RF (n=5) interneurons.

C. Bath application of muscarine significantly reduced action potential adaptation in type II RF interneurons (n=5), but not in type I FF (n=5), type II FF (n=8), type I RF (n=5) interneurons.

D. Bath application of muscarine significantly decreased slow AHP in type II FF (n=8) and type II RF interneurons (n=5), but not in type I FF (n=5) or type I RF (n=5) interneurons.

E. Bath application of muscarine did not significantly change action potential half width (AP half width) in any types of interneurons (type I FF (n=5), type II FF (n=8), type I RF (n=5), type II RF (n=5)).

3.3.4 M3, not M1 muscarinic receptors contribute to the muscarinic response of interneurons

We then tested which subtype of muscarinic receptors mediate the muscarinic currents found in some interneurons in the BL by bath applying selective M1 muscarinic receptor antagonist, telenzepine (TZP) (100 nM), or M3 muscarinic receptor antagonist, 4-DAMP (1 µM) with muscarine (10 µM). We found that 4-DAMP (1 µM) ($n=7$, $p<0.01$) but not TZP (100 nM) ($n=6$, $p>0.05$) blocked the muscarinic currents in RF interneurons (Figure 3.4A). Similarly, in FF interneurons, 4-DAMP (1 µM) significantly inhibited the muscarinic currents ($n=5$, $p<0.01$), however TZP (100 nM) did not affect them ($n=5$, $p>0.05$) (Figure 3.4B). We further confirmed the effectiveness of the batch of TZP we used by applying it to PFC layer V pyramidal cells which have been shown having M1 receptor mediated currents (Gulledge et al., 2009). We found that the same batch of TZP (100 nM) almost completely blocked the muscarinic currents in PFC layer V pyramidal cells ($n=5$, $p<0.01$) (Figure 3.4C).

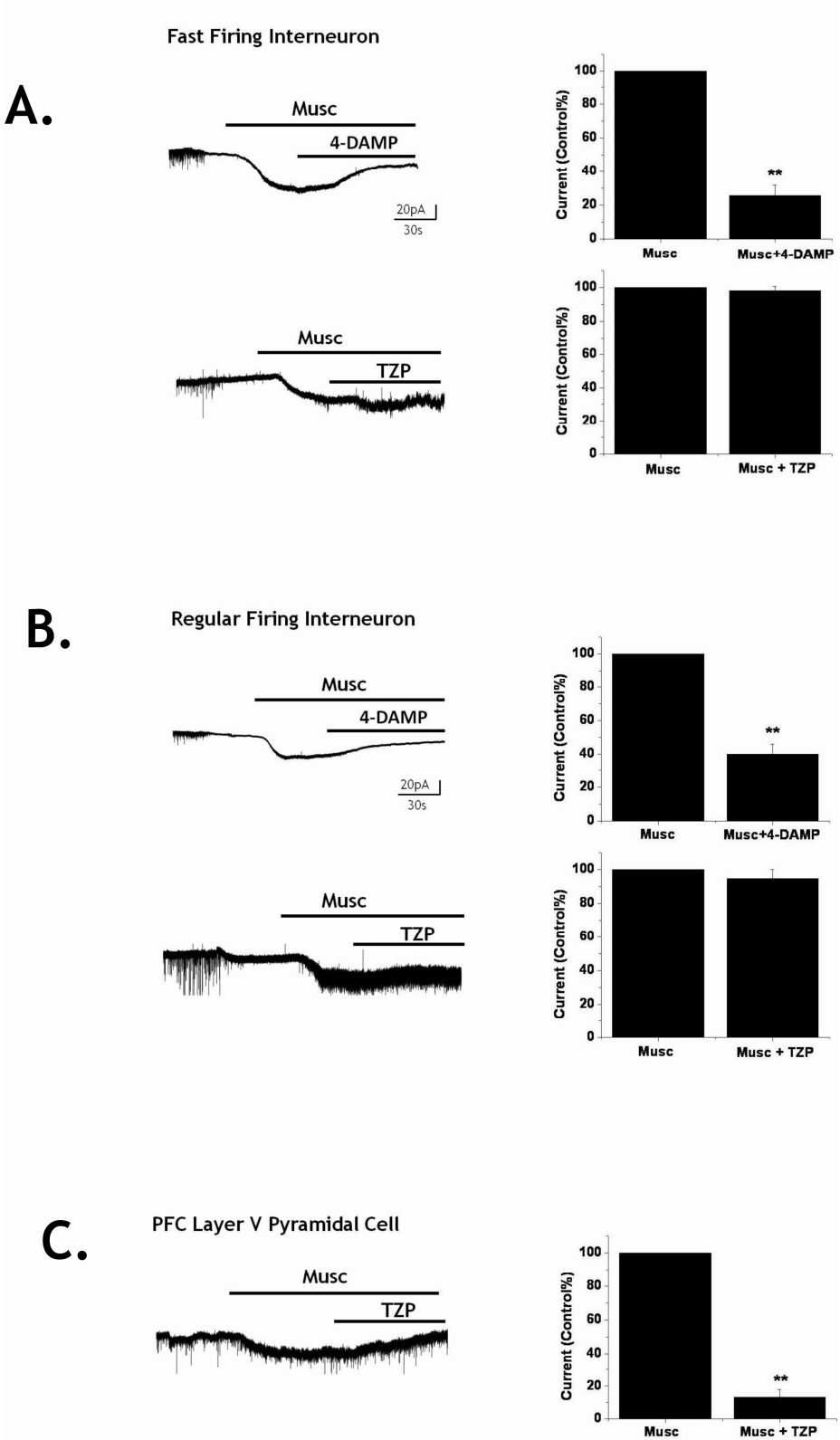

A.
Fast Firing Interneuron
Musc
4-DAMP
20pA
30s
Musc
TZP
Current (Control%)
100
80
60
40
20
0
**
Musc
Musc+4-DAMP
Musc
Musc + TZP
B.
Regular Firing Interneuron
Musc
4-DAMP
20pA
30s
Musc
TZP
Musc
Musc+4-DAMP
Musc
Musc + TZP
C.
PFC Layer V Pyramidal Cell
Musc
TZP
Musc
Musc + TZP

Figure 3.4 M3, not M1 muscarinic receptors contribute to the muscarinic response of interneurons

A. Left, representative examples of bath application of 4-DAMP (1μM) and telenzepine (TZP) (100nM) blocks and does not affect FF interneuron muscarinic current, respectively. Top right, the mean value of FF interneurons muscarinic current was significantly reduced ($p < 0.01$, n=7) and unchanged ($p > 0.05$, n=6) by 4-DAMP and TZP, respectively.

B. Left, representative examples of bath application of 4-DAMP (1μM) and telenzepine (TZP) (100nM) blocks and does not affect RF interneuron muscarinic current, respectively. Top right, the mean value of RF interneurons muscarinic current was significantly reduced ($p < 0.01$, n=5) and unchanged ($p > 0.05$, n=5) by 4-DAMP and TZP, respectively.

C. Left, representative example of bath application of TZP blocked PFC layer V pyramidal cell muscarinic response. Right, the mean value of PFC layer V pyramidal cells muscarinic response was significantly reduced by TZP ($p < 0.01$, n=5).

3.3.5 Neurochemical identification of subpopulations of BL interneurons

A total of 97 interneurons were processed for neurochemical identification. In each case biocytin filled the soma and the entire dendritic arborization of these neurons. They were identified as interneurons on the basis of having a nonpyramidal form and aspiny or spine-sparse dendrites (Figure 3.5). The extent of axonal biocytin filling varied from a total lack of filling (e.g., Fig. 3.5A) to extensive filling of a dense local axonal arborization (e.g., Figure 3.5C,D). 23% (22/97) of these morphologically identified interneurons were immunoreactive for one of the three interneuronal markers investigated (Figure 3.6). Immunostaining was usually confined to the soma, but was occasionally seen in proximal dendrites. Basic electrophysiological properties, including firing patterns, were determined for all 97 interneurons, including the 22 neurochemically-identified interneurons.

We first examined the correlation between firing patterns and neurochemical markers expression. Out of 14 PV+ interneurons, 5 were RF interneurons while 9 were FF interneurons. In contrast, 6 out of 7 SOM+ interneurons were RF and only 1 out of 7 SOM+ interneurons were FF. (Fig. 3.7A). These results demonstrate that PV+ interneurons are heterogeneous, and contain RF and FF interneuronal types, which agree with previous studies (Rainnie et al., 2006, Woodruff and Sah, 2007b). FF interneurons most likely are PV+. SOM+ interneurons are more homogeneous, which mostly have RF pattern. Response to muscarine is interneuron type dependent. Collectively, 8 out of 9 (89%) PV+ FF interneurons respond to muscarine, while only 1 out 5 (20%) PV+ RF interneurons had muscarinic current ($p<0.05$). (Figure 3.7B). All SOM+ interneurons but only 1 SOM+ RF interneuron do not exhibited muscarinic current ($p<0.01$). (Figure

3.7B). Taken together, we have identified a subset of interneurons, PV+ FF interneurons, selectively responding to muscarine.

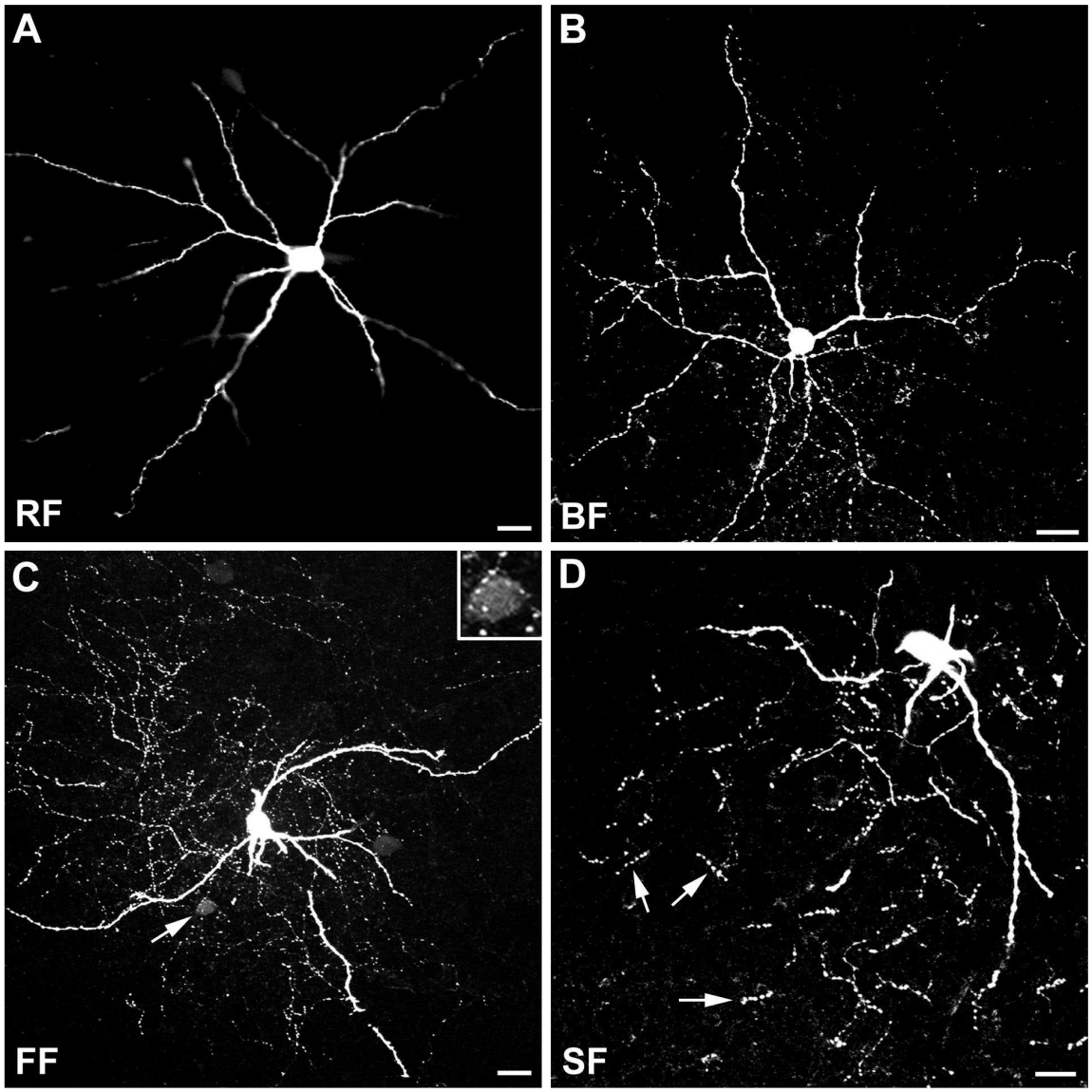


Figure 3.5 Photomicrographs illustrating the morphology of biocytin-filled BL interneurons with different firing patterns.

All images are Z series reconstructions except A.

A. Regular firing interneuron that exhibited no muscarinic response. Only the cell body and dendrites of this neuron were filled. This interneuron did not express any of the interneuronal markers investigated.

B. Burst firing interneuron that exhibited a muscarinic response. The fine punctate labeling between the dendrites represents the axonal arborization of this neuron. This cell exhibited CR-ir (not shown).

C. Fast firing interneuron that exhibited a muscarinic response. The thin beaded processes surrounding this neuron represent the axonal arborization. This cell exhibited PV-ir (see Fig. 6C). Some axonal segments formed curvilinear patterns suggesting that the axon is forming multiple contacts with cell bodies, typical of PV+ basket cells. Arrow indicates a neuronal cell body that was lightly stained, perhaps due to uptake of biocytin that had diffused from the region surrounding the recorded cell. The axon of the recorded cell made multiple contacts with this cell body (see inset).

D. Stutter firing interneuron that exhibited a muscarinic response. The thin beaded processes surrounding this neuron represent the axonal arborization. The linear arrangement of the terminal axon segments (arrows) suggests that they may innervate the axon initial segments of pyramidal cells, typical of axo-axonic chandelier cells. This interneuron exhibited PV-ir (see Fig. 6D). Scale bars = 20 µm.

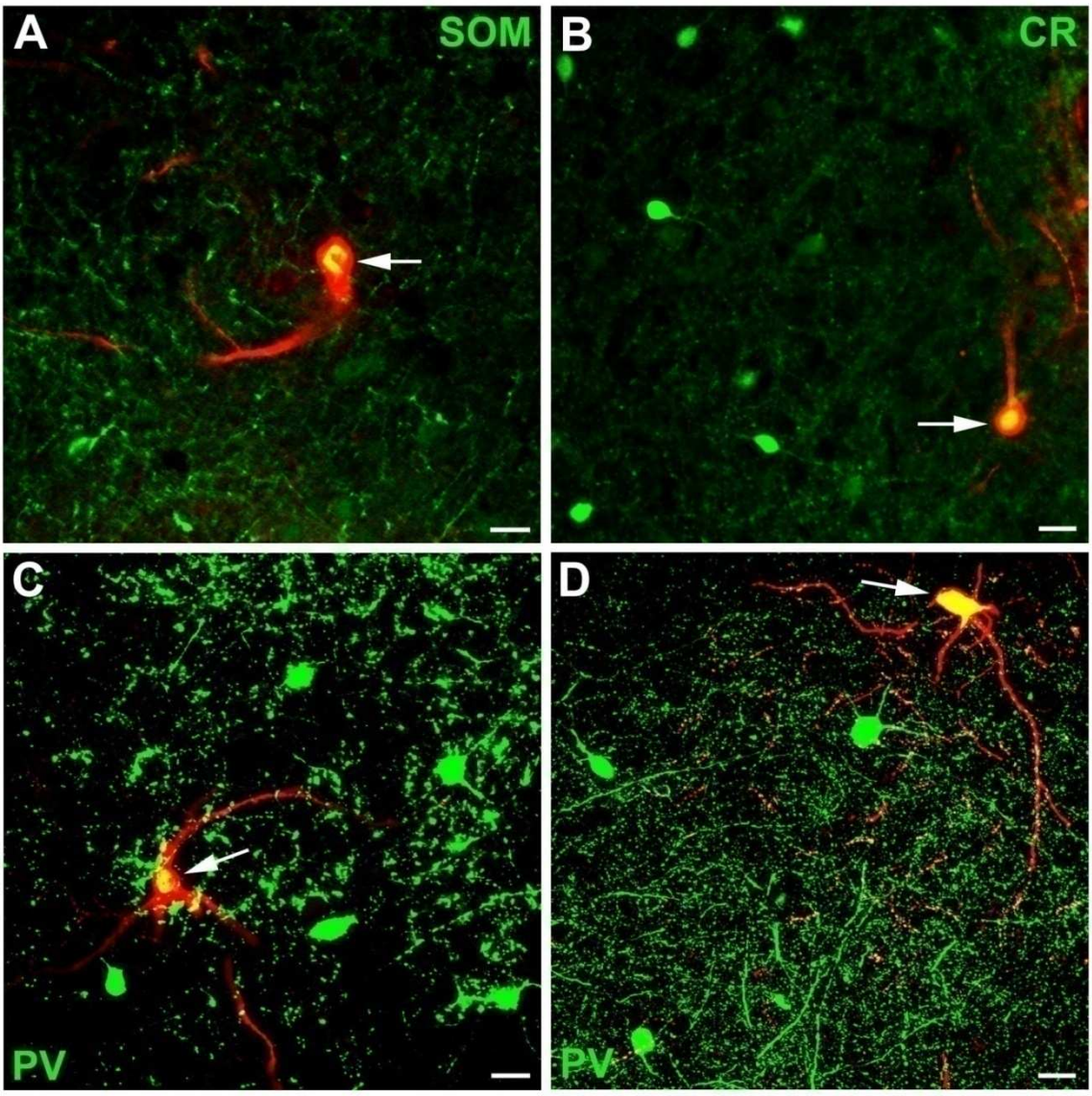

A
SOM
B
CR
C
PV
D
PV

Figure 3.6 Expression of interneuronal markers in biocytin-filled BL interneurons.

Although these were all triple-labeled preparations, only biocytin and the interneuronal marker expressed by the recorded neuron is visualized in these images. Biocytin is red and interneuronal markers (PV, SOM, or CR) are green. Yellow indicates colocalization of biocytin and the interneuronal marker.

A. Regular firing SOM+ interneuron that exhibited no muscarinic response.

B. Burst firing CR+ interneuron that exhibited a muscarinic response.

C. Fast firing PV+ interneuron that exhibited a muscarinic response (see Figure 3.5C for a Z series reconstruction of this cell without the marker label).

D. Stutter firing PV+ interneuron that exhibited a muscarinic response. This field was reconstructed from a Z series (see Figure 3.5D for a Z series reconstruction of this cell without the marker label). Scale bars = 20 µm.

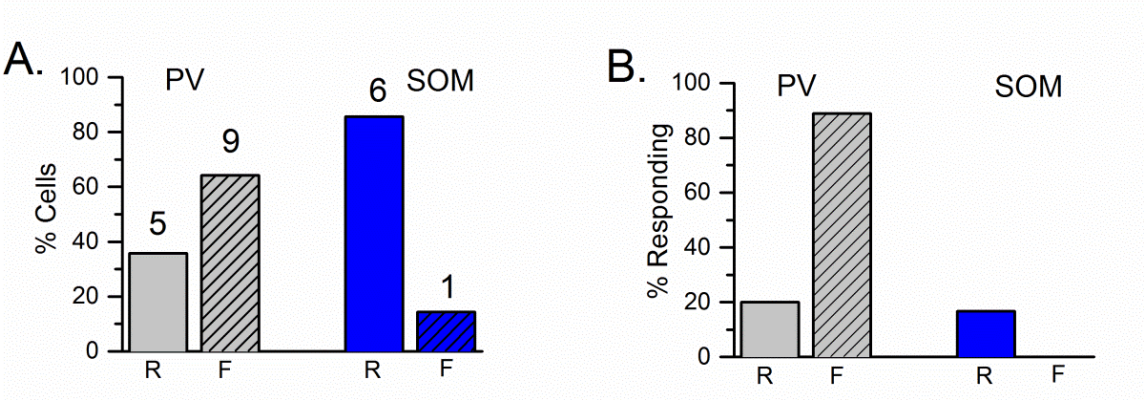


Figure 3.7 Muscarine preferentially depolarizes PV and FF interneurons, but not SOM interneurons.

A. a bar graph showing that out of 14 recorded PV interneurons 5 are regular firing and 9 are fast firing. Out of 7 recorded SOM interneurons there are 6 regular firing interneurons and only 1 fast firing interneuron.

B. a bar graph showing that there are only 20% of PV RF interneurons responding to muscarine (10 μM), while there are 89% of PV FF interneurons having muscarinic currents ($p<0.05$). Only 1 SOM RF interneuron out of total 7 SOM interneurons responds to muscarine (10 μM) ($p<0.01$).

3.4 DISCUSSION

In the present study, we used whole cell recording and immunohistochemistry to study the correlations among cell markers, electrophysiological properties, and response to muscarine. We found that PV FF but not SOM or RF interneurons are preferentially engaged by mAChRs. Muscarine modulates BL interneurons in a manner that is opposite to the cortex.

3.4.1 Correlations between firing patterns and neurochemical phenotypes in the BL interneurons

Interneurons in the BLA can be divided into several subpopulations based on either firing patterns or neurochemical markers expression (Spampanato et al., 2011). Previous studies have shown BL PV interneurons in mice are heterogeneous having diverse firing patterns (Woodruff and Sah, 2007b). Another classification study demonstrated that in lateral amygdala there was no strong correlation between firing patterns and neurochemical markers. However, SOM+ interneurons tended to be delayed stutter firing pattern (Sosulina et al., 2010). Another study found that in rat BL amygdala PV+ interneurons tended to be burst firing and stutter firing patterns (Rainnie et al., 2006). In the present study, we showed that PV+ interneurons included RF, FF, and SF pattern, however SOM+ interneurons tended to be RF pattern. This agrees with the previous studies in mouse but is slightly different from Rainnie's study. The difference could be caused by sampling issues. In this study, we mostly recovered RF and FF interneurons, while in Rainnie's study, the interneurons they recovered were BF and SF (Rainnie et al., 2006). Therefore, it is very likely PV interneurons include all four types

of firing patterns. In the present study, PV interneurons were found to be the most common type, which made up about 65% of the total neuromarker-identified interneurons. This also agrees with previous anatomical studies which suggested that about 50% interneurons in the BLA amygdala of rats were PV+ (McDonald and Betette, 2001, McDonald and Mascagni, 2001a). PV interneurons in BLA amygdala form synapses onto both the perisomatic and the distal dendritic domains as well as spines of the pyramidal cells (Muller et al., 2006). Perisomatic inhibition can tightly control action potential generation of pyramidal cells, which in turn can regulate the output of the pyramidal cells (Freund and Katona, 2007). Whereas interneurons which project to distal dendrites and spines of pyramidal cells modulate activity and plasticity of pyramidal cells, thereby can filter and regulate the information inputs to the pyramidal cells (Miles et al., 1996). Like in the cortex and hippocampus PV FF interneurons are engaged in perisomatic inhibition to generate neuronal oscillations, it might be possible that in BLA amygdala PV FF interneurons preferentially project to perisomatic domain of pyramidal cells, while PV RF interneurons project to distal dendritic domain of pyramidal cells. Further EM level studies need to be done to test this hypothesis. Unlike PV interneurons, SOM interneurons in the BLA amygdala preferentially form synapses onto distal dendritic domain of pyramidal cells (Muller et al., 2007a), which are exclusively engaged in regulation of inputs of pyramidal cells. Interestingly, we found that SOM+ interneurons tended to RF.

3.4.2 Muscarine differentially modulates interneurons in the BL amygdala

In the cortex and hippocampus, subpopulations of interneurons are sensitive to mAChRs agonists (Kawaguchi, 1997, Xiang et al., 1998, Gulledge et al., 2007,

Lawrence, 2008). In the cortex, Kawaguchi found that SOM+ interneurons and RF interneurons were noticeably depolarized by muscarinic agonists whereas FF or PV interneurons were not (Kawaguchi, 1997). Considering amygdala is a cortical like structure (McDonald, 1992b), it is very surprising that it is opposite from the cortex. This finding also agrees with one anatomical study showing that PV interneurons received basal forebrain cholinergic innervation (Muller et al., 2011). This indicates that ACh modulates the amygdala function differently from the neocortex. In the hippocampus, which subpopulations of interneurons respond to cholinergic signaling still remains to be determined (Parra et al., 1998, McQuiston and Madison, 1999, Widmer et al., 2006). However, one recent study found that in the hippocampus mAChRs agonists depolarize both PV+ and CCK+ basket cells but differentially modulates their electrophysiological properties (Cea-del Rio et al., 2010). Muscarine regulates CCK+ basket cells through M1 and M3 receptors, while modulates PV basket cells solely through M1 receptors (Cea-del Rio et al., 2010). In contrast, muscarine only changed slow AHP without affecting firing frequency, firing adaptation or action potential waveform of type II FF interneurons in the BL. Whereas it altered firing frequency, slow AHP and firing adaptation without affecting action potential waveform of type II RF interneurons. The differences might be caused by the BL interneurons have different ion channel profile such K^+ channels from the hippocampal interneurons. Moreover we found that muscarinic response in the BL interneurons were mediated by M3 but not M1 receptors. This finding agrees with the previous anatomical studies showing that M1 receptors were mostly expressed on pyramidal cells but not on interneurons (McDonald and Mascagni, 2010). It is also consistent with previous electrophysiology studies (Yajeya et al., 1999, Yajeya et al.,

2000). We do not exclude the possibility that cholinergic inputs would also modulate interneuronal function through other ways. For example, by activation of presynaptic muscarinic receptors on interneurons terminals, mAChRs activation would regulate GABA release. In fact, SOM+ interneurons axons express M2 receptors (McDonald and Mascagni, 2011).

3.4.3 Functional relevance of muscarinic modulation of PV FF interneurons in the BL

Neuronal network oscillations correlate with specific brain state and behavior, which are generated by interaction between pyramidal cells and interneurons (Klausberger et al., 2003, Klausberger et al., 2005). PV FF interneurons faithfully control the pyramidal cells fire action potentials through tight perisomatic inhibition. Therefore, PV+ FF interneurons are involved in generation of neuronal oscillations, which in turn regulate neuronal network function (Cardin et al., 2009a). For example, theta and gamma oscillations correlate with the learning state of the brain (Klausberger et al., 2003, Klausberger et al., 2005, Popescu et al., 2009) and enhance information processing (Sohal et al., 2009). In the BL amygdala neurons activities show oscillatory rhythms during fear learning (Bauer et al., 2007a, Popescu et al., 2009). Here we demonstrated that muscarine preferentially modulated PV FF interneurons in the BL, suggesting that cholinergic transmission can potentially regulate neuronal oscillations in the BL amygdala. In fact, ACh is released during learning (Lee et al., 2005) and thereby induces neuronal oscillations (Fisahn et al., 1998, Nagode et al., 2011). All the evidence suggests that cholinergic signaling can affect amygdala function at least through modulation of neuronal oscillations via exciting PV FF interneurons. Cholinergic transmission is also important for synaptic plasticity, particularly spike timing-dependent plasticity (Gu and

Yakel, 2011). Spike timing-dependent plasticity is involved in fear learning in the amygdala (Pape and Pare, 2010). The timing of ACh release relative to glutamate inputs determines LTP or LTD induced (Gu and Yakel, 2011). In addition to postsynaptic muscarinic receptors, presynaptic muscarinic and pre and postsynaptic nicotinic receptors are also involved in cholinergic modulation of spike timing-dependent plasticity (Gu and Yakel, 2011). Future studies on presynaptic mAChRs as well as nAChRs are needed to understand how cholinergic signaling modulates synaptic plasticity in the amygdala.

CHAPTER 4

GENERATION OF SYNCHRONIZED INHIBITION IN BL PNS BY ACTIVATION OF MACHRS

4.1 INTRODUCTION

Oscillatory activities recorded by electroencephalogram (EEG) and local field potential (LFP) has been shown to be correlated to distinct patterns of behaviors (Singer, 1999). These oscillations are thought to be important to integrate sensory inputs, allow binding of information from different brain areas and facilitate synaptic plasticity in target downstream structures. Neurons in the BL oscillate rhythmically during emotional processing. Synchrony at theta frequency between the BLA, hippocampus and prefrontal cortex are increased during fear memory acquisition and retrieval but declined during fear extinction learning (Sangha et al., 2009, Lesting et al., 2011). Disruption of theta coupling through electrical stimulation impaired fear conditioning and extinction (Lesting et al., 2011).

Inhibition has been shown to play a key role in generation of rhythmic oscillations. Intracellular recordings during theta activity have revealed that perisomatic inhibition contributes to intracellular theta oscillatory activity. Oscillatory activity in a neuronal network can be generated by coordinated GABAergic inhibitory IPSPs across

many neurons to synchronize their firing (Buzsaki and Chrobak, 1995, Benardo, 1997, Fisahn et al., 1998). Synchronized IPSPs set time window for pyramidal cells to fire and phase reset their firing by producing rebound excitation.

Similar as seen in the cortex and hippocampus, BL interneurons exhibit diverse morphological, electrophysiological, and synaptic properties. They have different firing patterns, including RF and FF. Based on neurochemical markers expression, there are four types: those containing PV (McDonald and Betette, 2001), those containing SOM (McDonald and Mascagni, 2002), those containing CCK with either calretinin or VIP (Mascagni and McDonald, 2003), and those containing only CCK (Mascagni and McDonald, 2003). Of these types, PV+ interneurons make up about 40% of total interneuronal population, which project to either to the somatic areas and distal dendrites of BL pyramidal cells (Muller et al., 2006). In contrast, SOM+ interneurons mainly synapse onto distal dendrites (Muller et al., 2007a). Those SOM+ distal dendritic projection interneurons are thought to modulate synaptic plasticity induction in BL PNs whereas perisomatic projection interneurons, such as PV+ interneurons may regulate BL PNs firing and output (Cobb et al., 1995, Miles et al., 1996).

Spontaneous large amplitude IPSPs/IPSCs at around 1 Hz that are synchronized across BL PNs have been reported (Popescu and Pare, 2011, Ryan et al., 2012). However, their origin and functional significance are not clear. In the cortex, metabotropic receptors, including mAChRs, agonists selectively depolarize a network of electrically coupled interneurons generate synchronized IPSP in neighboring neurons and coordinate the activity of local assemblies of pyramidal cells (Beierlein et al., 2000). The BL receives dense cholinergic innervation from basal forebrain, providing a basis for

mAChRs regulation of oscillatory behavior in this region (Carlsen et al., 1985). However, the mechanism through which BL neurons synchronize their activities is poorly understood. In the present study, we explored the role of mAChRs in generating synchronized firing of BL pyramidal cells and underlying mechanisms.

4.2 Materials and Methods

Brain slices were prepared from Sprague Dawley rats (15-30 days old) as described in chapter 2. Dual cells recordings were made in between an interneuron in current clamp and a PN in voltage clamp. IPSCs were recorded was symmetrical Cl^- internal solution containing (in mM) KCl 135, HEPES 10, Na-ATP 2, Na-GTP 0.2, $MgCl_2$ 2, EGTA 0.1, and PH 7.3. Carbenoxolone (100 µM) (Sigma, St Louis, MO) was used to block gap junctions.

4.3 Results

4.3.1 Muscarine induces theta oscillations in the BL PNs

When recorded in aCSF under current clamp, BL PNs in brain slices often show irregular membrane potential oscillation in low frequency and low power. (Figure 4.1A and Figure 4.1E,F). The average frequency at peak power of this oscillation is 1.0 ± 0.2 Hz with the power of 0.16 ± 0.03 mV2 /Hz (n=5). A brief (2 s) puff of muscarine (50 µM) to the slices induced rhythmic membrane potential oscillation with higher frequency and power (7.1 ± 0.4 Hz and 0.61 ± 0.05 mV2 /Hz, n=5) (Fig. 4.2B and Fig.4.1E,F), compared to control baseline. Blockade of glutamatergic transmission by bath application of CNQX (20 µM) and D-APV (50 µM) did not disrupt the muscarine-induce membrane

potential oscillations in BL PN, but slightly reduced the frequency of the oscillations without affecting the peak powers (5.1 ± 0.2 Hz and 0.58 ± 0.07 mV2 /Hz). (Figure 4.1C,E,F). This suggests that despite glutamatergic transmission that contributes to the membrane potential oscillations, muscarine is able to generate theta oscillation in BL pyramidal cells independent of glutamatergic synaptic inputs. However, they were completely blocked by bicuculline (20 µM) (Figure 4.1D,E,F), suggesting that the membrane fluctuations observed here may be rhythmic large GABAergic IPSPs induced by muscarine. We then recorded IPSCs in BL PNs under voltage clamp mode. Similar as seen in current clamp, in aCSF, in addition to a lot of small spontaneous IPSCs with amplitude of less than 50 pA, some large IPSPs were with large amplitude more than 200 pA) at 0.9 ± 0.3 Hz (n=9). (Figure 4.2A black waveform, Figure 4.2G). A 2 s puff of muscarine (50 µM) induced large rhythmic IPSCs at theta frequency (6.5 ± 1.7 Hz, n=9). (Figure 4.2A blue waveform, Figure 4.1G). Bath application of CNQX (20 µM) and D-APV (50 µM) eliminated spontaneous large IPSCs in baseline control but did not abolish muscarine-induced rhythmic IPSCs (5.6 ± 0.9 Hz, n=9). (Figure 4.2C,G). In contrast, bath application of either TTX (1 µM) (Figure 4.2E,G) or bicuculline (20 µM) (Figure 4.2F,G) was able to completely block both spontaneous, low frequency large IPSCs in baseline control and muscarine-induced theta frequency IPSCs.

Previous studies have shown that BL PNs can be depolarized and fire action potentials by mAChRs agonists when their membrane potentials are at around -60 mV slightly above their resting membrane potential (Washburn and Moises, 1992b). Moreover inhibition has been shown to be able to entrain pyramidal firing thereby provides a mechanism of PNs firing synchrony. Therefore, we hypothesized that

muscarine depolarizes BL PNs to fire and meanwhile muscarine-induced large IPSPs can entrain and phase-set BLA PNs firing. BL PNs were recorded in current clamp and their membrane potentials were adjusted to -60 mV by current injection. A 2 s puff of muscarine (50 μM) depolarized BL PNs to fire spikes which were often appeared in between two single IPSPs. Thus the frequency of APs was at theta frequency band (4.9 ± 1.1 Hz, n=6), which were set by the large rhythmic IPSPs. (Figure 4.3A,C). Blockade GABAergic transmission by bicuculline increased firing frequency to beta band (16.4 ± 2.8 Hz, $p<0.05$, n=6). (Figure 4.3B,C).

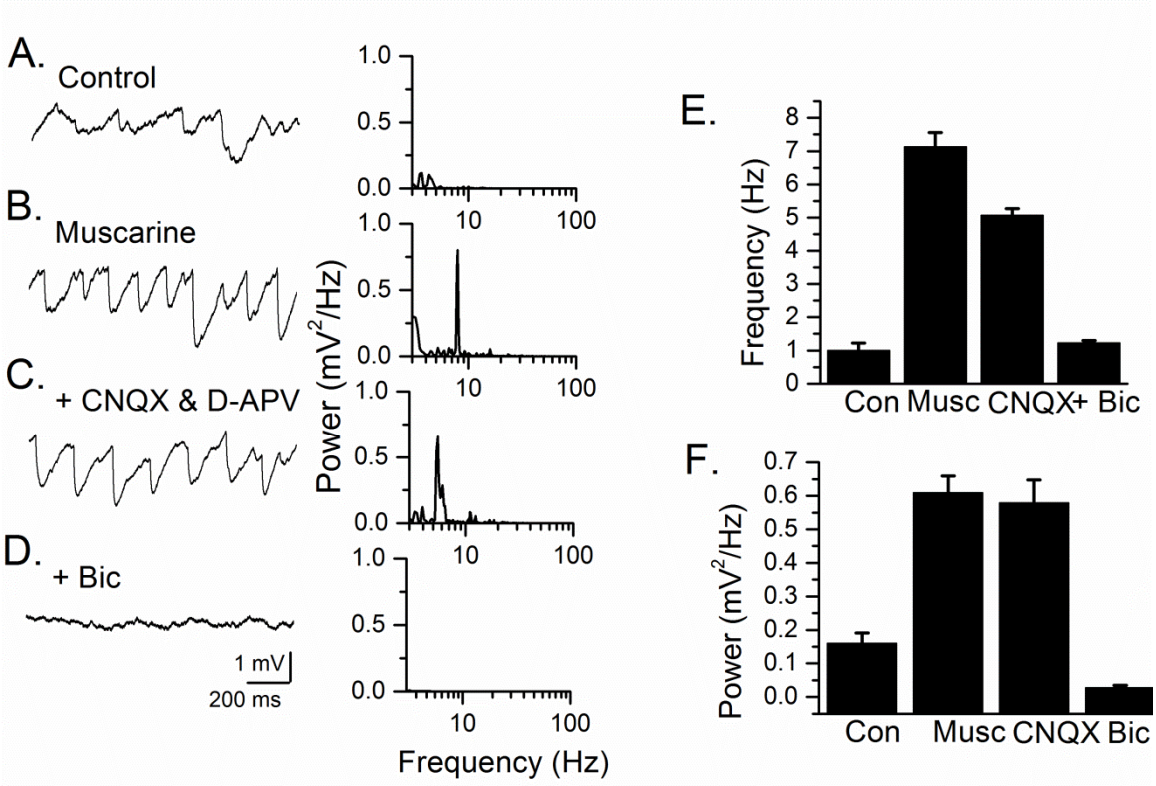


Figure 4.1 Muscarine-induced theta oscillations in the BL.

A. Control baseline. Membrane potential was recorded in a BL pyramidal cell under current clamp in aCSF. Power spectrum analysis is showing little membrane potential fluctuation.

B. Muscarine (10 µM) induces theta frequency oscillation. Membrane potential was recorded from the same cell in the presence of muscarine (10 µM). Power spectrum analysis shows the frequency of membrane fluctuation at peak power is 7.8 Hz.

C. Rhythm persists after addition of CNQX (20 µM) and D-APV (50 µM). Power spectrum analysis shows the peak at 5.5 Hz.

D. Bicuculline (20 µM) blocks the oscillation. Left (A-D): representative waveforms of membrane potential from a same BL pyramidal cell in the presence of different drugs. Right (A-D): Power spectrums showing the powers at different frequencies.

E and F. Two bar graphs showing the frequencies of the oscillations at peak power, in aCSF (1.0 ± 0.23 Hz at 0.16 ± 0.032 mV2 /Hz), in muscarine (10 µM) (7.1 ± 0.43 Hz at 0.61 ± 0.051 mV2 /Hz), in muscarine (10µM), CNQX (20 µM), and D-APV (50 µM) (5.1 ± 0.21 Hz at 0.58 ± 0.069 mV2 /Hz), in muscarine (10 µM) and bicuculline (20 µM) (1.2 ± 0.08 Hz 0.03 ± 0.007 mV2 /Hz). (n=5).

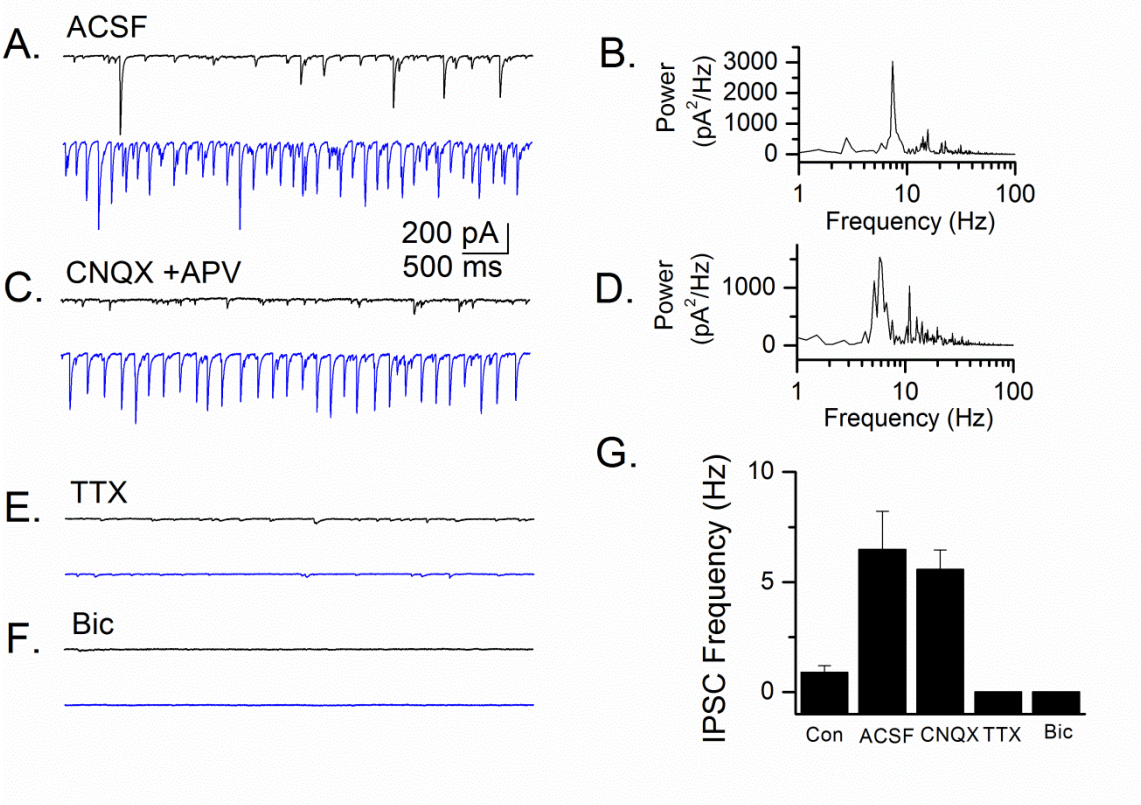


Figure 4.2 Muscarine induces IPSCs with large amplitude at theta frequency independent of glutamatergic transmission.

Recordings were made in symmetrical chloride internal solution.

A. a representative waveform of rhythmic large IPSCs in a BL pyramidal cell induced by in aCSF puff of muscarine (50 µM).

B. power spectrum analysis showing its frequency at peak power is at 7.3 Hz.

C. representative waveform showing muscarine-induced rhythmic IPSCs in the presence of CNQX (20 µM) and D-APV (50 µM).

D. power spectrum analysis showing its frequency at peak power is at 5.8 Hz.

E and F. representative waveforms showing muscarine-induced rhythmic IPSCs were blocked by TTX (1 µM) and Bicuculline (20 µM), respectively.

G. a bar graph showing the frequency of large rhythmic IPSCs in control baseline in aCSF and after puff of muscarine (50 µM) in aCSF, in the presence of CNQX (20 µM) and D-APV (50 µM), TTX (1 µM), and Bicuculline (20 µM). (n=5).

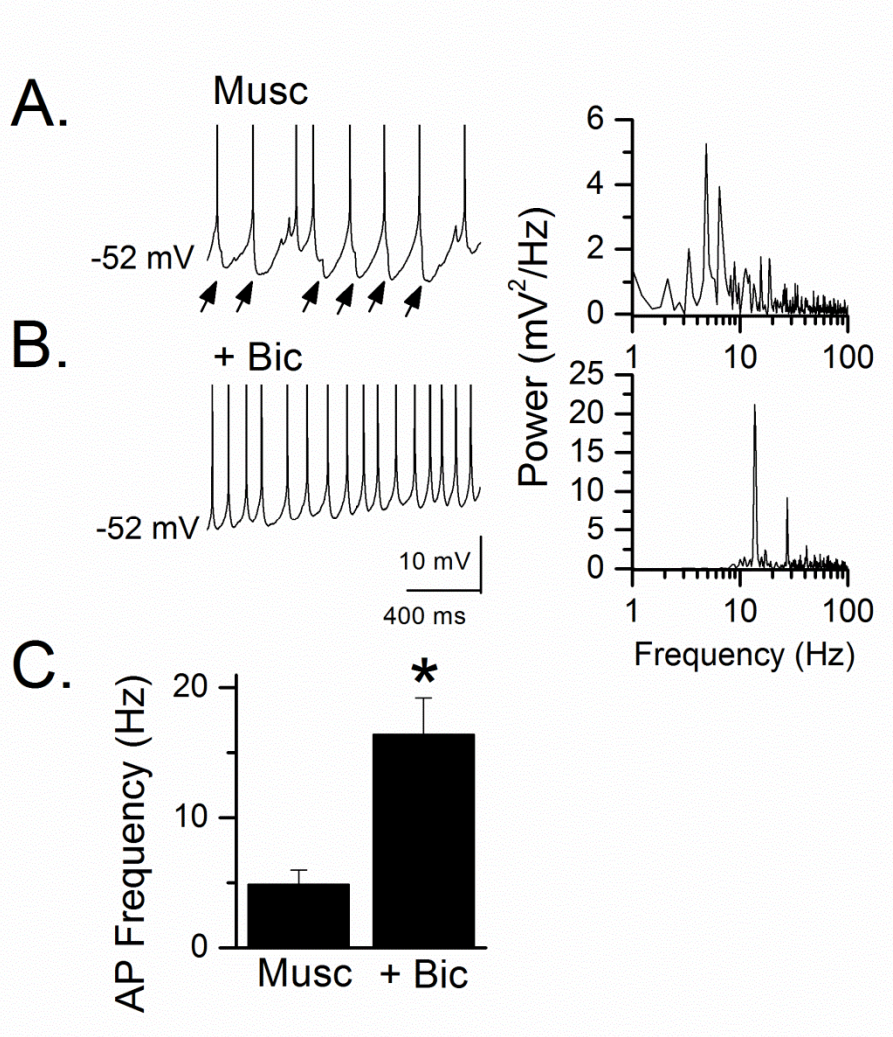


Figure 4.3 Firing of pyramidal cells was entrained at theta frequency by muscarine-induced IPSPs. Responses recorded in low chloride.

A. Left: puff application of muscarine (50 µM) depolarizes a PN in BL to fire APs. APs are entrained by IPSPs (arrows). Right: power spectrum analysis showing the APs frequency is at theta band.

B. Bicuculline blocks these compound IPSPs (Left), increasing AP firing frequency to beta/gamma band (Right).

C. a bar graph showing in the presence of bicuculline (20 µM) muscarine-induced AP frequency is significantly higher than in control. ($p<0.05$, $n=5$).

4.3.2 FF interneurons are responsible for generation of muscarine-induced rhythmic IPSCs in BL PNs

The sensitivity of muscarine-induced rhythmic IPSCs to TTX and bicuculline suggests that this is mediated by GABA release from interneurons firing caused by muscarine. The evidence shown in chapter 3 points that PV+ FF interneurons are the ones that fire APs caused by muscarine and generate rhythmic IPSCs in BL PNs. To test this hypothesis, we performed dual-cell recordings of IN-PN pairs. Interneurons were recorded in current clamp, while PNs were recorded in voltage clamp. As expected, RF interneurons did not fire upon a 2 s puff of muscarine, despite muscarine-induced rhythmic IPSCs were reliably observed in simultaneously recorded PNs. (Figure 4.4A,B,C). In contrast, in FF-PN pairs, all FF interneurons were depolarized by a 2 s puff of muscarine and fired long lasting APs, which were highly correlated with the large IPSCs in simultaneously recorded PNs. (Figure 4.4D,E,F). Cross correlation analysis suggests that FF but not RF interneurons were responsible for generation of muscarine-induced rhythmic IPSCs in BL PNs. (0.34 ± 0.02, $n=6$ VS 0.04 ± 0.01, $n=18$, $p<0.01$) (Figure 4.4G,H,I). We also noticed that the FF interneuron AP peaks were not perfectly aligned up with the peaks of IPSCs in BL PNs, which may be due to synaptic time delay of synaptic transmission. As FF depolarization by muscarine is mediated through M3 receptors, muscarine-induced rhythmic IPSCs in BL PNs should also be sensitive to M3 receptors antagonists. Indeed, they were abolished by bath application of 4-DAMP (1 μM) ($p<0.05$, $n=5$) but not TZP (100 nM) ($p>0.05$, $n=5$) (Figure 4.5).

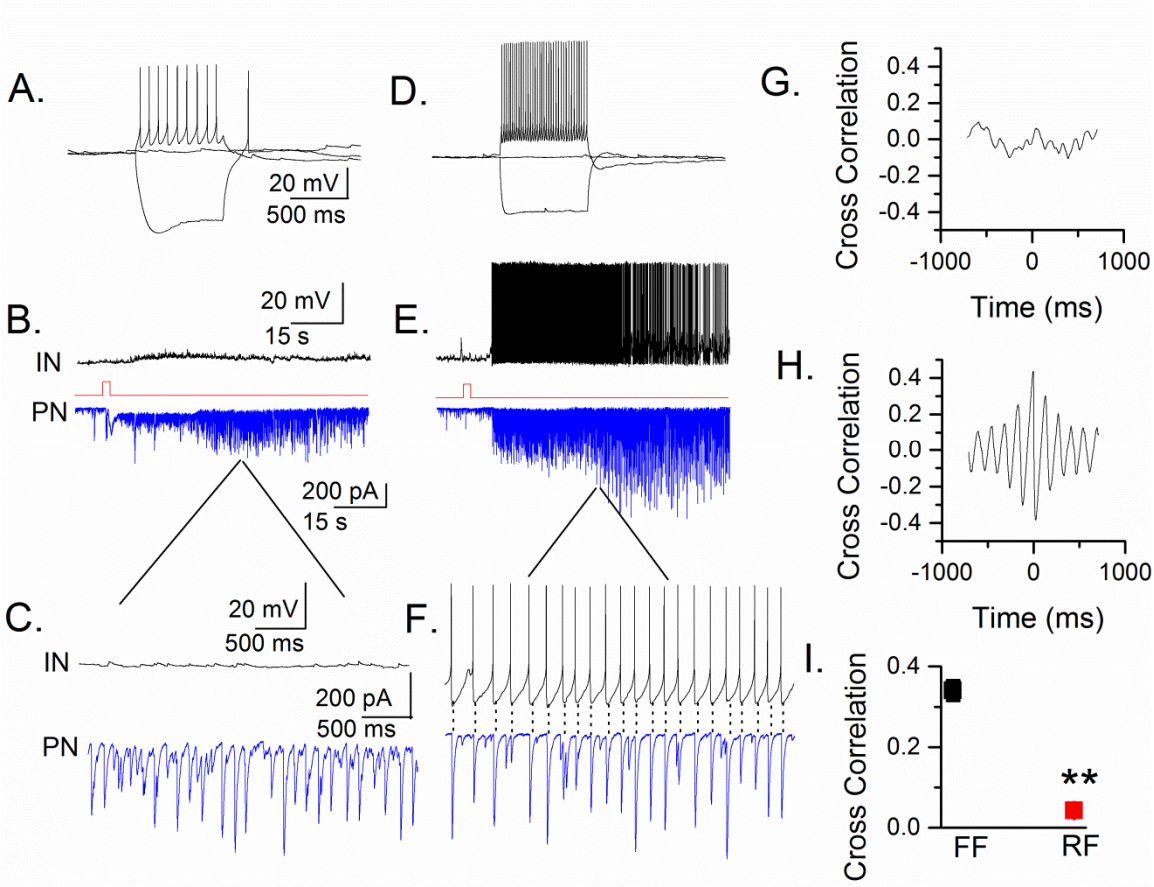

A.
20 mV
500 ms
D.
G.
Cross Correlation
0.4
0.2
0.0
-0.2
-0.4
-1000
0
1000
Time (ms)
B.
20 mV
15 s
IN
PN
200 pA
15 s
E.
H.
Cross Correlation
0.4
0.2
0.0
-0.2
-0.4
-1000
0
1000
Time (ms)
C.
20 mV
500 ms
IN
200 pA
500 ms
PN
F.
I.
Cross Correlation
0.4
0.2
0.0
**
FF
RF

Figure 4.4 FF interneurons are responsible for generating muscarine-induced large IPSCs in BL pyramidal cells.

A. and D. recorded interneurons showing RF pattern and FF pattern, respectively.

B. and E. dual-cell recording of a RF interneuron (in A) and a pyramidal cell, and a pair of FF interneuron (in D) and a pyramidal cell, respectively. Interneuron was recorded in current clamp (black). Pyramidal cell was recorded in voltage clamp (blue). The red trace indicates puff application of muscarine (50 μM).

C. and F. expanded waveforms from B and E, respectively. Dotted lines in F. indicate the APs in the FF interneuron is synchronized with the large IPSCs in the BL pyramidal cell.

G. and H. Cross correlations of the RF-PN pair in shown in B. (G.)and the FF-PN pair shown in E. (H.).

I. a graph showing cross correlation of FF-PN pairs is significantly higher than RF-PN pairs. ($p<0.01$, $n=6$).

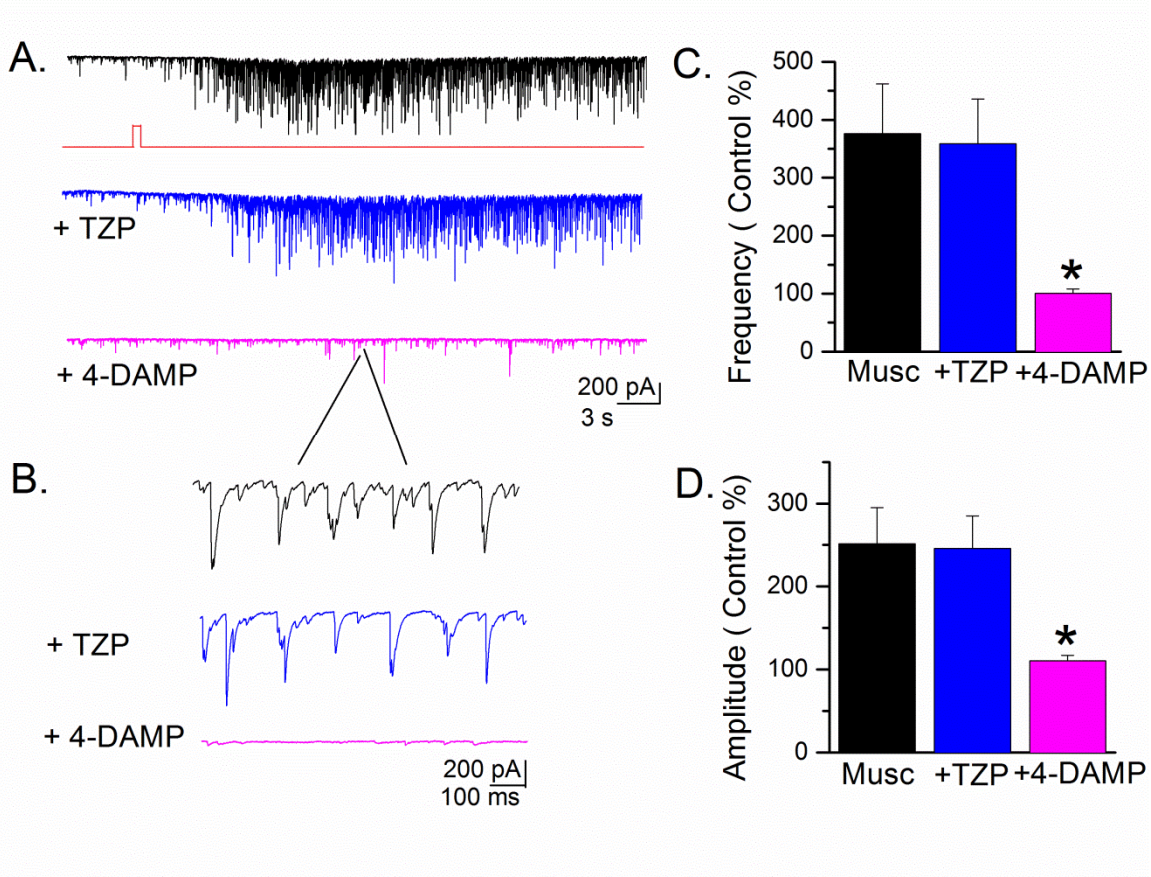


Figure 4.5 Rhythmic IPSCs are blocked by M3 but not M1 antagonist.

A. representative waveforms from a recorded BL pyramidal cell in voltage clamp (Holding potential is -70 mV) showing that muscarine-induced rhythmic IPSCs (in black) were blocked by 4-DAMP (1 µM) (in pink) but not TZP (100 nM).

B. expanded waveforms shown in A.

C. and D. bar graphs showing both frequency (C.) and amplitude (D.) of muscarine-induced rhythmic IPSCs were significantly reduced by 4-DAMP (1 µM) but not TZP (100 nM).

4.3.3 Muscarine generates synchronized inhibition in BL PNs

It has been shown that inhibition can be synchronized within neuronal assemblies (Beierlein et al., 2000). Large sIPSCs seen in control baseline were shown being synchronized in simultaneously recorded BL PNs (Ryan et al., 2012). It is critical to know whether muscarine-induced rhythmic IPSCs are synchronized among BL PNs, which would enable them to generate synchronized firing of a population of PNs in the BL. Therefore we performed dual-cell recording of nearby PN-PN pairs. We found that muscarine-induced rhythmic IPSCs in these pairs were highly synchronized (Figure 4.6A,C). PV interneurons are interconnected by gap junctions (Muller et al., 2005, Woodruff and Sah, 2007b). It has been shown gap junctions were required for generation of synchronized inhibition by a network of interneurons in the cortex (Beierlein et al., 2000). Thus we tested whether this is the case in the BL. Bath application of Carbenoxolone (CBX) (100 µM) did not affect the synchrony (Figure 4.6B,D,I). Cross correlation analysis revealed that CBX (100 µM) application did not significantly change the cross correlation values of PN-PN pairs ($p>0.05$, $n=5$) (Figure 4.6C,D,J), suggesting that synchrony of muscarine-induced rhythmic IPSCs between nearby PN-PN pairs did not require gap junctions. We then recorded PN-PN pairs which were far away from each other. Similar as nearby PN-PN pairs, synchronized muscarine-induced IPSCs were observed in far apart PN-PN pairs (Figure 4.6E,G). In contrast to nearby PN-PN pairs, CBX (100 µM) disrupted the synchrony (Figure 4.6F,K). The averaged correlation value was significantly reduced in the presence of CBX (100 µM) (0.50 ± 0.06 VS 0.21 ± 0.05, $p<0.05$, $n=5$). (Figure 4.6G,H,L). This indicates that gap junctions are required for synchronization within large but not small neuronal network.

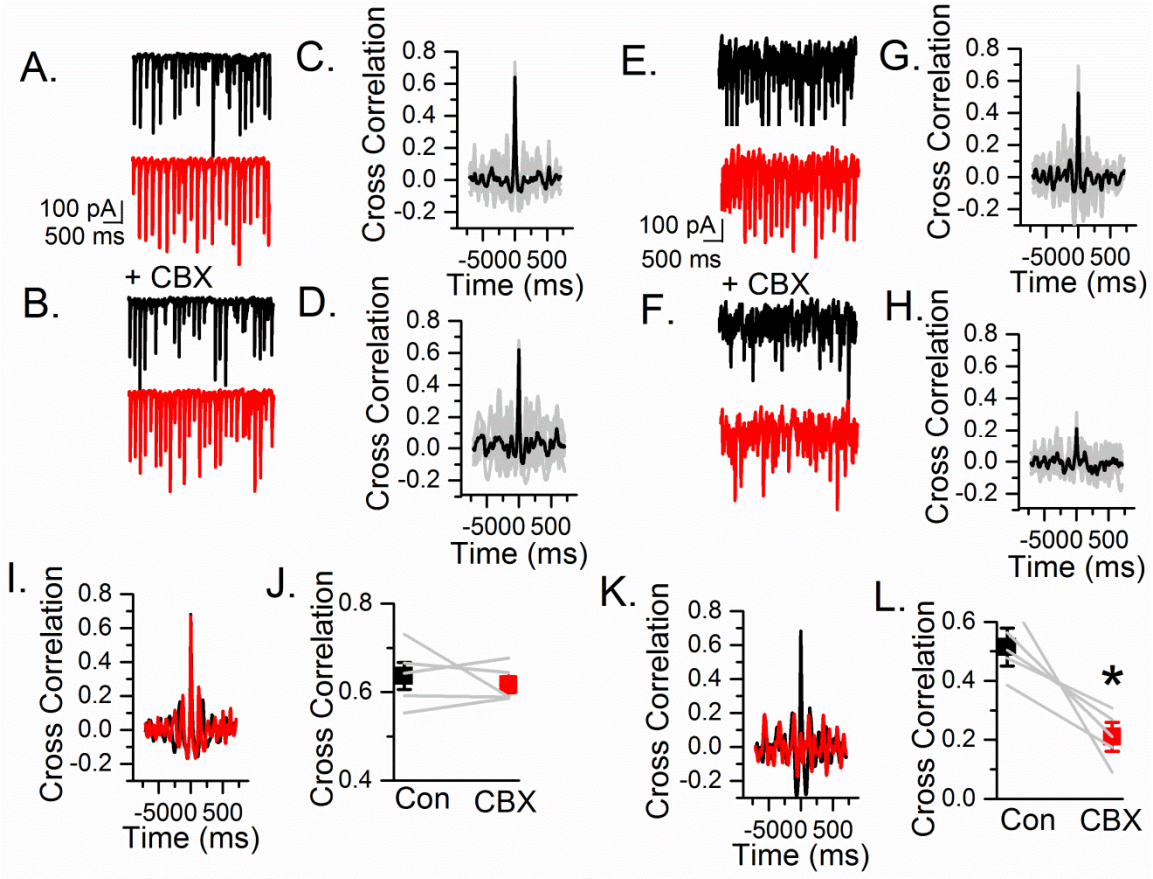

A.
100 pA
500 ms
+ CBX
B.
C.
Cross Correlation
0.8
0.6
0.4
0.2
0.0
-0.2
-5000 500
Time (ms)
D.
E.
100 pA
500 ms
+ CBX
F.
G.
H.
I.
J.
Con
CBX
K.
L.
*

Figure 4.6 Muscarine generates synchronized inhibition in BL PNs.

A. and B. representative waveforms from a simultaneously recorded nearby PN-PN pair before, and after CBX application, respectively.

C. Cross correlation of muscarine-induced IPSCs in a pair of PNs shown in A. and B. shows high synchrony before (black trace) and after (red trace) CBX application.

D. and E. Superimposed cross correlations from 5 pairs of simultaneously recorded nearby BL PNs (grey) in (D) and without (E.) the presence of CBX (100 µM), with the population average indicated in black.

F. There is no significant difference of cross correlation values between with and without the presence of CBX (100 µM).

G. and H. representative waveforms from a simultaneously recorded faraway PN-PN pair before, and after CBX.

I. Cross correlation of muscarine-induced IPSCs in these two PNs shows a high correlation before CBX (100 µM). application and reduced correlation in the presence of CBX (100 µM).

J. and K. Superimposed cross correlations from 5 pairs of simultaneously recorded faraway BL PNs (grey) in (J) and without (K.) the presence of CBX (100 µM), with the population average indicated in black.

L. The cross correlation value is significantly lower in the presence of CBX (100 µM). ($p<0.05$, $n=5$).

4.4 DISCUSSION

In this study we demonstrated that muscarine activation of mAChRs generated synchronized rhythmic large IPSCs in BL PNs, which were able to entrain BL PNs firing and set their firing frequency. We further showed that muscarine-induced rhythmic IPSCs were contributed by FF interneurons selectively recruited by activation of M3 receptors by muscarine. Gap junctions were required for synchrony in large neuronal networks but not confined ones.

As has been previously reported, large sIPSCs/sIPSPs in BL PNs at low frequency were observed in control baseline (Popescu and Pare, 2011, Ryan et al., 2012). There are some similarities between large sIPSCs in control baseline and muscarine-induced large IPSCs shown in this study. First, both appear to have large amplitude often is several fold of average amplitude of sIPSCs in baseline. Second, interneurons firing is required for generation of both. Third, both of them are highly synchronized in BL PNs. However, muscarine-induced rhythmic IPSCs reported here are fundamentally different from large low frequency sIPSCs observed in control baseline. First, they are rhythmic and are at much higher theta frequency band, whereas large low frequency sIPSCs are irregular and at lower delta frequency band. Delta frequency oscillations are often observed during idle states, whereas theta oscillations indicate emotional arousal. It is possible that muscarine-induce rhythmic IPSCs play a role in emotional processing. Second, they were generated by interneuron firing directly driven by mAChRs activation but not glutamatergic transmission. This suggests that cholinergic signaling can set frequency of synchronized BL PNs firing by controlling excitability of PV FF interneurons.

Synchronization of PN assembly firing can be achieved either by receiving common excitatory glutamatergic inputs or by perisomatic inhibition (Tiesinga and Sejnowski, 2009). In the latter case, inhibition sets time window to allow PNs to fire and also generates rebound excitation after hyperpolarization and thereby phase-reset PNs firing (Cobb et al., 1995, Woodruff and Sah, 2007a). Muscarine-induced rhythmic IPSPs entrain and synchronize BL PNs firing possibly by both mechanisms, because it was often found each PN AP was between two consecutive IPSPs and APs were often generated soon after IPSP-caused hyperpolarization.

Interneurons in the BL can be divided into different groups based on their firing patterns and neurochemical markers that they express (Rainnie et al., 2006, Woodruff and Sah, 2007b, Spampanato et al., 2011). We have shown, in this study, PV+ interneurons are heterogeneous, including RF and FF interneurons, which agrees with previous findings. In contrast, the majority of SOM+ interneurons were identified as RF. In short, FF interneurons most likely express PV but not SOM. In line with this, previous anatomical studies have shown in the BL PV+ interneurons project either to PN soma and proximal dendrites or to PN distal dendrites (Muller et al., 2006), suggesting they are heterogeneous type, while SOM+ interneurons are homogeneous and mostly project to PN distal dendrites (Muller et al., 2007a). It has been suggested that PV FF interneurons are basket cells (McDonald et al., 2005, Muller et al., 2006, Rainnie et al., 2006, Woodruff and Sah, 2007b). Indeed, some PV FF interneurons whose axons were successfully filled with biocytin showed basket cell-like morphology (Figure 3.5C).

It has been shown, in the cortex and hippocampus, selective type of interneurons response to mAChRs agonists (Kawaguchi, 1997, Yi et al., 2014). For example, in the

cortex, SOM+ and RF interneurons are depolarized by mAChRs agonists, while PV+ and FF interneurons do not respond to them (Kawaguchi, 1997). However, in the BL amygdala, we found that PV+ FF interneurons but not SOM+ or RF interneurons were preferentially depolarized by muscarine, which is opposite from the findings in the cortex. As expected we further demonstrated that FF interneurons were responsible for generation of muscarine-induced rhythmic IPSCs. In the cortex, however, RF interneurons mediate synchronized inhibition induced by metabotropic receptors agonists including mAChRs agonists (Beierlein et al., 2000), which agrees with the fact that RF but not FF interneurons are sensitive to mAChRs activation. In the hippocampus, CCK interneurons are involved in rhythmic IPSCs induced by mAChRs agonists (Nagode et al., 2011, Nagode et al., 2014). Although we were not able to identify the neurochemical phenotype of these FF interneurons due to neuronal contents washout during recording, it was very likely that they were PV+ based on the finding that the majority of FF interneurons are PV+, which is also supported by previous studies. Moreover, they are unlikely to be CCK+ or VIP+ interneurons. CCK+ interneurons in the amygdala mostly show RF pattern (Sosulina et al., 2010). VIP+ interneurons are often specialized being inter-interneuronal inhibitory interneurons (Mascagni and McDonald, 2003, Pi et al., 2013). Taken together, those FF interneurons are very likely to be PV+ interneurons. RFs in the cortex are interconnected as a network by gap junctions, which are necessary for producing synchronized inhibition (Gibson et al., 1999, Beierlein et al., 2000). In the BL PV interneurons also contain gap junctions and are preferentially interconnected among the ones with same firing pattern (Muller et al., 2005, Woodruff and Sah, 2007b). Synchrony between BL nearby PN-PN pairs does not require electrical coupling in PV+

FF interneurons, suggesting that a single PV FF interneuron may be able to synchronize a confined BL PN assembly firing. However, we do not exclude the possibility that a synchronized interneuronal network formed by a small number of PV FF interneurons with reciprocal chemical synaptic connections contribute to it. In the BL, a single PV interneuron diverge onto about a hundred of PNs (McDonald et al., 2005). One can imagine that only a single or few PV FF interneurons would be able to synchronize hundreds of PNs firing. Therefore selective modulation of PV FF interneuron excitability by cholinergic transmission would be a very effective way to control a large number of PNs firing. In inferior olive, a single interneuron synchronizes a neuronal ensemble controlling one whisk (Long et al., 2002). One can speculate that in the BL a single or a few PV FF interneurons innervate a functional PNs ensemble and thereby controlling just them would be able to efficiently phase reset the ensemble firing to generate synchronized outputs, which are supported by previous studies. In the BL, mAChRs activation depolarize PNs when they are slightly above resting membrane potential but do not do so when they are at resting membrane potential (Washburn and Moises, 1992b). This phenomenon was also repeated in our study. Moreover inhibition-mediated rebound excitation only happens when PNs are moderately depolarized (Cobb et al., 1995). We posit that a few PNs simultaneously receive a common glutamatergic input and are depolarized by subsequent EPSPs. ACh would selectively further depolarize these PNs but not others which were not depolarized to fire. Meanwhile a single or few PV FF interneurons innervating them would also been activated and fire to produce synchronized inhibition. Since only these PNs are depolarized, rebound excitation and subsequent phase resetting would only be observed on them. In this way, cholinergic

transmission would be able to amplify inputs to neuronal ensemble and enhance their output through modulation of PV FF interneurons.

In contrast, when PN-PN pairs were far apart from each other, gap junctions were required for the synchrony between them. PV basket cells axons often localize at nearby area (McDonald et al., 2005), thus in order to provide synchrony in a larger network they may need to transmit firing to other neighboring PV FF interneurons through gap junctions. By modulation of gap junctions it would be possible to regulate the extent of synchrony in the BL.

Muscarine depolarization of PV FF interneurons were mediated through M3 receptors but not M1 receptors. This agrees with previous anatomical studies shown that in the BL M1 receptors are mostly expressed on PNs but not on interneurons (McDonald and Mascagni, 2010).

Asynchronous unitary excitatory synaptic inputs are usually unable to reliably depolarize target cells to fire APs, which would affect information flow between brain structures (Long et al., 2002). Consequently, it is critical to synchronize PNs firing for generation of output so that the produced EPSPs would be able to temporally summate to trigger APs in the downstream postsynaptic cells. It has been shown that enhanced synchrony of BL PNs play a role in facilitating communication between perirhinal and entorhinal cortex (Pare et al., 2002), which is thought to be important for the emotional enhancement of memory. It is also involved in adaptively learning behaviors by influencing neuronal oscillations in striatum (Popescu et al., 2009). Therefore, neuronal oscillations originated from BL promote emotional memory formation and consolidation.

ACh release is increased particularly during emotional learning (Letzkus et al., 2011). Here we provided a novel mechanism of generation of theta oscillations in the BL by mAChRs activation, which is very different from the findings in the hippocampus and cortex.

CHAPTER 5

CHOLINERGIC MODULATION OF GLUTAMATERGIC AND GABAERGIC TRANSMISSION IN BL

5.1 INTRODUCTION

It is well established that the lateral amygdalar nucleus (LA) is important for fear conditioning (LeDoux, 2000). It receives inputs from the cortex and thalamus that permit the association of information regarding unconditioned stimuli such as footshock with conditioned stimuli such as a tone resulting in the potentiation of CS inputs and the subsequent generation of fear behavior by the CS alone (LeDoux, 2000). However, recent studies indicate that the basolateral nucleus (BL), the nucleus is also involved in the acquisition, expression, and extinction of conditioned fear responses (Goosens and Maren, 2001, Anglada-Figueroa and Quirk, 2005, Herry et al., 2008), as well as the enhancement of memory formation by emotional arousal (McGaugh, 2004). The BL receives the densest cholinergic innervation from the basal forebrain. Numerous studies have reported that mAChRs activation in the BL is critical for fear learning and consolidation (Vazdarjanova and McGaugh, 1999, Power et al., 2003b, Malin and McGaugh, 2006). Post-training infusions of mAChRs antagonists into the BL, or lesions of the BF cholinergic projections to the amygdala, produce impairments in several types of emotional or motivational learning (Power et al., 2003b). mAChRs activation in the

BL is critical for consolidation of memories for both contextual fear conditioning and its extinction (Vazdarjanova and McGaugh, 1999, Boccia et al., 2009). In fact, it has been suggested that the degeneration of the cholinergic projections to the BL in Alzheimer's disease is important for the memory disturbances seen in this disorder (Kordower et al., 1989, Power et al., 2003b). The BL receives afferent glutamatergic inputs from many brain regions including sensory cortex, thalamus including midline thalamus, hippocampus and mPFC (Turner and Herkenham, 1991, Mcdonald et al., 1996, McDonald and Mascagni, 1997, Pitkanen et al., 1997, McDonald, 1998, Kishi et al., 2006, Vertes, 2006) Synaptic plasticity of these glutamatergic inputs to BL pyramidal cells contributes to fear conditioning and extinction.

Previous studies have shown that acetylcholine, signaling through muscarinic receptors, suppresses glutamate release at internal recurrent pathways in cortex and hippocampus, but not at afferent glutamatergic pathways into these brain regions (Giocomo and Hasselmo, 2007). This action is thought to be important for information processing as it enhances the signal/noise ratio of external input. The functional effects of mAChRs on glutamatergic and GABAergic transmission in BL have not been studied. Anatomical studies have shown M1 and M2 mAChRs are located on subpopulations of both dendritic spines and excitatory and inhibitory terminals (Muller et al., 2013). The differential expression of mAChRs on distinct pre- and postsynaptic sites suggests that there may be organizing principles to cholinergic regulation of BL function. In the present study, we examined the role of muscarinic signaling in regulating glutamatergic and GABAergic transmission in the BL.

5.2 Materials and Methods

AAV-CAMKII-hChR2(H134R)-EYFP (2 µL) were injected to midline thalamus (2.0 mm posterior and 0.0 lateral to the bregma) or prelimbic mPFC (3.5 mm anterior and 0.5 lateral to the bregma) of male Sprague Dawley rats (around 30 days old). They were ready for electrophysiology experiments 6-8 weeks after injection. Methods of Optogenetic stimulation were described in chapter 2.

5.3 Results

5.3.1 Muscarine suppresses inputs from external and internal capsule to the BL

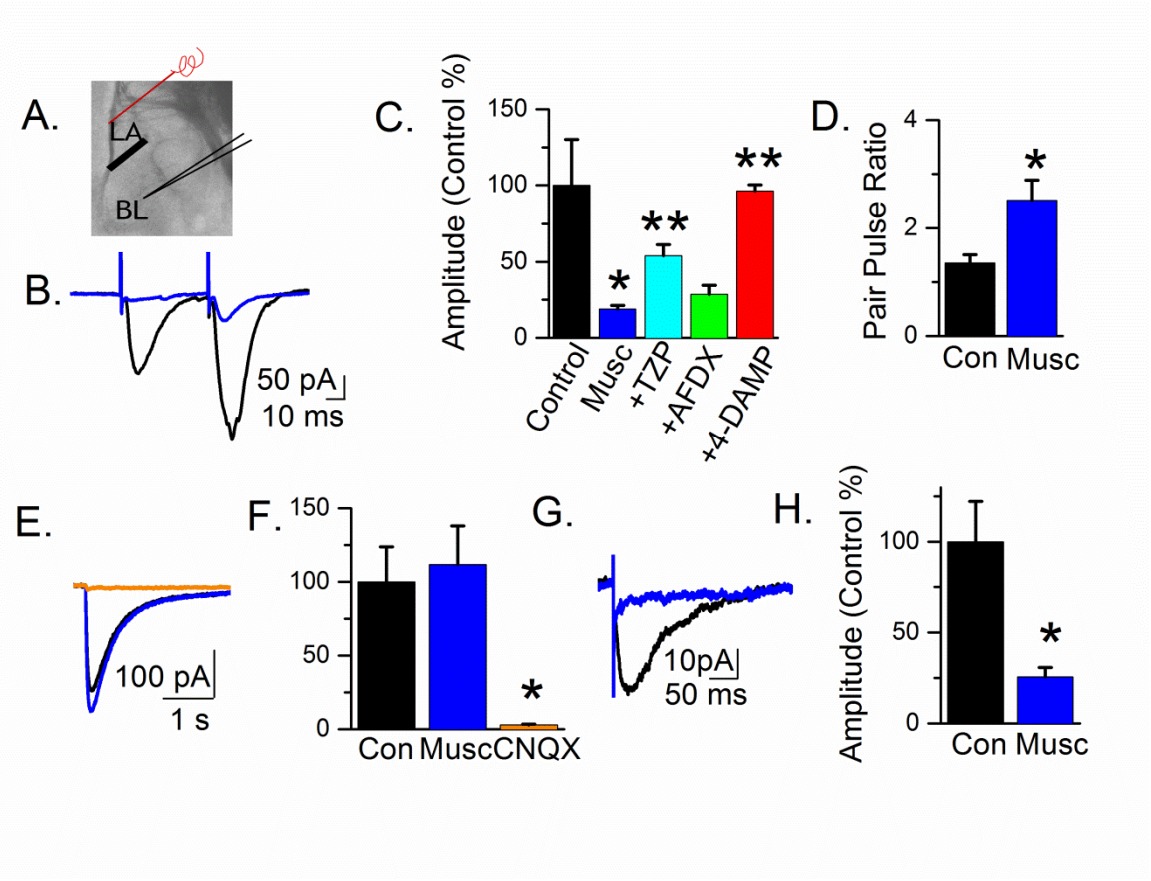
Glutamatergic axonal fibers coming from multiple cortical areas get into the BL though external capsule. To test the effect of muscarine on glutamatergic transmission from this pathway eEPSCs were recorded from BL PNs were induced by paired stimulation (50 ms interval) of external capsule in the presence of picrotoxin (100 µM) and CGP (10 µM) to block GABAa and GABAb receptors. (Figure 5.1A,B). eEPSCs from this pathway showed paired pulse facilitation (Figure 5.1B). Bath application of muscarine (10 µM) caused a significant reduction in the amplitude of the eEPSCs with increased paired pulse ratio in BL PNs. (Figure 5.1B). On average, application of muscarine (10 µM) decreased the amplitude of eEPSCs by 81% ± 3% (n=7) compared to baseline control. Paired pulse ratio was increased from 1.4 ± 0.2 in baseline to 2.5 ± 0.4, suggesting that the attenuation of eEPSCs from external capsule pathway was through a presynaptic mechanism ($p<0.05$, n=7). (Figure 5.1D). Importantly, the amplitude of eEPSCs was not altered during a same period of perfusion without muscarine, indicating that the suppression of eEPSC was not due to rundown. Glutamatergic inputs from

multiple thalamic nuclei to the BL pass by internal capsule (LeDoux, 2000). We next tested the effect of muscarine on glutamatergic transmission of internal capsule pathway. Similar to muscarinic effects seen in the external pathway, muscarine significantly attenuated eEPSCs induced by stimulation of internal capsule and increased paired pulse ratio (Figure 5.2). To further investigate the mechanism of suppression of glutamatergic transmission from external and internal capsule pathways, AMPA current was evoked by puffing kainate (100 μM), a AMPA/kainate receptors agonist, onto recorded BL PNs. (Figure 5.1E). Application of muscarine (10 μM) did not affect the amplitude of evoked AMPA currents in BL PNs ($p>0.05$, $n=7$), indicating that postsynaptic modifications were not involved in muscarine-induced reduction of glutamatergic transmission of external and internal capsule pathways (Figure 5.1F). Amplitude of NMDA currents recorded in BL PNs evoked by stimulation of external capsule was suppressed by 74% ± 5% compared to control, which was similar to the percentage of inhibition of AMPA-mediated eEPSC from the same pathway by muscarine (10 μM). This further demonstrated that muscarine-mediated suppression of glutamatergic inputs from external and internal capsule to the BL PNs is through a presynaptic but not a postsynaptic mechanism.

There are several subtypes of muscarinic receptors expressed in the BL, including M1 and M2 (McDonald and Mascagni, 2010, 2011, Muller et al., 2013). To determine which subtypes mediate the reduction of glutamatergic transmissions in both external and internal capsule pathways, we examined the effects of application of muscarine (10 μM) along with TZP (100 nM), AFDX-116 (1 μM), or 4-DAMP (1 μM). AFDX-116 (1 μM) did not affect muscarine-mediated suppression (external capsule: $p>0.05$, $n=5$) (Figure

5.1C), while TZP (external capsule: $p<0.01$, $n=7$; internal capsule: $p<0.01$, $n=7$) and 4-DAMP (external capsule: $p<0.01$, $n=5$; internal capsule: $p<0.01$, $n=5$) partially and fully inhibited muscarinic inhibition, respectively. (Figure 5.1C, 5.2C). This suggests that both M1 and M3 receptors are involved in muscarine-mediated presynaptic inhibition of glutamatergic transmission from external and internal capsule pathways. Noticeably, we found there is variability in muscarinic effect on both pathways between experiments (Figure 5.2E).

Glutamatergic inputs from cortical and thalamic pathways also synapse onto BL interneurons to mediated feed forward inhibition (Sah et al., 2003, Ehrlich et al., 2009). Feed forward inhibition plays an important role in gating LTP induction in the BLA (Bissiere et al., 2003). Therefore, we examined the effects of muscarine on glutamatergic transmission to BL interneurons from external and internal pathways. BL interneurons were separated into two groups based on their firing patterns, FF and RF. (Figure 5.3B, 5.4B). Recorded putative interneurons were filled with biocytin and confirmed as interneurons by performing post-hoc immunochemistry. (Figure 5.3A, 5.4A). For both FF and RF interneurons, eEPSCs induced by stimulation of external or internal capsule were only observed in about half of the recorded ones, suggesting that some interneurons are involved in feed forward inhibition, while others mediate feedback inhibition. Similar as BL PNs, we found that glutamatergic transmission from external or internal pathways to BL FF and RF were significantly suppressed by application of muscarine (10 μM), which was regulated by a M1 and M3 receptors-mediated presynaptic mechanism. (Figure 5.3C,D,E,F,G, 5.4C,D,E,F,G).


A.
LA
BL
B.
50 pA
10 ms
C.
Amplitude (Control %)
150
100
50
0
*
**
**
Control
Musc
+TZP
+AFDX
+4-DAMP
D.
Pair Pulse Ratio
4
2
0
*
Con Musc
E.
100 pA
1 s
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Amplitude (Control %)
100
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Figure 5.1 Muscarine Suppresses Input from External Capsule to the BL by acting on presynaptic M1 and M3 mAChRs.

A. A diagram showing the positions of stimulating and recording electrodes.

B. EPSCs evoked by paired stimulation of the external capsule before (black) and after (blue) muscarine (10 μM) application.

C. Muscarine (10 μM) significantly suppressed the evoked EPSC amplitude (n=7, $p<0.05$). This effect was partially reversed by the M1 mAChR antagonist, telenzepine (TZP, 100 nM; n=7, $p<0.01$) and completely blocked by the M3 mAChR antagonist, 4-DAMP (1 μM; n=5, $p<0.01$). The M2 mAChR antagonist, AF-DX 116 (1μm; n=5, $p>0.05$) had no effect.

D. Muscarine (10 μM) significantly increased the EPSC paired pulse ratio (n=5, $p<0.05$), suggesting that its effects were presynaptic.

E. AMPA current evoked by puffing kainate (100 μM) onto the recorded PN before (black) and during muscarine (10 μM; blue) or CNQX (100 μM; red) application.

F. Muscarine (10 μM) did not suppress the AMPA current ($p<0.05$, n=7), whereas this current was blocked by CNQX (100 μM; $p<0.01$, n=7).

G. NMDA current evoked by EC stimulation before (black) and after (blue) muscarine application.

H. Muscarine suppressed NMDA current (n=3, $p<0.05$).

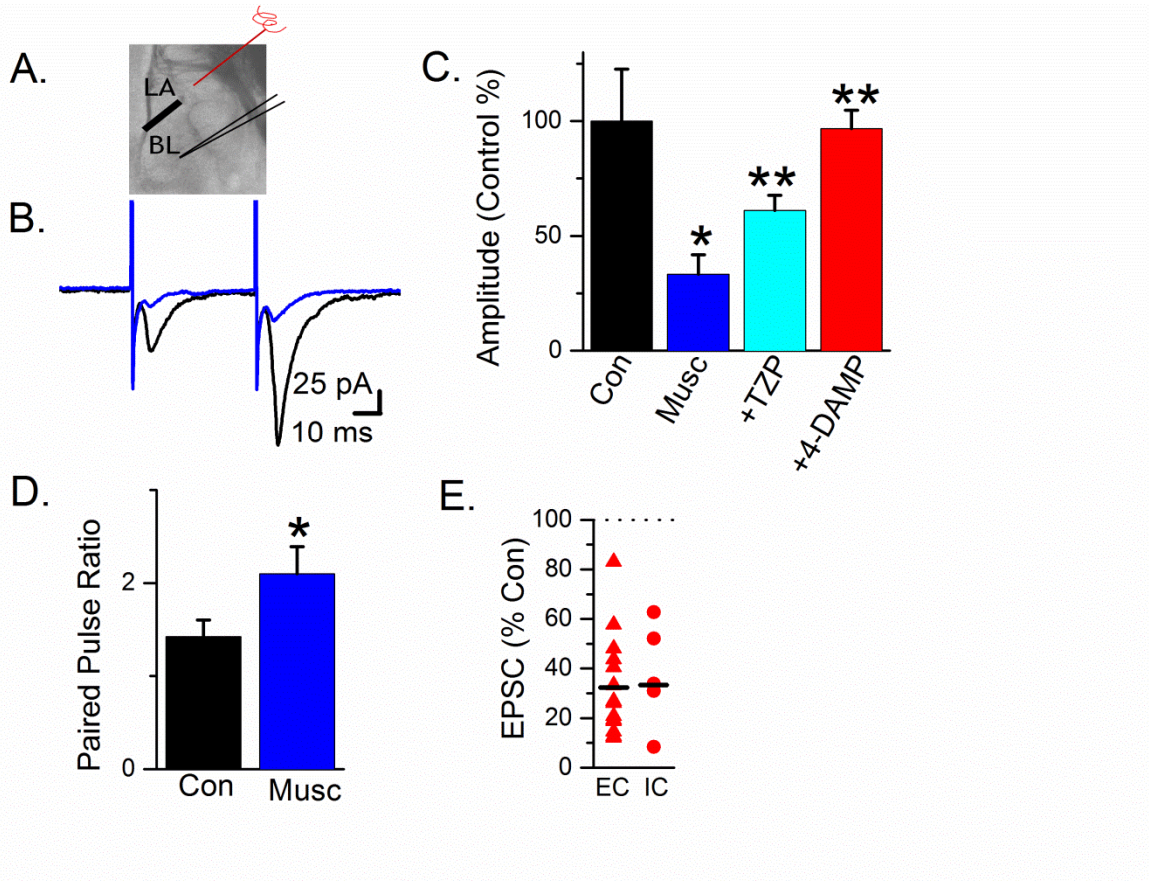

Figure 5.2 Muscarine Suppresses Input from internal Capsule to the BL by acting on presynaptic M1 and M3 mAChRs.

A. A diagram showing the positions of stimulating and recording electrodes.

B. EPSCs evoked by paired stimulation of the internal capsule before (black) and after (blue) muscarine (10 μM) application.

C. Muscarine (10 μM) significantly suppressed the evoked EPSC amplitude ($n=5$, $p<0.05$). This effect was partially reversed by TZP (100 nM; $n=7$, $p<0.01$) and completely blocked by 4-DAMP (1 μM; $n=5$, $p<0.01$)

D. Muscarine (10 μM) significantly increased the EPSC paired pulse ratio ($n=5$, $p<0.05$), suggesting that its effects were presynaptic.

E. Muscarine produced similar, but variable inhibition of EPSCs evoked by stimulating external capsule (EC) or internal capsule (IC). The black bar indicates the average inhibition.

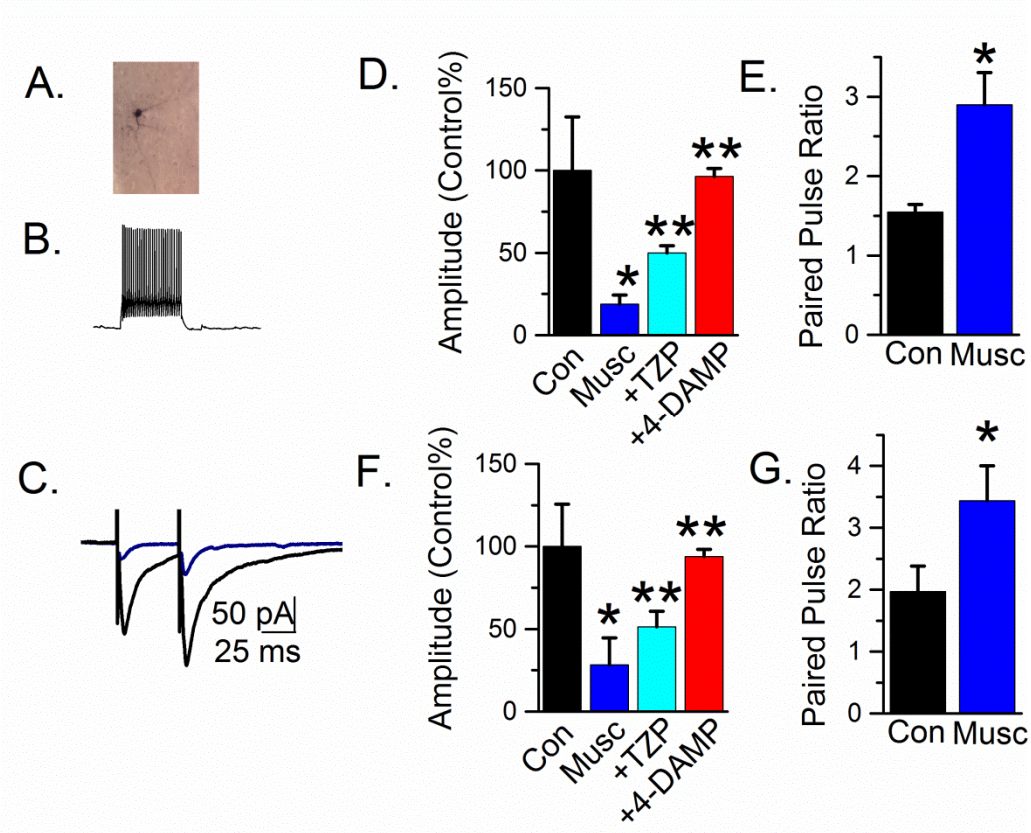

Figure 5.3 Muscarine Suppresses Cortical and Thalamic Input to BL FF Interneurons by Acting on Presynaptic M1 and M3 mAChRs.

A,B. A representative BL FF interneuron.

C. EPSCs from this interneuron evoked by paired stimulation of the external capsule before (black) and after (blue) muscarine (10 μM) application.

D,F. In FF interneurons muscarine (10 μM) suppressed the amplitude of the EPSC evoked by stimulation of both the external capsule (D., n = 5, $p<0.05$) and internal capsule (F., n = 5, $p<0.05$). The effect of muscarine in both pathways was partially reversed by TZP (100 nM; n=5, $p<0.01$) and completely blocked by 4-DAMP (1 μM; n=5, $p<0.01$).

E,G. Muscarine (10 μM) significantly increased the EPSC paired pulse ratio in both EC (E.) and IC (G.) pathways (n=5, $p<0.05$).

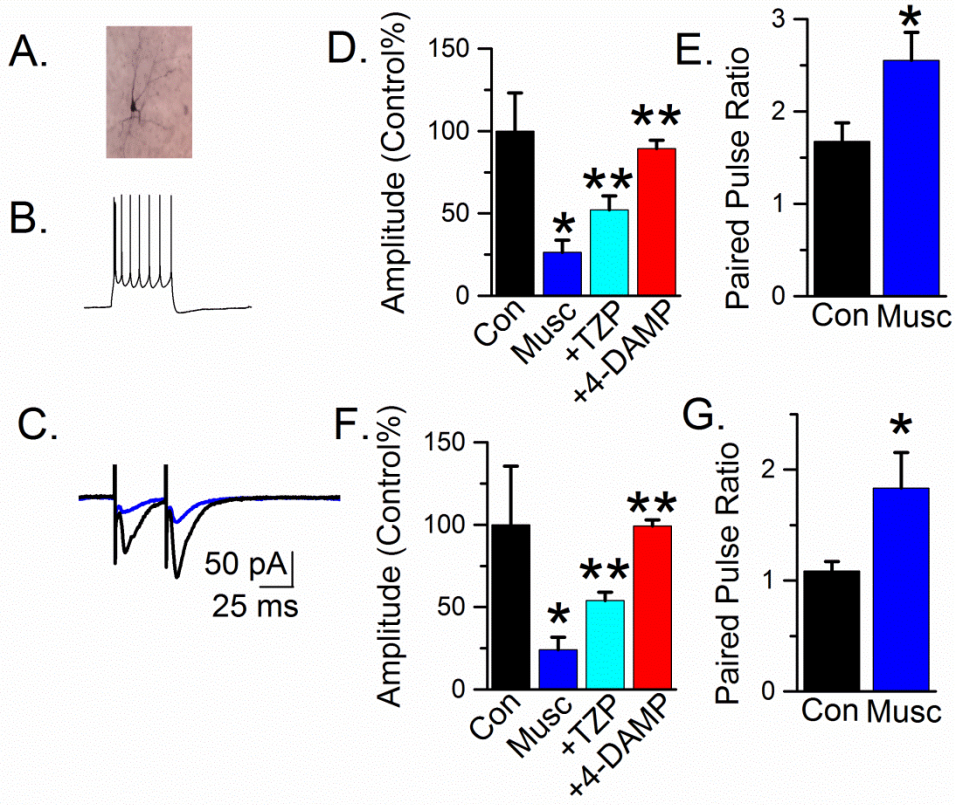

Figure 5.4 Muscarine Suppresses Cortical and Thalamic Input to BL RF Interneurons by Acting on Presynaptic M1 and M3 mAChRs.

A,B. A representative BL RF interneuron.

C. EPSCs from this interneuron evoked by paired stimulation of the external capsule before (black) and after (blue) muscarine (10 μM) application.

D,F. In RF interneurons muscarine (10 μM) suppressed the amplitude of the EPSC evoked by stimulation of both the external capsule (D., n = 5, $p<0.05$) and internal capsule (F., n = 5, $p<0.05$). The effect of muscarine in both pathways was partially reversed by TZP (100 nM; n=5, $p<0.01$) and completely blocked by 4-DAMP (1 μM; n=5, $p<0.01$).

E,G. Muscarine (10 μM) significantly increased the EPSC paired pulse ratio in both EC (E.) and IC (G.) pathways (n=5, $p<0.05$).

5.3.2 Muscarine enhances synaptic transmission within BL and from LA

BL PNs receive glutamatergic excitatory inputs from neighboring PNs and LA PNs. Communication between local PNs is thought to be important in formation of fear memory traces (Han et al., 2007, Han et al., 2009). Then we tested the effects of muscarine on glutamatergic transmission from internal inputs. Puff kainate (100 μM) depolarized PNs to fire action potentials.(Figure 5.5A,B). Bath application of muscarine (10 μM) increased the firing frequency by inhibition of sAHP (Figure 5.5B). Glutamate release from these activated BL PNs could be detected as EPSCs from recording a BL PN away from the puffing site (Figure 5.5B). Each EPSC corresponded to glutamate release caused by each action potential from neighboring PNs. Thus the EPSCs frequency represents neighboring PNs firing frequency and the average of amplitude of glutamatergic transmission from a population of activated neighboring PNs is calculated as the mean of EPSCs amplitude. Importantly, EPSCs responded to kainate puff were blocked by application of TTX (1 μM), confirming that they were not AMPARs activation by diffused kainate but rather directly caused by kainate-evoked neighboring PNs firing (data not shown). Application of muscarine (10 μM) significantly increased EPSCs frequency ($p<0.05$, $n=5$) but did not change the amplitude ($p>0.05$, $n=5$). (Figure 5.5C,D). Next we tested the effects of muscarine on glutamatergic transmission from LA PNs to BL PNs by puffing kainate to LA PNs. Same as BL recurrent glutamatergic transmission, muscarine did not affect the EPSCs amplitude ($p>0.05$, $n=5$) but increased frequency ($p<0.05$, $n=5$) (Figure 5.5C,D). These results indicate that muscarine does not suppress internal glutamatergic transmission within BL and from LA but rather amplifies excitatory interactions within the BL PNs and between BL and LA PNs.

Some BL interneurons are also involved feedback inhibition. Therefore we examined the effects of muscarine on feedback excitatory drives to BL interneurons. We found about half of the recorded interneurons receive feedback glutamatergic transmission, including both FF and RF interneurons. In these recorded feedback interneurons muscarine increased the EPSCs frequency ($p<0.05$, $n=5$) but did not alter the amplitude ($p>0.05$, $n=5$).

In short we demonstrated that unlike the inhibitory effects on the external and internal capsule pathways muscarine does not suppress but rather amplifies local recurrent glutamatergic transmission from BL and LA PNs to BL PNs and interneurons.

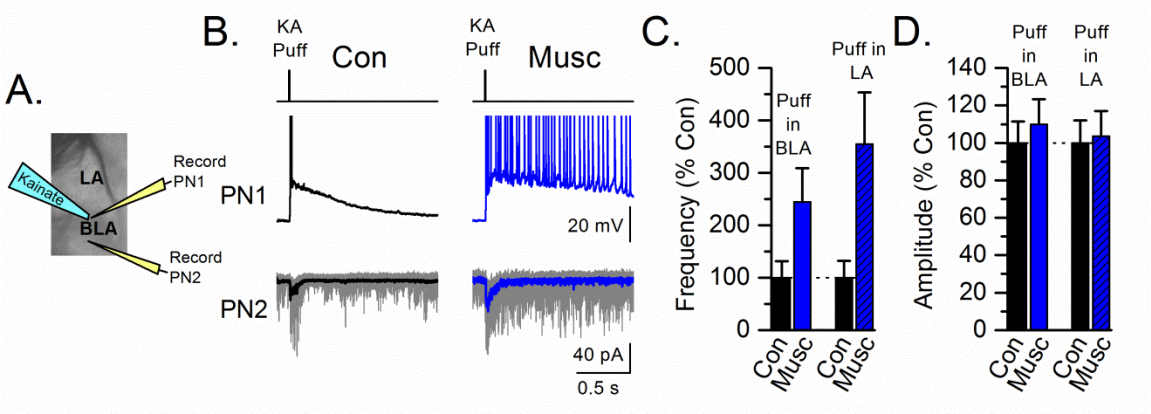

Figure 5.5 Muscarine does not inhibit LA-evoked or recurrent EPSCs in BL.

A,B. A kainate puff near one cell of PN cell pair in BL caused APs which, in turn, evoked an EPSC in the second cell. Muscarine dramatically enhanced firing in the first cell in response to the kainate puff, resulting in a large increase in EPSC frequency in the second cell. Superimposed EPSCs in PN2 from ten sweeps are shown (grey) with the average EPSC highlighted (black).

C,D. Muscarine enhanced EPSC frequency (C), but not amplitude (D) in PN2. Similar results were obtained when kainate puffs were delivered to the LA.

5.3.3 Muscarine has no effect on sEPSCs or mEPSCs in BL PNs

Then we examined the effects of muscarine on overall glutamatergic transmission to the BL PNs. sEPSCs and mEPSCs were recorded in BL PNs. We found that application of muscarine (10 μM) did not affect either sEPSCs or mEPSCs frequency or amplitude ($p>0.05$, $n=5$) (Figure 5.6). Two possibilities could cause that sEPSCs or mEPSCs were not affected by muscarine (10 μM). One is that there are some glutamatergic pathways suppressed by muscarine as shown above while other pathways were potentiated by muscarine may exist. The other possibility is that due to technique limits we could only sample a portion of total glutamatergic transmission, which may have mainly consisted of recurrent inputs which were not suppressed by muscarine (10 μM). Since M1R+ terminals mainly synapse onto dendritic spines (Muller et al., 2013), recordings made at the somas may not be able to detect the small currents generated at compartmental spines due to cable properties.

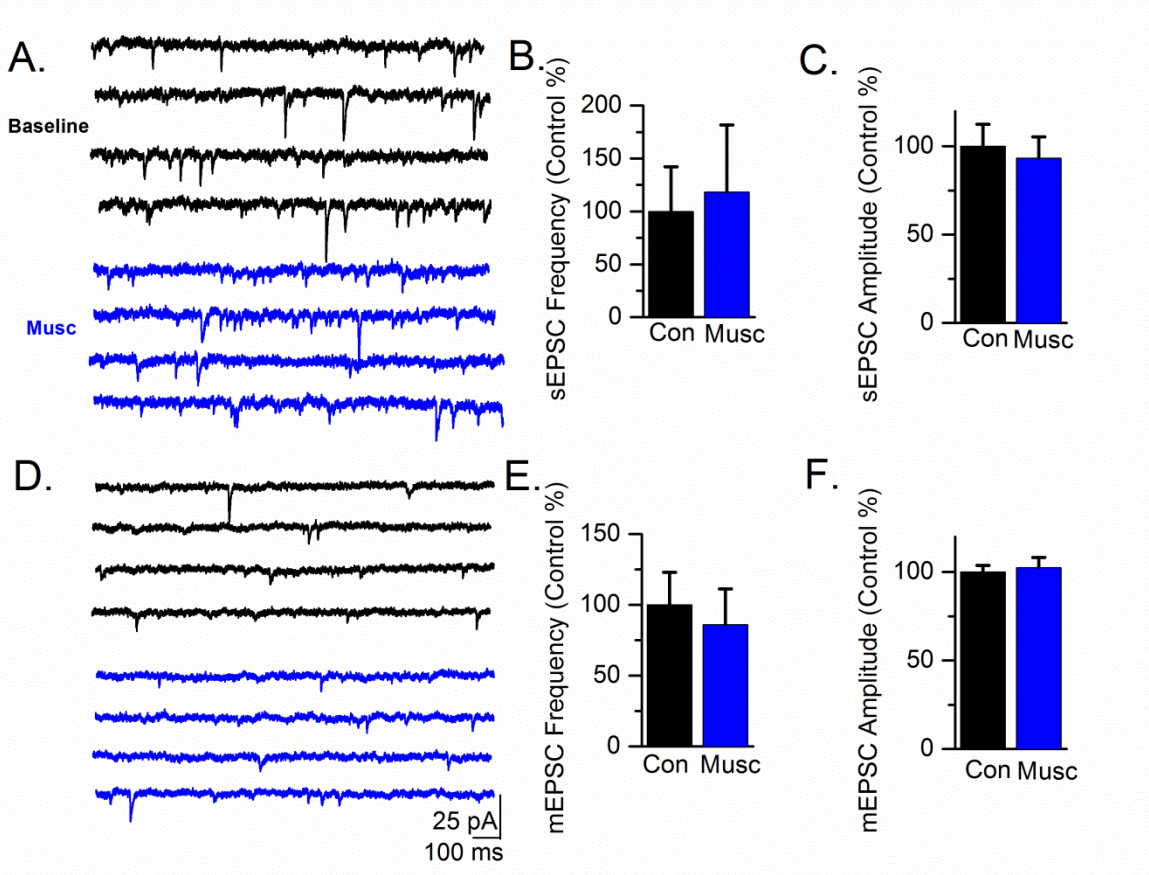

Figure 5.6 Muscarine Does Not Affect sEPSCs or mEPSCs in BL PNs.

A,D. Spontaneous (A.) and Miniature (D.) EPSCs recorded from BL PNs before and after muscarine (10 μM) application.

B,C,E, and F. Muscarine (10 μM) did not affect either sEPSCs or mEPSCs frequency or amplitude (n=5, $p>0.05$).

5.3.4 Muscarine inhibits glutamatergic inputs from midline thalamus and mPFC at different extents

Since both external and internal capsule carry various intermingled axonal fibers, to investigate whether muscarine differentially modulates specific pathways to the BL we utilized optogenetics to study projections from midline thalamus and mPFC. Projections from midline thalamus are important for fear conditioning (Vertes, 2006, McNally et al., 2011), while the ones from mPFC are necessary for fear extinction (Milad and Quirk, 2002). Neither of them get to the BL through external or internal capsule (McDonald, 1998, Vertes, 2004, 2006). We injected AAV-CaMKIIa-hChR2(H134R)-EYFP to the midline thalamus or mPFC of rats. After 6-8 weeks ChR2 were expressed in pyramidal cells in injected regions and in the axonal fibers coming from those neurons at injected sites in the target regions, including BL. (Figure 5.7). eEPSCs from midline thalamus or mPFC projections could be induced by shining a pulse of blue light (470 nM) to the BL. (Figure 5.8A). We found that bath application of muscarine (10 µM) inhibited eEPSCs of these two pathways to different extents. eEPSCs from midline thalamus pathways were inhibited only by 34% ± 11% by muscarine (10 µM) through M1 receptors (muscarine: $p<0.05$, n=5; +TZP: $p>0.05$, n=5) (Figure 5.8C,D), while the ones from mPFC projections were inhibited almost completely by 92% ± 0.5% ($p<0.001$, n=2) (Figure 5.8A,B). This provides the evidence that cholinergic transmission differentially modulates glutamatergic inputs from different brain regions.

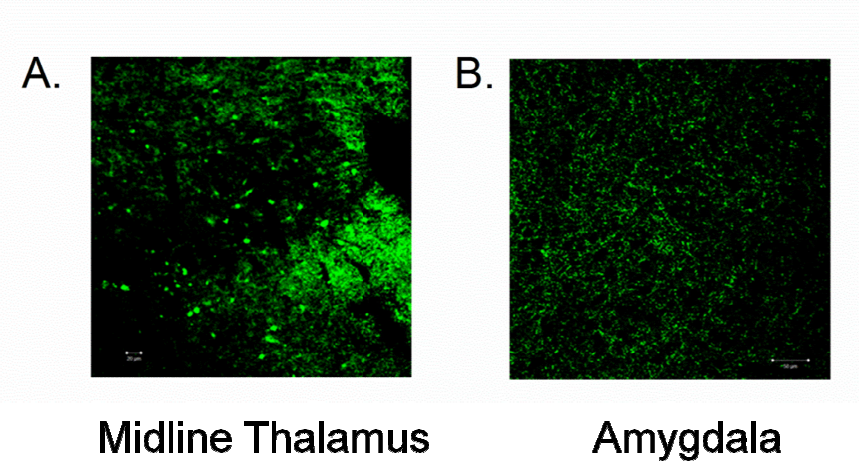

Figure 5.7 Confocal images showing virus expression.

A. Viruses injected in the midline thalamus were expressed in pyramidal cells. Green: EYFP.

B. Some midline thalamic axons in the BL expressed with hChR2(H134R)-EYFP.

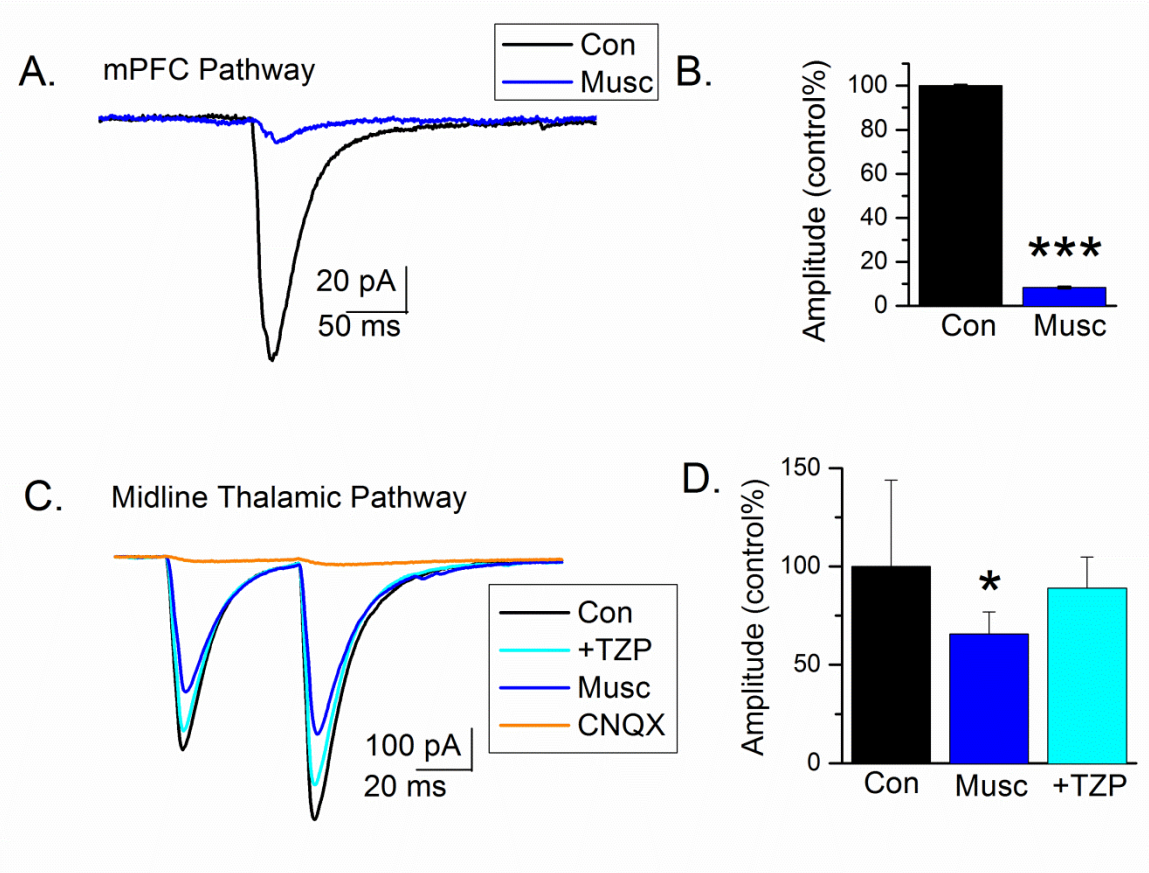

Figure 5.8 Muscarine differentially modulates Projections from mPFC and midline thalamus

A. EPSCs evoked by optogenetic stimulation of mPFC axons in BL before (black) and after (blue) muscarine (10 μM) application.

B. Muscarine (10 μM) potently suppressed the eEPSC amplitude (n=2, $p<0.001$).

C,D. EPSCs evoked by optogentic stimulation of midline thalamus axons in BL before (black), after (blue) muscarine (10 μM) application. Muscarine (10 μM) only slightly suppressed the eEPSCs ($p<0.05$, n=5). This effect was blocked by TZP (100 nM) ($p>0.05$, n=5).

5.3.5 Frequency gating of glutamatergic transmission by muscarine

Previous behavioral studies have shown mAChRs activation in the BL enhances fear learning and consolidation whereas blockade of mAChRs has the opposite effects (Power et al., 2003a, Power et al., 2003b). So far, we have found that muscarine inhibits external glutamatergic inputs from the pathways tested to the BL. Muscarine-mediated suppression of these inputs which are involved in fear learning and extinction would contradict the behavioral findings. To resolve this paradox we proposed that muscarine by inhibition of glutamate release increases the reliability of excitatory transmission at these inputs by reducing synaptic depression during a stimulus train. To test this hypothesis, eEPSCs were recorded at BL PNs evoked by a train of 10 electrical stimuli given at external capsule. When comparing the amplitude of the 1st EPSC to the 10th EPSC in control, we found synaptic transmission was depressed in a frequency dependent manner (Figure 5.9A). Application of muscarine (10 μM) transformed the depression into frequency dependent facilitation, which has the maximal effect at gamma frequencies (Figure 5.9A,C). Indeed, the amplitude of the 10th EPSCs in muscarine (10 μM) was significantly larger than the 1th ones in control at gamma frequencies (Figure 5.9B). These results suggest that glutamatergic inputs arriving at gamma frequencies would be strengthened during periods of high cholinergic tone, whereas weak or asynchronous signals would be suppressed.

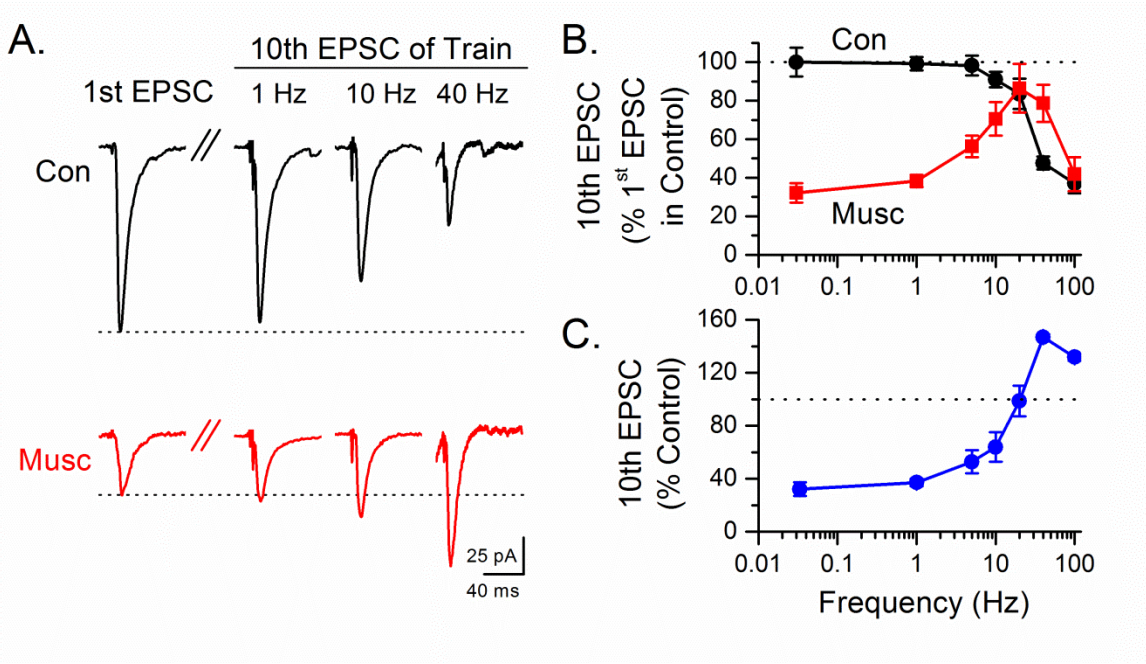

Figure 5.9 Frequency Gating of Glutamatergic transmission by muscarine

A. Sample traces from a PN showing the response to the first and 10th stimulus of the train at different frequencies in control and muscarine. Note that at 40 Hz the 10th EPSC in muscarine is larger than the 10th EPSC in control.

B. Averaged data showing the amplitude of the 10th EPSC in control (black) or muscarine (red) expressed as a percentage of the first EPSC in control.

C. The amplitude of the 10th EPSC in muscarine expressed as a percentage of the 10th EPSC in control. Note that in muscarine the 10th EPSC is enhanced at gamma frequencies.

5.3.6 Muscarine differentially suppresses GABAergic transmission in the BL

Fear learning is tightly gated by GABAergic inhibition in the BLA (Bissiere et al., 2003, Ehrlich et al., 2009). Cholinergic transmission may be able to facilitate fear conditioning by modulation of local GABAergic transmission in the BL. We next tested the effects of muscarine on GABAergic transmission in the BL. In agreement with the expression of M2 mAChRs on GABAergic terminals (Muller et al., 2013),we found that muscarine acts on M2 mAChRs to inhibit the evoked IPSC in BL PNs (muscarine: $p<0.05$, $n=5$; +AFDX: $p>0.05$, $n=5$). (Figure 5.10E,F). The inability of muscarine to completely inhibit the IPSC at saturating concentration suggests that M2 mAChRs are present on only a subpopulation of inhibitory terminals. In agreement, paired recording from a connected IN-PN cell pair revealed that muscarine suppressed the unitary IPSC in the PN, but produced a large increase in the frequency and amplitude of spontaneous IPSCs (Figure 5.10A,B). This suggests that presynaptic mAChRs on the terminals of the recorded interneuron suppressed GABA release from that cell. In contrast, muscarine depolarized other interneurons, which lacked mAChRs on their terminals, to increase action potentials firing and generate spontaneous IPSCs. These findings are in line with previous studies in hippocampus which have reported differential suppression of GABA release from distinct interneuron subpopulations (Fukudome et al., 2004, Neu et al., 2007, Szabó et al., 2010). By analyzing the frequency, amplitude and kinetics of individual sIPSCs, we found that muscarine selectively recruited IPSCs with larger amplitude and faster decay. (Figure 5.10G,H). This suggests that muscarine may inhibit dendritic inhibition and meanwhile enhance somatic inhibition.

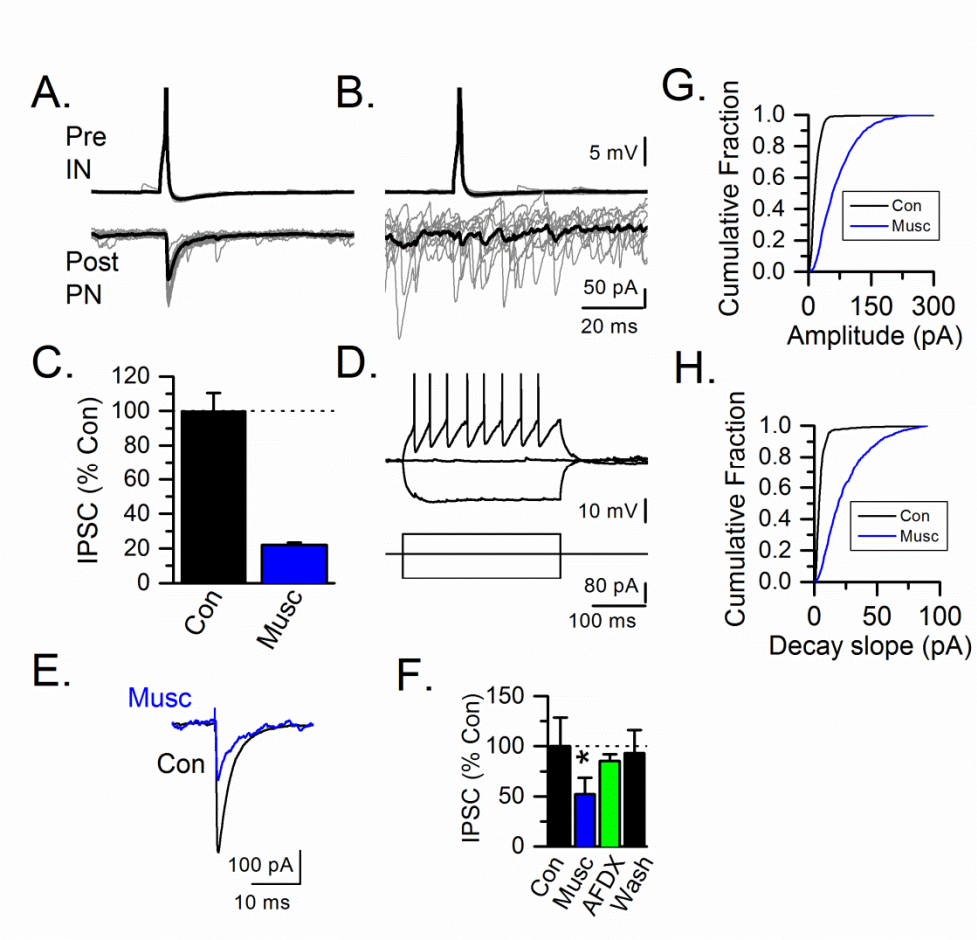

Figure 5.10 Differential Suppression of IPSCs by mAChRs.

A,B. Paired recording from a IN→PN. Ten superimposed sweeps (grey) are shown for the PN with the averaged response in black. Muscarine suppressed the IPSC evoked by the presynaptic IN but increased sIPSCs in the PN.

C. Averaged suppression of the evoked IPSC in 3 connected cell pairs.

D. Firing pattern of the IN shown in A & B, indicating that it is a regular firing IN.

E. Evoked monosynaptic IPSCs are inhibited ~50% by muscarine.

F. IPSC suppression by muscarine is blocked by AFDX116, a selective M2R antagonist.

G,H. Cumulative fraction plots show that the IPSCs are larger (D.) and faster (E.) in the presence of muscarine.

5.3.7 Muscarine facilitates LTP induction in the BL

We have demonstrated that muscarine makes BL PNs more receptive to the external glutamatergic inputs arriving at gamma frequency band and meanwhile inhibits GABAergic inhibition. We therefore hypothesized that by doing this muscarine facilitates LTP induction in the BL. LTP was successfully induced by high frequency stimulation of external capsule. (Figure 5.11A, black traces). In the presence of atropine LTP was blocked (Figure 5.11A, red traces), suggesting that tonic ACh or released ACh caused by high frequency stimulation is required for LTP induction in the BL. Furthermore, muscarine bath applied only during the period of LTP induction significantly facilitated LTP comparing to control (Figure 5.11A, blue traces). In all cases, paired pulse ratio of EPSCs after LTP remained the same as in baseline (Figure 5.11B), indicating that induced LTP was due to postsynaptic modification.

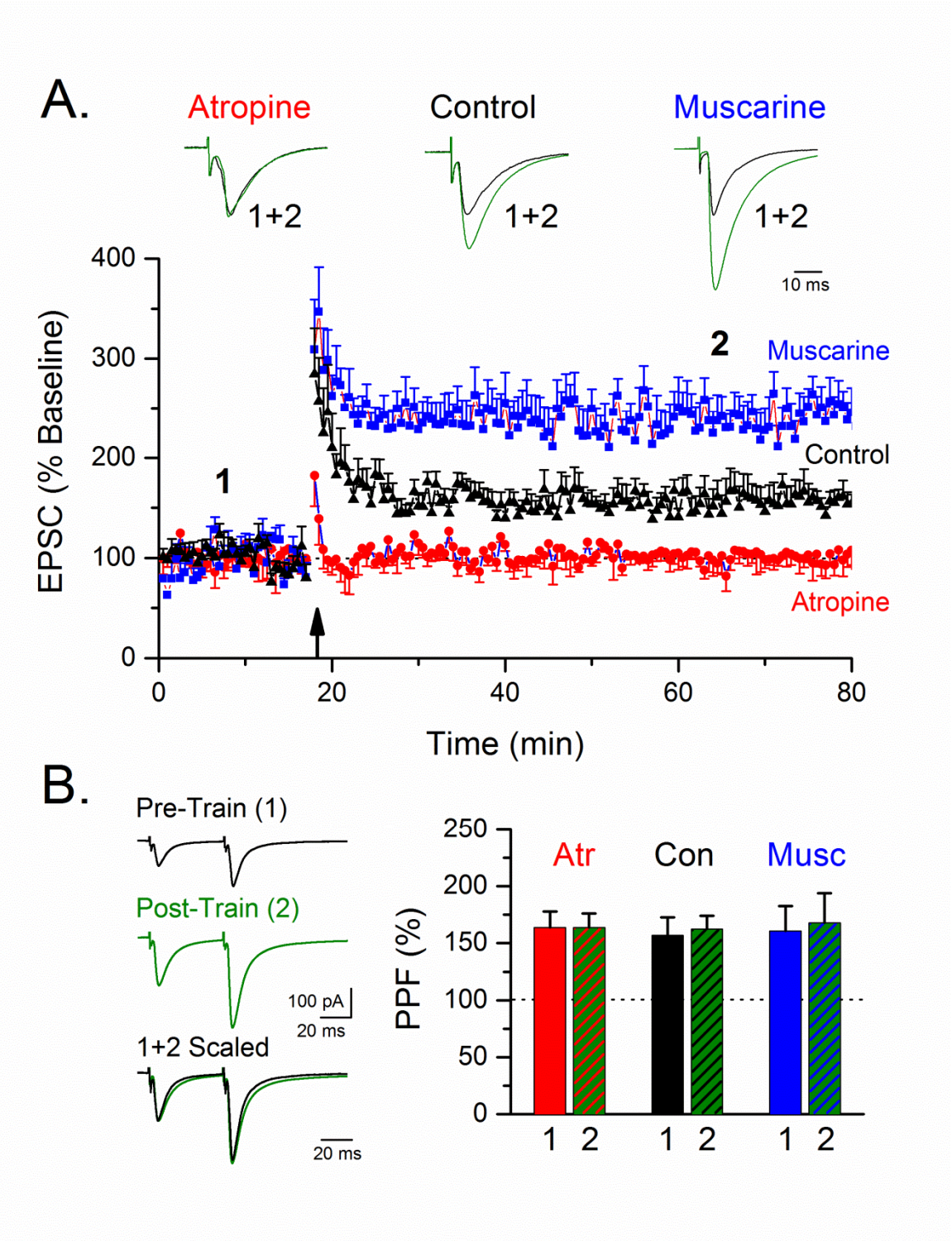
A.
Atropine
Control
Muscarine
1+2
1+2
1+2
10 ms
EPSC (% Baseline)
400
300
200
100
0
1
2
Muscarine
Control
Atropine
0
20
40
60
80
Time (min)
B.
Pre-Train (1)
Post-Train (2)
100 pA
20 ms
1+2 Scaled
20 ms
PPF (%)
250
200
150
100
50
0
Atr
Con
Musc
1 2
1 2
1 2

Figure 5.11 Muscarine facilitates LTP induction in the BL.

A. A high frequency stimulus train (shown by the arrow) produces LTP (black triangles; n=5). Application of atropine (5 µM) during the LTP-inducing train blocked LTP (red circles; n=5). In the presence of muscarine during the LTP-inducing train potentiated LTP (blue squares; n=5). Waveforms are representative EPSCs at the indicated time points.

B. In all cases, paired pulse ratio of EPSCs after LTP remained the same as in baseline. Waveforms are representative EPSCs at the indicated time points.

5.4 DISCUSSION

In the present study, we demonstrated that muscarine suppresses external but not recurrent glutamatergic transmission to the BL in a frequency dependent manner and attenuates feedforward inhibition and local GABAergic transmission, thereby facilitates LTP induction in BL PNs. mAChRs modulate glutamatergic neurotransmission in the BL in a manner markedly different from that in cortex or hippocampus.

mAChR-mediated inhibition of glutamatergic transmission from external capsule pathway observed in this study is consistent with previous findings (Yajeya et al., 2000). In addition, we found that there was noticeable variability in its effect between experiments, which could be caused by several possible reasons. External capsule contains axonal bundles from many different brain areas. Therefore electrical stimulation of external capsule could activate axonal fibers originated from projection neurons in multiple cortical regions. The particular axons activated could vary between experiments. Thus, the incomplete inhibition by muscarine and variability in its effect between experiments could be caused by the differential expression of mAChRs (Muller et al., 2013) at distinct cortical inputs. Functional differences between BL PNs have been reported previously. Fear neurons and extinction neurons are activated during high and low fear state, respectively (Herry et al., 2008, Senn et al., 2014). They receive differential inputs from the ventral hippocampus and prelimbic cortex respectively (Herry et al., 2008, Senn et al., 2014). Therefore, differences between BL PNs could also contribute to the variability of muscarinic effects. If this is the case, it would be interesting to test whether cholinergic transmission could differentially modulate contextual conditioning and extinction. The variability could also be due to variations

between experimental animals. For example, variations of fear extinction ability between experimental animals have been found (Wilson et al., 2013). It is possible that there are distinguishable differences of mAChRs expression level between animals.

Optogenetics which has been extensively utilized in numerous literatures makes investigation of synaptic transmission from specific brain regions possible(Tye and Deisseroth, 2012). By using this technique we found that muscarine only inhibited midline thalamus inputs by about 50%, while mPFC inputs were almost completely suppressed. This evidence supports the idea that cholinergic transmission could modulates glutamatergic inputs to BL in a pathway specific manner. Both the midline thalamic nuclei (Turner and Herkenham, 1991, Vertes, 2006) and mPFC (McDonald, 1998) provide robust inputs to the BL, but little or none to the LA. Midline thalamus is critical for regulating fear learning by signaling unexpected aversive events (McNally et al., 2011), whereas mPFC mediates fear extinction (Myers and Davis, 2007, Herry et al., 2010). One can imagine that when the BL has high cholinergic tone, such as during fear learning, midline thalamic inputs would be facilitated if they arriving in theta or gamma frequency band, whereas mPFC projections would be shut off due to potent muscarinic inhibition. In this way, information flows can be coordinated depending upon the brain states.

Several subtypes of mAChRs are expressed in the BL, including M1 and M2 receptors (McDonald and Mascagni, 2010, 2011, Muller et al., 2013). In this study, we found that both M1 and M3 receptors were involved in inhibition of external glutamatergic inputs by muscarine. M1 and M3 receptors could be expressed on same axonal terminals or could be on separate axonal terminals originated from distinct brain

regions. The latter is favored by the fact that midline thalamic pathway was solely mediated by M1 receptors. Anatomical studies found that M1 receptors are located only on subpopulations of both dendritic spines and excitatory and inhibitory terminals (Muller et al., 2013). Electron microscope studies demonstrated that many M1R+ terminals formed asymmetrical synapses onto BL PNs spines and cholinergic terminals were often observed to be adjacent to M1R+ terminals and spines (Muller et al., 2013). These anatomical findings suggest that there may be differential mAChR subtypes regulation of glutamatergic transmission from different brain regions, which is consistent with the results in the present study.

Muscarinic inhibition of external glutamatergic inputs to the BL seemed contradict to the fact that facilitation of fear learning and consolidation by mAChRs activation in the BL (Power et al., 2003b). However, we propose that glutamatergic inputs arriving at theta or gamma frequencies would be strengthened during periods of high cholinergic tone, whereas weak or asynchronous signals would be suppressed. While muscarine inhibited external inputs to BL PNs during a single or low frequency stimuli, it increased the reliability of excitatory transmission at these synapses by preserving transmitter vesicles during a stimulus train. This effect was greatest at stimulus frequencies in the gamma band (30-90 Hz). This could protect the BL from asynchronous noises, while increasing the response to relevant information. We suggest that when mAChRs are activated, neuronal ensembles that oscillate at gamma frequencies would be more likely to communicate with the BL. This may contribute to the observed increase in functional coupling between BL and target neurons during gamma oscillations in vivo (Bauer et al., 2007a, Popescu et al., 2009). Dense cholinergic innervation of the

BL may be essential for its ability to bind spatially distributed information represented in multiple brain regions. Decreased cholinergic tone in Alzheimer's disease may impair information binding of BL and allows asynchronous signals to trigger BL circuits to cause emotional disturbances.

In contrast to inhibition of external glutamatergic inputs, muscarine did not suppress recurrent synaptic transmission, suggesting that during periods of high cholinergic tone mAChRs would filter external inputs, while leaving inputs from LA and recurrent inputs from BL unchanged. This may serve to increase the signal to noise ratio for LA input to BL, allowing the LA PNs to more strongly influence BL PNs at frequencies below gamma frequency band. In addition, the preservation of recurrent excitation within the BL during periods of high cholinergic tone may be important in establishing and extending neuronal ensembles carrying same information (Han et al., 2007). These observations are very different from those in other brain regions, such as piriform cortex where mAChRs suppress recurrent excitation and keep afferent input unchanged (Hasselmo and Bower, 1992). The lack of mAChR suppression of afferent inputs to piriform cortex supports the role of this region in associative memory (Hasselmo and Bower, 1992). In contrast, strong cholinergic innervation of the BL may be important in filtering external glutamatergic inputs, thereby keeping the BL from weak or asynchronous signals.

Muscarine suppressed eIPSCs but increased sIPSCs frequency and amplitude, suggesting that mAChRs activation differentially suppresses GABA release from interneuron subpopulations. This may allow mAChRs to regulate distinct aspects of network activity in the BL. For example, suppression of GABA release from dendritically

projecting interneurons could contribute to muscarinic regulation of synaptic plasticity. Muscarine increased the number of sIPSCs with larger amplitude and faster decay. These sIPSCs very likely came from perisomatic projecting interneurons, which might be depolarized by application of muscarine. M1 and M2 receptors are located in both perisomatic and dendritic inhibitory terminals (Muller et al., 2013), suggesting that both perisomatic and dendritic projecting terminals could be suppressed by muscarine. However, since perisomatic projecting basket cells are mainly fast firing interneurons, mAChRs at their inhibitory terminals may make GABA release more reliable during high frequency firing, as observed at glutamatergic terminals. This could explain why the sIPSCs with large amplitude and fast kinetics were not suppressed by muscarine. Suppression of dendritic inhibition could facilitate LTP induction, while enhancement of perisomatic inhibition promotes oscillation and coordinates outputs from BL PNs.

Frequency dependent modulation of external glutamatergic inputs and disinhibition of feedforward and dendritic GABAergic inhibition by muscarine may be responsible for facilitation of LTP induction in the BL by mAChRs activation, shown in the present study. This agrees with previous studies. For example, application of scopolamine in medial and lateral amygdala blocked LTP induced by high frequency stimulation (Watanabe et al., 1995). Substantial evidence from other brain regions supports the ability of mAChRs to regulate LTP. In the hippocampus, activation of mAChRs by muscarinic agonists or released ACh enhances LTP evoked by tetanic stimulation (Burgard and Sarvey, 1990, Maeda et al., 1993, Ovsepian et al., 2004, Shinoe et al., 2005) and also facilitates spike timing dependent plasticity (STDP) (Seol et al.,

2007, Sugisaki et al., 2011). mAChRs activation also lowers the threshold for LTP induction (Ovsepian et al., 2004).

Taken together, we demonstrated how mAChRs activation could protect the BL from weak or asynchronous signals while enhancing the response to meaningful information in distinct afferent pathways. The ability of mAChRs to filter out or select vital information and facilitates LTP may be key to the development of improved therapies for Alzheimer's disease, drug addiction, anxiety disorders and schizophrenia.

CHAPTER 6

GENERAL DISCUSSION

6.1. FINDINGS OF THE STUDY

1. Muscarine preferentially depolarizes PV+ FF interneurons but not SOM+ or RF interneurons in the BL through activation of M3 receptors.

2. Muscarine generates rhythmic synchronized inhibition at theta frequency band across BL PNs by selectively recruiting PV+ FF interneurons. This inhibition is able to synchronize BL PNs firing which may be responsible for generation of theta oscillations in the BL. The synchrony among far apart but not adjacent BL PNs requires gap junction.

3. Muscarine suppresses external inputs to the BL PNs in a frequency dependent and pathway specific manner, while it enhances recurrent synaptic transmission in the BL and from LA. Muscarine also inhibits both feedforward and local GABAergic inhibition in the BL. Due to these effects muscarine facilitates LTP induction in the BL.

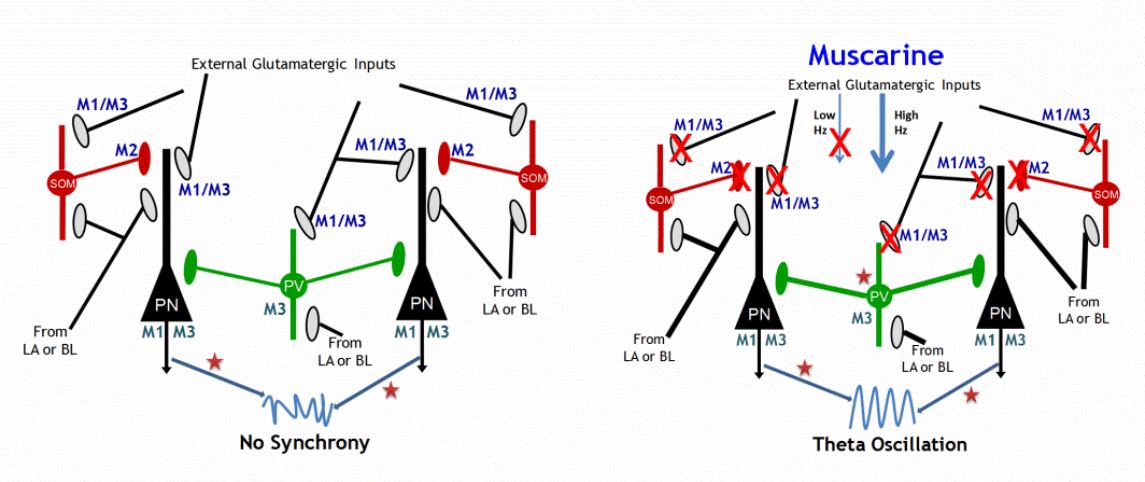

Figure 6.1 Mechanism of regulation of BL neuronal oscillation and LTP by mAChRs activation

6.2 COMPARISONS OF MUSCARINIC MODULATION IN THE AMYGDALA AND IN OTHER BRAIN REGIONS

Cholinergic signaling modulates the BL of amygdala in a way that is strikingly different from other brain structures. In the BL, PV+ FF interneurons are depolarized by muscarine to generate synchronized inhibition across BL PNs, while in the cortex, SOM+ and RF interneurons are preferentially excited by mAChRs agonists (Kawaguchi, 1997). Since different types of interneurons perform distinct functions, cholinergic transmission may modulate these two brain areas differently. However, same types of interneurons in these two brain structures seem to share same projecting patterns and may have same functions in the local circuits (Muller et al., 2006, 2007a). SOM+ and RF interneurons which project to the distal dendrites and spines of pyramidal cells may regulate receiving information inputs, while PV+ FF interneurons synapse on to somas may modulate information output. Therefore, in the cortex, cholinergic modulation of interneurons may play a role in sharpening sensory information perceiving from the thalamus (Hasselmo and Sarter, 2011). This is in line with extensive behavioral evidence that cholinergic signaling in the cortex mediates bottom up regulation (Hasselmo and Sarter, 2011). Cholinergic modulation of glutamatergic transmission in the BL is opposite to that in the cortex and hippocampus. In the BL amygdala, activation of mAChRs suppresses external inputs but keeps recurrent glutamatergic transmission intact, whereas in the cortex and hippocampus, cholinergic signaling inhibits internal glutamatergic transmission and enhances afferent inputs (Giocomo and Hasselmo, 2007). This difference could be explained by functional differences between the amygdala and the cortex and hippocampus. The cortex needs to constantly receive and process information the body

senses from the thalamus. By suppressing internal glutamatergic interactions and enhancing thalamic glutamatergic transmission, cholinergic signaling in the cortex makes the cortical pyramidal cells well tuned to specific stimuli and increases signal noise ratio (Giocomo and Hasselmo, 2007). In contrast, the amygdala is specialized in emotional processing, which ignores spontaneous, emotionally irrelevant information. By inhibition of asynchronous, spontaneous external inputs to the BL cholinergic transmission protects the amygdala from being persistently disturbed. However, when emotionally relevant information coming in at theta or gamma frequency band, cholinergic signaling makes the synaptic transmission more reliable. Enhancement of recurrent glutamatergic transmission would further amplify incoming emotional signals. In short, in the cortex and hippocampus, cholinergic signaling may act as a gain modulator, while it functions like a high pass filter in the amygdala.

6.3 FUNCTIONAL RELEVANCE OF NEURONAL OSCILLATIONS IN THE BL

Much evidence has pointed to neuronal oscillations as a mechanism for mediating interactions among functionally related neuronal ensembles in distributed brain areas (Freeman, 1978, Gray et al., 1989, Singer, 1993, Buzsaki, 2005, Fries, 2009, Colgin, 2013). However, it is still unknown that how these brain state related neuronal oscillations are generated and how they are synchronized between brain circuits. In this project, we provided a potential mechanism of generation theta oscillations in the BL. Two models of generation of neuronal oscillations, pyramidal-interneuron gamma (PING) and interneuron-pyramidal gamma (ING), have been proposed as potential mechanisms of origination of neuronal oscillations (Whittington et al., 2000, Whittington and Traub, 2003, Tiesinga and Sejnowski, 2009). PING model suggests that

synchronized projection from pyramidal cells to interneurons drives rhythmic inhibition and thereby generates neuronal oscillation (Tiesinga and Sejnowski, 2009). In contrast, ING model is proposed that synchronized interneurons firing drives neuronal oscillation (Whittington et al., 2000). In other words, according to PING model the origin of synchrony is from synchronized pyramidal cells firing, while oscillations of ING model are initiated directly by interneurons. Cholinergically induced oscillations in hippocampal slices are thought to be a PING mechanism (Fisahn et al., 1998). In the BL, we found that muscarine-induced synchrony is based on ING mechanism. We do not exclude the possibility that PING mechanism is also involved in vivo. Why neuronal firing needs to be synchronized and what the functional relevance of it? When a set of synaptic inputs arrive to their targets at approximately the same time quantified the timing precision of the input spikes, they form a volley (Tiesinga et al., 2008). When glutamatergic inputs arrive in volleys, they become much more effective especially when their timing precision is high (Azouz and Gray, 2000). Coupling between brain circuits can promote their communications. For example, if the inputs always arrive at the peaks of the oscillations in the target area, they would be much likely cause the target neurons to fire and form LTP. Otherwise, target neurons would not respond to the stimuli. How do two spatially distributed neural circuits get synchronized? The answer is not clear. However, here we proposed some potential mechanisms of the synchrony between brain structures. In order to be synchronized, two neural circuits must be interconnected or indirectly connected via a third brain area. If two neural circuits are directly interconnected, oscillation generated in one circuit would drive the oscillation in the other. In this case, muscarine-induced neuronal oscillation in the BL would be able to drive downstream

circuits activities to be synchronized at similar frequency. In line with this, during adaptive learning, synchronization was observed between BLA and striatum (Popescu et al., 2009). More importantly, the synchrony was driven by neuronal oscillations in the BLA (Popescu et al., 2009). The other way of generating synchrony between two brain areas may be through mediating by a third brain structure. For example, amygdala is interconnected with multiple brain areas so that it is well suited to act as a mediator. Amygdala could send projections to PV interneurons in both mPFC and hippocampus. By activating the PV interneurons in both areas at the same time would be able to make pyramidal cells in mPFC and hippocampus be synchronized. Indeed, during fear conditioning, fear retrieval, extinction and extinction retrieval, synchrony was found between these three brain structures (Seidenbecher et al., 2003, Narayanan et al., 2007, Szinyei et al., 2007, Sangha et al., 2009, Pape and Pare, 2010).

6.4 POTENTIAL SIGNIFICANCE OF PV+ FF INTERNEURONS IN THE BL

In this project, we have shown that a single or few PV FF interneurons were able to phase a group of BL PNs. We speculated that each functionally specialized neuronal ensemble may be innervated by a single or small network of PV FF interneurons. Controlling a single PV FF interneuron would be a very efficient way to manipulate a neuronal ensemble activity. Simply phase resetting the neurons in an ensemble would facilitate their output without increasing their firing rate. If that is the case, the information about the connectivity of individual PV interneurons would be very valuable.

6.5 FUTURE DIRECTIONS

1. Both mAChRs and nAChRs utilize ACh as the endogenous agonist. Cholinergic signaling acts on both types of receptors. Therefore it would be important to know the role of nAChRs in cholinergic modulation of amygdala function. Without this information we would not be able to predict what cholinergic transmission really does in the amygdala in vivo.

2. In addition to cholinergic projections, the basal forebrain also sends GABAergic axons to the amygdala. It would be interesting to know how these two projections work together in modulation of amygdala function.

3. Information about cholinergic neurons activity and fluctuation of ACh concentration during fear conditioning and extinction would be valuable to predict the role of ACh in the amygdala function.

4. Studies on variability on individual BL PNs and animals would provide critical information about etiology of anxiety disorders.

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