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Individual Differences in Markers of Cholinergic Signaling Correlating to Fear and Extinction Learning

By

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Bachelor of Science
Winthrop University, 2015

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I would like to say a special thank you to my wonderful mentors, past and present, for their never ending knowledge and guidance. Thank you to my lab mates, friends, and family for their continued encouragement during the entirety of not just my Master's education, but all of my years of education, and for volunteering to be subjects in this study.

Abstract

Posttraumatic stress disorder (PTSD) results when individuals are exposed to a life threatening event, assault, serious injury, or other traumatic incident. Individuals with PTSD are impaired in their ability to extinguish fear memories, resulting in intrusive symptoms that impair their ability to live otherwise healthy lives. It remains unclear why some individuals exposed to traumatic events develop PTSD while others do not. Acetylcholine has been shown to play a critical role in fear learning, but its role in fear extinction is not well understood. This study utilized a rat model of fear learning and extinction to determine if individual differences in fear and extinction learning are correlated with markers of cholinergic signaling. This study examined M1 muscarinic acetylcholine receptor (M1 mAChR) and acetylcholinesterase (AChE), both heavily expressed in the basolateral amygdala (BLA), a region that has been heavily implicated in the acquisition, consolidation, and recall of fear and extinction memories. The goal of the present study was to determine if individual differences in these proteins involved in cholinergic signaling in the BLA potentially underlie the individual differences observed in the fear learning and extinction processes. Rats were conditioned using a Pavlovian fear conditioning and extinction paradigm and behavior was analyzed by measuring extent of freezing behavior during each stage of the trial. Grouped differences were found in ability to undergo fear extinction learning and to recall the fear extinction memory.

Coronal brain sections were processed for immunofluorescence, labeling for M1 mAChR and AChE, and imaged in order to measure extent of protein expression. Significant correlations were observed between individual's BLA M1 mAChR densities and ability to undergo fear acquisition and ability to recall fear extinction memories. This lead to the conclusions that M1 mAChR are functioning in the BLA in the processes of fear memory acquisition and extinction memory consolidation and that high expression of M1 mAChR allows for improved ability to undergo fear memory acquisition, resulting in a deficit in fear extinction. No significant correlations were observed between BLA AChE expression and any fear or extinction learning phase. These results add to the growing body of literature implicating M1 mAChR in fear and extinction learning. Therapeutic strategies aimed at altering muscarinic signaling in the amygdala could be implemented in order to enhance fear extinction in animals and patients with PTSD.

Table of Contents

Acknowledgements iii

Abstract iv

List of Figures viii

List of Abbreviations x

Chapter 1: Introduction 1

A. Fear Learning and Extinction 1

B. Anatomy of Fear Learning and Extinction 4

C. Cholinergic Regulation of Fear Learning and Extinction 7

D. Objective, Hypothesis, and Aims 11

Chapter 2: Methods 13

A. Animal Model of Fear Conditioning and Extinction Learning 13

B. Tissue Preparation, Immunofluorescence, & Image Collection & Analysis 16

C. Statistical Analysis 19

Chapter 3: Results 21

A. Behavior Results 21

B. Fluorescent Imaging Results and Analysis 27

C. Correlation between Behavior and Image Analysis 34

Chapter 4: Conclusions 43

Chapter 5: Future directions 48

References 50

Appendix A: Supplemental Data and Figures. 57

List of Figures

Figure 2.1: Fear and Extinction Behavior Paradigm 14

Figure 3.1: Group 1- Grouped Differences in Freezing Behavior During Fear and Extinction Paradigm.. 23

Figure 3.2: Group 2- Grouped Differences in Freezing Behavior During Fear and Extinction Paradigm.. 26

Figure 3.3: M1 mAChR Immunofluorescence 29

Figure 3.4: Basolateral Amygdala M1 mAChR+ Cell Density 30

Figure 3.5: Acetylcholinesterase Immunofluorescence... 33

Figure 3.6: Correlation- M1 mAChR vs AChE Protein Expression Levels 34

Figure 3.7: Correlation- M1 mAChR expression levels vs fear learning processes......... 36

Figure 3.8: Correlation- M1 mAChR expression levels vs the extinction learning processes 38

Figure 3.9: Correlation- AChE expression levels vs fear learning processes................... 41

Figure 3.10: Correlation- AChE expression levels vs extinction learning processes....... 42

Figure A.1: M1 mAChR CEA expression 57

Figure A.2: S1BF image collection and comparison 58

Figure A.3: Correlation- M1 mAChR expression levels vs contextual fear recall and delayed extinction acquisition.. 59

Figure A.4: Correlation- AChE expression levels vs contextual fear recall and delayed extinction acquisition 59

List of Abbreviations

Abbreviation	Definition
ACh	Acetylcholine
AChE	Acetylcholinesterase
ANOVA	Analysis of variance
BLA	Basolateral amygdaloid nuclei[*]
BLC	Basolateral complex, consisting of BLA, LA and BM
BLV	Ventral basolateral amygdaloid nuclei[*]
BM	Basomedial nucleus, consisting of the BMA and BLV nuclei
BMA	Basomedial amygdaloid nuclei[*]
CA1	CA1 field of the hippocampus[*]
CaMK	Calcium/calmodulin dependent protein kinase
CEA	Central nucleus, including medial and central subdivisions
CS	Conditioned stimulus
IL	Infralimbic cortex[*]
ITC	Intercalated cells/nuclei
LA	Lateral amygdaloid nuclei
M1 mAChR	M1 subtype of the muscarinic acetylcholine receptor[#]
mAChR	Muscarinic acetylcholine receptors[#]
PFC	Prefrontal cortex

PL .... Prelimbic cortex

Pri .... Periform cortex*

PTSD .... Posttraumatic stress disorder

PV .... Parvalbumin

S1BF .... Barrel field of the somatosensory cortex*

TB/TBS .... Tris-buffered/tris-buffered saline solution

US .... Unconditioned stimulus

VAChT .... Vesicular acetylcholine transporter

* : as defined by Paxinos and Watson, The Rat Brain in Stereotaxic Coordinates, 6th revised edition, 2008

: no distinction between molecular and pharmacological designation, unless otherwise stated

Chapter 1: Introduction

A. Fear Learning and Extinction

Fear is the natural, seemingly instantaneous response to a stimulus that is perceived as potentially harmful or threatening. It keeps us safe. It causes soldiers to swerve when they see a bomb in the road during a battle; it causes us to reach for the phone or a frying pan when we come across a stranger sifting through our drawers in a dark kitchen; and it causes us to run screaming when we happen across a bear while walking in the woods. It keeps animals safe too. It causes a deer to run when it hears a hunter approaching; it causes a fish to swim when it senses movement in the current as a shark speeds toward it; and it causes a rat to freeze when it hears a snake slithering toward it. All of these are appropriate responses to frightening or potentially threatening situations which keep individuals safe.

Fear-inducing situations such as these can be very impactful and can cause strong association memories to form by associating environmental cues such as sights, sounds, and even internal stimuli with the fearful situation. Fear-association memories can then later be triggered in a different situation by the same cues, causing fear memories to be activated, which induce a biological and behavioral fear response, allowing an individual to defend itself.

What about when fear responses are generalized and become inappropriate? When throwing a friend a party, you don’t expect them to call the police or reach for a frying pan when their family and friends yell “surprise”. A paper bag on a city road should not cause a veteran to swerve into oncoming traffic. The fear of being presented with a potentially threatening situation should not keep an individual from being able to leave the safety of their home. These inappropriate responses to non-threatening situations are a hallmark of posttraumatic stress disorder.

Posttraumatic stress disorder (PTSD) is a disorder that results when an individual is exposed to a life threatening event, assault, serious injury, or other traumatic incident. Strong associations are made which cause re-experiencing, avoidance behavior, negative mood changes, and hyper-arousal (American Psychiatric Association, 2013, p 271-280; Wilson and Reagan, 2016). Associations formed when experiencing a traumatic event can be so strong that individuals are unable to differentiate between a fearful stimulus and a similar harmless stimulus, causing inappropriate behavioral or physiological responses. However, not all individuals exposed to a traumatic event, even the same traumatic event, will develop PTSD. Where some individuals are able to undergo extinction learning, or the learning that a once fear-inducing stimulus is no longer an indication of danger, other individuals have a more difficult time undergoing extinction. The necessity to better understand and treat PTSD is very evident, with the yearly prevalence in America being 3.5% and veteran prevalence being higher than 13% (Kessler et al., 2005; Tamelian and Jaycox, 2008; for review see Wilson and Reagan 2016). The neurological differences between good and poor extinguishers are of great intrigue. The development of animal models has been an extremely useful tool in the understanding of individual differences

of fear learning and fear extinction (Fendt and Fanselow, 1999; Zoladz and Diamond, 2016; Wilson and Reagan, 2016; Wilson and Fadel, 2017).

Classical or Pavlovian conditioning, a technique often used in fear learning and fear extinction animal behavioral models, is the learned association between a neutral stimulus, such as a tone (conditioned stimuli, CS) and a biologically relevant stimulus, such as food or pain (unconditioned stimuli, US). This is a crucial ability for survival and allows individuals to associate safe and harmful situations with unconditioned stimuli, and is thus conserved across higher organisms (Pavlov, 1927; for review see Milad and Quirk, 2012 and Orsini and Maren, 2012).

This study takes advantage of this natural phenomenon in order to study fear learning, fear extinction, and the neurochemical mechanisms and functional receptors involved. The fear learning and extinction behavior protocol induces an association between a tone (CS) and a shock (US). Pairing of a weak stimulus, CS, with a strong stimulus, US, causes an overall strengthening of neural pathways, a property called associativity (Orsini and Maren, 2012). After an association has been made, the CS is repeatedly presented to induce fear extinction. Fear extinction is a learning process which occurs upon repeated re-exposure of the CS in the absence of the US, resulting in the CS no longer inducing a fear response. Upon re-exposure, the brain can respond in two different ways: the original fear memory can be reconsolidated, where the fear is strengthened and remains intact, or an extinction memory can be formed. Extinction occurs when the memory retrieval induces the formation of a new associated memory between the CS and the absence of the US, causing a reduced or absent conditioned fear response. This new memory does not modify or replace the original fear memory, but rather competes with it, masking its

expression, which can be demonstrated by observed instances of spontaneous recovery, renewal, or reinstatement (Baldi and Bucherelli, 2015; Baldi and Bucherelli, 2010; Myers and Davis, 2007; Quirk and Mueller, 2008). There is a natural, observable variation in individual ability to undergo fear extinction, as seen commonly in individuals with PTSD (Horn et al., 2016).

This study investigates some of the suspected underlying mechanisms of fear and extinction learning, as well as the individual differences in ability to undergo fear and extinction learning. This study is unique in that no pharmacological manipulations were made and protein expression is directly correlated to freezing behavior in order to extrapolate how protein expression level relates to fear learning, extinction learning, and specific learning phase.

B. Anatomy of Fear Learning and Extinction

Brain structures involved in the processing of fear and fear learning, including the prefrontal cortex (PFC), thalamus, hippocampus, and the amygdala, are conserved across species (Milad and Quirk, 2012). Environmental information is sent to the amygdala from the thalamus, PFC, and hippocampus (Fendt and Fanselow, 1999). This includes information about context, past experiences, and any perceived environmental stimuli, including sights, sounds, tactile information, etc. Sensory information is transmitted from the thalamus to the lateral amygdaloid nuclei (LA), where contextual information from the hippocampus is sent to the basolateral anygdaloid nuclei (BLA). Intrinsic connections between the LA and BLA allow for passage of information from the LA to the BLA to the central nucleus (CEA), as well as direct excitatory connections from the LA to the CEA. BLA neurons also have projections to the intercalated (ITC) nuclei, which then

synapse on to the BLA-CEA projection, allowing for filtering of information passed from the BLA to the CEA (Orsini and Maren, 2012). The CEA projects to the hypothalamus and brain stem, initiating behavioral and physiological responses, including freezing or running, autonomic responses, and inducing stress and startle responses (Sah and Westbrook, 2008). This initial response to threatening stimuli occurs much quicker than situational evaluation can occur, which allows individuals to respond seemingly instantaneously in preparation for fight or flight action (Principles of Neural Science, page 1478; Milad and Quirk, 2012).

Long-term memory formation and consolidation then occurs, allowing the animal to recall details about the threatening situation, should it be presented again. Synaptic plasticity caused by associative cued-fear learning can be observed in both the LA and the BLA, shown by enhanced excitatory postsynaptic potentials, increasing synaptic plasticity between BLA and CEA fear-out-put circuits (Sah and Westbrook, 2008; Orsini and Maren, 2012). Consolidation of the fear memory in the amygdala is required for stable long-term memory storage, and requires new protein synthesis. This can be demonstrated by giving intra-amygdalar protein synthesis inhibitors after fear conditioning, which prevents memory consolidation and subsequent recall (Maren et al. 2003).

Cued-fear extinction occurs upon repeated exposure of the CS in the absence of the US, similar to the practice of exposure therapy (Orsini and Maren, 2012). Extinction was first considered to be a specific type of learning when Pavlov observed spontaneous recovery of appetitive responses in dogs over time (Pavlov, 1927).

Like cued-fear learning, cued-fear extinction learning relies on multiple brain regions, which connect and communicate to make a plastic network. The amygdala, PFC, and hippocampus are the major players in this network, and, while each function in acquisition, consolidation, and retrieval, each has a set of major functions. The hippocampus functions in extinction context recall, the PFC mediates extinction consolidation, and the amygdala is thought to be where extinction memories are acquired and stored (Baldi and Bucherelli, 2015; Power et al., 2003b; Orsini and Maren, 2012). Hippocampal CA1 and ventral subiculum regions project to the LA, BLA, CEA, and the PFC. The BLA projects to each sub-region of the hippocampus and PFC. These dense reciprocal projections between the amygdala and hippocampus allow for fast and effective communication, which has been shown to be crucial for retrieval of context-aspects of extinction memories (Herry et al., 2008; Orsini and Maren, 2012). The PFC is subdivided into the prelimbic cortex (PL), which projects to the BLA and CEA, and the infralimbic cortex (IL), which projects to the basomedial amygdala (BM), ITC cells, and CEA (McDonald et al., 1996; Orsini and Maren, 2012). IL suppression of BLA through inhibitory circuits, including ITC cells, causes suppression of fear response (Quirk et al., 2003; Likhtik et al., 2008; Akirav et al., 2006; Sah and Westbrook, 2008; Orsini and Maren, 2012). Once extinction has been acquired, information about the CS, tone specifically, is relayed to the amygdala, not by the thalamus, but by the auditory cortex, indicating that after extinction there is a redistribution or rearrangement of information about the CS throughout the fear circuit (Pape and Pare, 2010; Orsini and Maren, 2012).

Cue based extinction recall involves a network of brain regions, which include the hippocampus, PFC, and the amygdala. The hippocampus functions in resolving meaning

of the presented CS using contextual cues (Quirk and Mueller, 2008). The PFC, specifically the IL, has been shown to be important for retrieval of extinction memory and suppression of fear (Myers and Davis, 2007). The importance of the IL in this process has been shown in electrophysiology and inactivation studies, where presentation of the extinguished CS, specifically, causes IL firing and BLA inactivation before extinction retrieval results in a fear response (Herry and Garcia, 2002; Milad and Quirk, 2002; Sierra-Mercado et al., 2006). Herry et al. (2008) and Senn et al. (2014) showed that the BLA contains separate populations of cells, fear neurons and extinction neurons, which are active specifically during fear or extinction, respectively. The BLA cell population which project to the PL is involved in fear and is activated during high fear situations, whereas the cell population projecting to the IL is involved in extinction and is activated during extinction behavior (Herry et al., 2008; Senn et al., 2014). While many brain regions are critical for the learning and expression of fear and extinction memories, this study's main focus was the amygdala, specifically the BLA due to its central role in each aspect of the fear learning and extinction process outlined above.

C. Cholinergic Regulation of Fear Learning and Extinction

Now that the neural structures and connections have been outlined, what role does the cholinergic system, and specifically M1 muscarinic receptors, play in fear learning and extinction? It has been suggested that acetylcholine (ACh) is important for learning and memory (reviewed in Power et al., 2003b, Wilson and Fadel, 2017, and Gold, 2003). Increases in ACh were shown to increase learning and cognitive function while decreases were shown to diminish such function (Power et al., 2003b). Gold (2003) review's literature in support of the idea that ACh controls the activity and extent of contributions

of various brain regions during learning in a variety of situations. It has been shown that the amygdala is the regulatory region modulating extinction learning and memory formation occurring in other regions, and that these regions compete over control of what is learned in the processing of information (Gold, 2003). However, Thiele et al. argues in a 2013 review that the local distribution and contribution of muscarinic signaling is what determines the cognitive tasks a brain region controls, rather than the release of acetylcholine alone. As thoroughly examined in Wilson and Fadel's 2017 review, current evidence suggests that fear extinction learning is regulated by activation of acetylcholine's metabotropic, muscarinic receptors (mAChR). Current research indicates that mAChR activation is crucial for fear acquisition, consolidation, and potentially recall, as well as extinction memory consolidation, and potentially cued fear extinction acquisition (Wilson and Fadel 2017).

It has been demonstrated that there is dense cholinergic presence in the brain regions involved in fear learning outlined above. The hippocampus and amygdala, specifically the BLA, were shown by Muller, Mascagni, and McDonald to have very dense cholinergic projections originating in the basal forebrain (2011). These projections terminate heavily on pyramidal neurons of the BLA, which postsynaptically express high levels of M1 mAChR. McDonald and Mascagni (2010) demonstrated through immunoperoxidase labeling studies that, while M1 mAChR is present throughout the basolateral complex of the amygdala (BLC), the anterior subdivisions of the BLA and LA contain the highest levels of mAChR expression of any amygdalar nuclei. This study also showed M1 mAChR expression is most dense in the cell bodies of pyramidal neurons within these regions with light expression in the neuropil. Choline acetyltransferase and

acetylcholinesterase (AChE) expression in the BLA has been shown to be some of the densest in the brain, further indicating the importance of cholinergic function in this region (Ben-Ari et al., 1977). This would lead one to assume that this region and its functions are largely modulated by cholinergic neurotransmission and cholinergic receptors.

As outlined in Wilson and Fadel's 2017 review, multiple drug studies have determined mAChR are important for fear learning. By giving mAChR antagonists prior to fear conditioning systemically or intracerebrally, studies have indicated that mAChR function is important for acquisition of cued and contextual fear (Rudy, 1996; Young et al., 1995; Feiro and Gould, 2005; Jiang et al., 2016; Fornari et al., 2000; for review see Wilson and Fadel, 2017). Drug studies examining fear consolidation, specifically, have generated varied results: several studies indicate that mAChR activation is not crucial for cued fear consolidation (Young et al., 1995; Anagnostaras et al., 1995; Wilson and Fadel, 2017), whereas several studies have shown that mAChR antagonists decrease contextual fear consolidation (Bucherelli et al., 2006; Passani et al., 2001; Wilson and Fadel, 2017) and mAChR agonists increase contextual or cued fear consolidation (Vazdarjanova and McGaugh, 1999; Power et al., 2003a; Young and Thomas, 2014; Wilson and Fadel, 2017). Young, Bohenek, and Fanselow, however, found that administration of a mAChR inhibitor actually increased consolidation of the fear memory (1995). A recent paper by Patricio et al. (2017) found that M1 mAChR are required for context fear memory recall. Collectively, these results indicate that ACh and mAChR are important for fear acquisition, and ACh may be important for consolidation, but mAChR are thought not to

be. These studies indicate a necessity for further studies elucidating the function of mAChR in the fear memory processes.

Literature examining the role of mAChR in extinction learning is also analyzed in Wilson and Fadel (2017). As of 2007, only 2 studies had looked at the role of cholinergic transmission in fear extinction, so the body of work surrounding this process is much smaller (Myers and Davis, 2007). However, the studies conducted thus far indicate that ACh and mAChR are important for extinction acquisition and consolidation in multiple different brain regions. Santini et al. (2012) highlighted the importance of mAChR by injecting the non-selective mAChR inhibitor scopolamine systemically and into the IL before and after extinction learning. Systemic injections both before and immediately after extinction were shown to impair extinction consolidation, shown by poor recall of extinction memory. Intra-IL injections were shown to impair extinction when administered before extinction learning, but not when administered after, indicating that mAChR in the IL are important for acquisition of extinction memory but not consolidation. Additionally, when given a general mAChR agonist systemically before or after extinction learning, recall of extinction memory was facilitated. Together, these data indicate that mAChR are important for extinction memory acquisition and consolidation, and IL mAChR are important for extinction memory acquisition alone (Santini et al. 2012). Boccia et al. (2009) examined the role of mAChR in the BLA in contextual extinction memory consolidation. While not addressing cued-extinction memory, Boccia et al. found that bi-lateral BLA injection of a general mAChR agonist, oxotremorine, immediately following extinction training improved the rat's ability to undergo consolidation of contextual extinction memories (2009). In a different type of

conditioning paradigm, Schroeder and Packard (2004) tested the effect of systemic and intra-BLA oxotremorine on amphetamine-induced conditioned place preference extinction consolidation. This study found that both systemic and intra-BLA treatment given post-extinction training facilitated extinction learning, further indicating that mAChR are functioning in extinction consolidation (Schroeder and Packard, 2004). Zelikowsky et al. found that post-extinction training mAChR inhibition by systemic scopolamine injection impaired rats' ability to undergo extinction consolidation (2013). Together, these findings seem to indicate that mAChR are important in extinction learning consolidation and mAChR inhibition prevents this process, where mAChR enhancement improves consolidation. My data, along with previous work, allows for the solidification of the hypothesis that mAChR are highly functional in the BLA's role in extinction acquisition and extinction memory consolidation. The Mott and McDonald laboratories are currently undergoing collaborative efforts to better understand muscarinic signaling within the amygdala, and this study aids in that effort.

D. Objective, Hypothesis, and Aims

The *objective* of this study was to generate a group of animal that demonstrated grouped and individual differences in ability to extinguish learned fear and to examine the expression levels of two crucial proteins involved in cholinergic signaling in animals that underwent a fear learning and fear extinction paradigm. This was done in order to understand if M1 mAChR and AChE BLA expression correlate to the animal's ability undergo the processes necessary for fear and extinction learning. Previous research by Joshua McElroy in Dr. Mott and Dr. Wilson's lab found a positive correlation between expression of the cholinergic markersM1 mAChR and vesicular acetylcholine transporter

(VAChT) in the basolateral amygdala and the ability of an animal to undergo extinction learning. These findings led us to propose the following hypothesis:

We *hypothesized* that BLA level of the cholinergic proteins M1 mAChR and AChE would positively correlate with extinction learning. Additionally, we hypothesized that expression of these proteins would demonstrate variations between individuals. To test these hypotheses, the following aims were proposed and accomplished: *Aim 1*: Determine if animals demonstrate grouped and individual differences when tested in fear learning and fear extinction paradigm, and *Aim 2*: Determine if there is a correlation between M1 mAChR and AChE expression in the BLA and individual differences in fear and extinction learning.

Chapter 2: Methods

The experiments conducted for this study included the generation of 2 groups of rats: one groups of 8 rats and a second group of 12 rats. A fear conditioning paradigm was used to condition fear to a US, tone, and induce fear extinction learning. Brain sections from each animal were fluorescently labeled for proteins involved in the cholinergic pathway. Labeled tissue was imaged using confocal and widefield fluorescence microscopy. The images were then analyzed and the data collected was analyzed alongside the data generated during behavioral conditioning trials.

A. Animal Model of Fear Conditioning and Extinction Learning

The fear conditioning and extinction paradigm used for this project was previously described in Sharko et al. (2016). The two groups of rats fear conditioned were done so separately and were slightly different and thus will be referred to and presented as separate. Group 1 (n=8) underwent ferret odor exposure trials, which were performed one week prior to fear conditioning. The ferret odor exposure behavioral trial was conducted by placing each individual rat into a Plexiglas cylinder that contains a small piece of fabric hanging inside the cylinder that has been soaked in ferret scent. Rats were kept in the cylinder for 60 minutes while freezing behavior was recorded using FreezeScan software (CleverSys Inc., Reston, VA). These data showed no significant differences

between individuals or groups (data not shown). Group 2 (n=12) were not exposed to ferret (predator) odor while all other paradigm parameters were kept the same between the two groups. Previous research conducted in the Wilson lab found that ferret scent exposure did not change the outcome of the observed behavioral patterns and thus it was decided not to conduct such behavioral trial on group 2 rats (unpublished data).

1. Subjects

Two groups (referred to as group 1 and group 2) of adult (9 weeks old) male Long Evans outbred rats 175-200 grams upon arrival were used for this study (n=8 and 12, respectively). Rats were singly housed and maintained a 12-hour light-dark cycle with free access to food and water. Upon arrival, rats were handled and weighed daily (weight change data not shown) for at least one week prior to the fear conditioning to habituate to experimenter.

2. Fear Conditioning and Extinction Behavior Paradigm

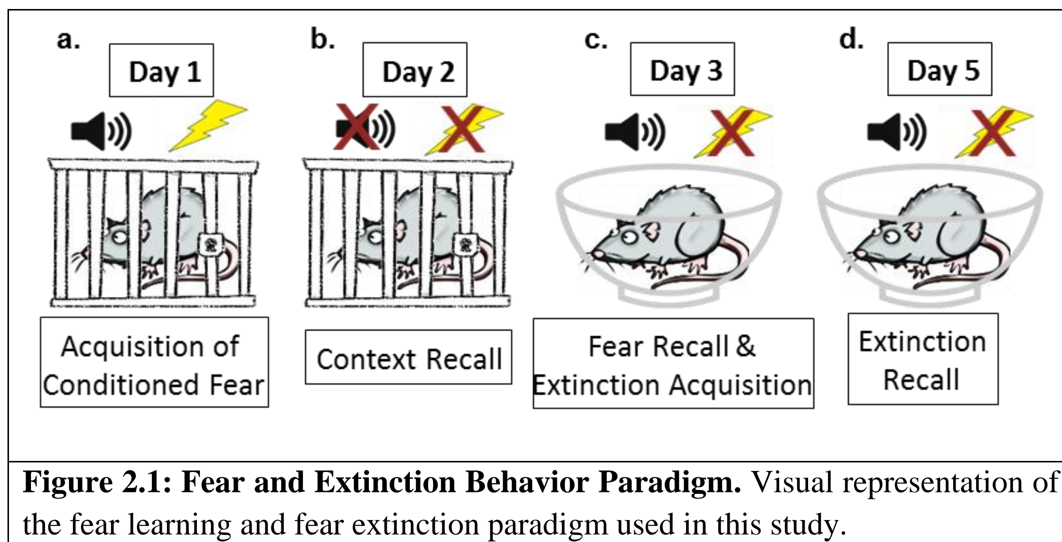

Figure 2.1: Fear and Extinction Behavior Paradigm. Visual representation of the fear learning and fear extinction paradigm used in this study.

Day 1: Acquisition of Conditioned Fear: Rats were individually placed in a shock box (Med Associates Inc., Vermont) (Context A, shock box), with a floor of evenly spaced

stainless steel rods which were connected to a shocking apparatus which delivered the foot shock (Figure 2.1.a). The shock box was inside a sound-attenuating box containing a ventilation fan and light. Unconditioned freezing behavior was recorded for the first 3 minutes of time in the box. Rats were then conditioned to the unconditioned stimulus (tone) with three 10 second tones (80db, 2kHz) co-terminating with a mild foot shock (1mA, 1 sec) at 60 second intervals. The shock box was cleaned between trials with 5% ammonium hydroxide.

Day 2: Context Recall: On day 2, 24-hours after fear acquisition, rats were placed back into the original shock box (Context A) for 8 minutes without the presentation of tone or shock to assess context conditioned freezing (Figure 2.1.b).

Day 3: Fear Recall & Extinction Acquisition: On day 3, 48-hours after fear acquisition, rats were assessed for cue conditioned freezing and within-session extinction learning using a novel chamber (Context B) with visual and olfactory cues distinct from those of the shock box (Figure 2.1.c). Animals were brought into the testing facility in a different manner (cages carried individually as opposed to in pairs and pushed on a cart) and tested in a different testing room. Context B was a Plexiglas bowl placed in a sound-attenuating box with a ventilation fan and light, cleaned with 70% ethanol between animals, lined with bedding, and scented with lemon extract (20μL). After unconditioned freezing in the novel environment was assessed for 3 minutes, rats were presented with twenty 10 second tones (80db, 2 kHz) at 60 second intervals.

Day 5: Extinction Recall: On day 5, 48-hours after extinction learning, animals were placed back in the extinction learning chamber (Context B, all other visual and olfactory

cues consistent with testing on day 3) and presented with twenty 10 second tones (80 db, 2kHz) to assess for fear extinction learning recall (Figure 2.1.d).

B. Tissue Preparation, Immunofluorescence, & Image Collection & Analysis

Two hours after the start of extinction recall on day 5, animals were anesthetized by 5% isoflurane inhalation for 5 minutes, transcardially perfused with 100mL of cold 0.1M phosphate buffered saline (pH 7.4) then 300mL cold 4% paraformaldehyde in 0.1M phosphate buffered saline (pH 7.4). Brains were immediately removed and post-fixed for 2 days in 4% paraformaldehyde in 0.1M phosphate buffered saline (pH 7.4) at 4°C. Brains were moved to 15% sucrose for 1 day and 30% sucrose until saturated. Coronal sections at 50µm were cut on a microtome and stored at -20°C in anti-freezing solution (30% ethylene glycol and 30% sucrose in 0.1M phosphate buffer, pH 7.4) until immunofluorescence processing. One tissue section per rat was labeled for M1 mAChR and the neighboring section was labeled for AChE. Tissue sections labeled and imaged ranged from Bregma -2.05mm to Bregma -2.30mm, according to Paxinos and Watson, The Rat Brain in Stereotaxic Coordinates (2008), all of which contained anterior BLA. All tissue was processed, imaged, and analyzed blindly together.

Immunofluorescence labeling with M1 mAChR antibody was used to measure protein expression in the amygdalar complex. Tissue was washed for 10 minutes 3 times in 0.05M tris-buffered saline solution (TBS) (pH 7.6). Tissue was exposed to pre-block for 30 minutes, consisting of 0.5% Triton and 10% normal goat serum in 0.05M TBS. Tissue was washed for 5 minutes 3 times in 0.05M TBS. Tissue was incubated at room temperature overnight in 0.5% triton, 2% NGS, anti-M1 mAChR primary antibody

(1:500; rabbit polyclonal; mAChR-M1-Rb-Af340; AB_2571791; Frontier Institute co., ltd.). Frontier Institute's M1 mAChR antibody specificity was confirmed by Narushima et al. using an M1 knock-out brain (2007). The next day, tissue was washed for 10 minutes 3 times in 0.05M TBS, then incubated for 2 hours, protected from light, in 0.5% triton, 2% normal goat serum, goat anti-rabbit conjugated Alexa Fluor 546 secondary antibody (1:400; A-11035; Thermo Fisher). Tissue was washed for 10 minutes 2 times in 0.05 TBS and exposed to DAPI staining solution (GTX16206; Lot# 821700090; GeneTex Inc.) for 10 minutes. Tissue was washed for 5 minutes 2 times in 0.05M TBS, 2 times for 5 minutes in 0.05M tris-buffered solution (TB) (pH 7.4), mounted on 0.5% gelatinized slides, and allowed to dry. Slides were coverslipped using ProLong Diamond Antifade Mountant (P36970; Invitrogen, Thermo Fisher) and kept flat at 4°C until imaging. Controls for antibody specificity were conducted by exposing one piece of experimental tissue only to secondary antibody, goat α-rabbit conjugated Alexa Fluor 546 secondary antibody. This tissue, imaged under identical parameters as that for experimental tissue, showed no detectable staining (data not shown).

All M1 mAChR image collection was conducted using a Leica SP8 multiphoton confocal microscope system equipped with the Leica Application Suite X (2.0.1.14392) (Leica Microsystems). Laser and detector settings were kept consistent throughout image collection (format: 1024x1024, speed: 400Hz, image size: 369.05µm x 369.05µm, pixel size: 360.75µm x 360.75µm, optical section: 4µm, 3.59 Airy Unit, z-step size: 2.5µm, solid state diode 552nm laser settings: laser intensity: 3.2%, emission spectrum: 560nm-590nm, gain: 840.0V, offset: -30.0%). A gridded Z-series through the BLA of each tissue section was collected with 40x objective, an optical zoom of 0.75, at 3.59 Airy

Units. Mosaic merge and maximal projection settings were optimized for each image using the accompanying LAS AF 3 software. Merged maximal projection images were used to perform all image analysis.

One neighboring tissue section from each rat was labeled for acetylcholinesterase (AChE), which allowed for both measured AChE protein expression and to identify the BLA in M1 mAChR labeled tissue, seeing that AChE cleanly and clearly labels the BLA. The labeling protocol used was identical to that outlined above, with the exception of the serum used, which here was BSA. The primary antibody used was anti-acetylcholine mouse monoclonal antibody (1:75; ZR3 clone, MA3-041, Thermo Fisher) with a chicken anti-mouse Alexa Fluor 647 (1:400; A-21463; Thermo Fisher).

All AChE image collection was conducted using an Invitrogen EVOS FL Auto cell imaging system, equipped with a Cy5 LED light cube (Thermo Fisher). Grid images were collected at 20X with Cy5 light intensity at 65%, exposure 0.1 msec, and gain set to 1.0V. Images were auto-merged by the Invitrogen EVOS FL Auto 2.0 Imaging system; merged images were used to perform all image analysis.

Fiji ImageJ 1.51h was used to analyze all images (National Institute of Health, USA). Several data points were collected from each image (not all data shown). Images were analyzed by converting image to a red-green-blue stack (RGB stack) and recording the histogram mean of the red channel alone for M1 mAChR and AChE images, measuring the average pixel intensity. Using the adjacent tissue section labeled for AChE as a reference, the BLA of M1 mAChR images was circled using the freehand selection tool and the histogram mean was recorded for the BLA in the red channel. Using a standard size oval, a small consistent region of the CEA in each image was selected and the

histogram mean was recorded. To generate a value of M1 mAChR expression for each animal, the histogram mean of M1 mAChR measured in the BLA was divided by the histogram mean of M1 mAChR measured in the CEA. The same was done for AChE images. All values generated for each rat are an average of each hemisphere (unless otherwise stated) on one tissue section from each animal. Only image analysis of group 2 is shown. The histogram mean values showing average pixel intensity collected and used for individual rat image analysis of M1 mAChR labeled tissue and AChE labeled tissue can be found in Table A.1 and Table A.2, respectively.

C. Statistical Analysis

All freezing behavior was recorded and assessed using FreezeScan software (CleverSys Inc., Reston, VA). FreezeScan software parameters were designated to detect freezing behavior as the animal not moving except breathing. Data were collected in 60 second bins and presented as a percent of freezing behavior during each 1 minute bin of each trial. For analysis of behavior data, groups were separated into high and low responders based extinction acquisition; the last 10 minutes of day 3 cue-conditioned freezing & extinction acquisition the animals were divided into high and low responders based on a median split of the average percent freezing. This distinction allows for examination of grouped differences in ability to undergo fear extinction learning. Rats in group 1 with an average percent freezing below 22% during extinction learning were determined to be low responders (good extinction learning), where rats above 22% freezing were determined to be high responders (poor extinction learning) (n=4 per group). Rats in group 2 with an average percent freezing below 34% during extinction learning were determined to be low responders (good extinction learning), where rats

above 34% freezing were determined to be high responders (poor extinction learning) (n=6 per group). Statistical analysis was conducted using Graph Pad Prism (Prism 5 for Windows, version 5.02). High versus low responders in each group were compared by two-way analysis of variance (ANOVA; high vs. low freezing) with repeated measures across time bins (significance level $p<0.05$). Bonferroni post-tests were conducted to compare individuals over time. Graphs showing grouped high vs low responder freezing across the experiment (Figure 3.1.e, 3.2.e) were generated by taking the average time bin for each animal during each stage and comparing by two-way ANOVA (high vs low freezers) with Bonferroni post-tests to compare over time. Amygdalar nuclei protein expression was analyzed by a paired t-test (Figure 3.3.d, 3.5.e).

Liner regression and correlation analysis was conducted using Graph Pad Prism in order to analyze correlations between freezing behavior and protein expression. Both receptors' expression was compared separately to freezing behavior across various time points throughout the behavior paradigm which represented different stages of the fear and extinction learning process, outlined in Table 3.1. Average freezing behavior during learning phases was correlated with AChE or M1 mAChR protein expression in the BLA. This was used to ascertain correlation between specific learning phases and cholinergic protein expression. Rats were not grouped into high and low responder groups for receptor expression analysis, but were considered individually in order to examine individual differences in the fear and extinction learning process. Pearson correlation and linear regression analysis (95% confidence interval) were conducted comparing AChE and M1 mAChR BLA/CEA values to average percent freezing during each designated learning process (TABLE 3.1).

Chapter 3: Results

A. Behavior Results

Two separate groups of rats were submitted to the fear learning and fear extinction paradigm described above, referred to as group 1 and group 2 (analyzed and discussed separately). High and low responder groups were determined by a median split of the average percent freezing of the last 10 minutes of day 3 cue-conditioned freezing& extinction learning, grouping rats into within session extinction (low responders) versus those who did not undergo within session extinction (high responders).

1. Group 1 Behavior Results:

Although there were observed individual differences, all rats in group 1 acquired the conditioned fear on day 1 of the behavior paradigm ($F[1,6]= 0.01$; $p=0.94$) (Figure 3.1.a, e). Group 1 then shows high individual variation and overall poor context recall in both high and low responder groups, indicating poor fear memory recall ($F[1,6]= 1.69$; $p=0.24$) (Figure 3.1.b, e). Group 1 rats showed varied cue-conditioned freezing and extinction learning on day 3, but did not show significant differences between groups ($F[1,6]=1.20$; $p=0.316$) (Figure 3.1.c). Cued-fear recall and extinction learning, on day 3, can be broken into 2 different phases: the first few tone exposures (tones 1-5, minutes 5-9) which indicate cue-fear recall in response to experiencing the CS in a new context, and the last 10 CS/tone presentations when the rats were undergoing within-session extinction

acquisition, learning that the CS is no longer associated with the US. Group 1 shows varied cue-fear memory recall across individuals but did not show significant differences between groups ($F[1,6]=0.60$; $p=0.698$) (Figure 3.1.c, e). When examined alone, the last 10 tone presentations of cue exposure, indicating fear extinction learning, show significant differences between high and low responders ($F[1,6]=8.60$; $p=0.026$) (Figure 3.1.c, e). Animals were grouped into high vs low responder groups based on the median split of freezing during this behavioral stage (median value = 21.77%). Group 1 rats showed individual variation during extinction recall on day 5, but did not show significant differences between groups ($F[1,6]=2.17$; $p=0.316$) (Figure 3.1.d). Extinction recall, on day 5, can also be broken into 2 different phases: the first few tone exposures (tones 1-5, minutes 2-6) which indicate recall of the previously learned extinction memory, and the last 10 tone presentations indicating within session extinction learning for those individuals who had not yet undergone extinction learning, or extinction learning reinforcement. Group 1, however, had very few individuals recall the previously acquired fear extinction memory and was not significantly different between high and low responder groups ($F[1,6]=1.55$; $p=0.431$) (Figure 3.1.d, e). Finally, both high and low responders undergo within session extinction learning ($F[1,6]=2.75$; $p=0.148$) (Figure 3.1.d, e).

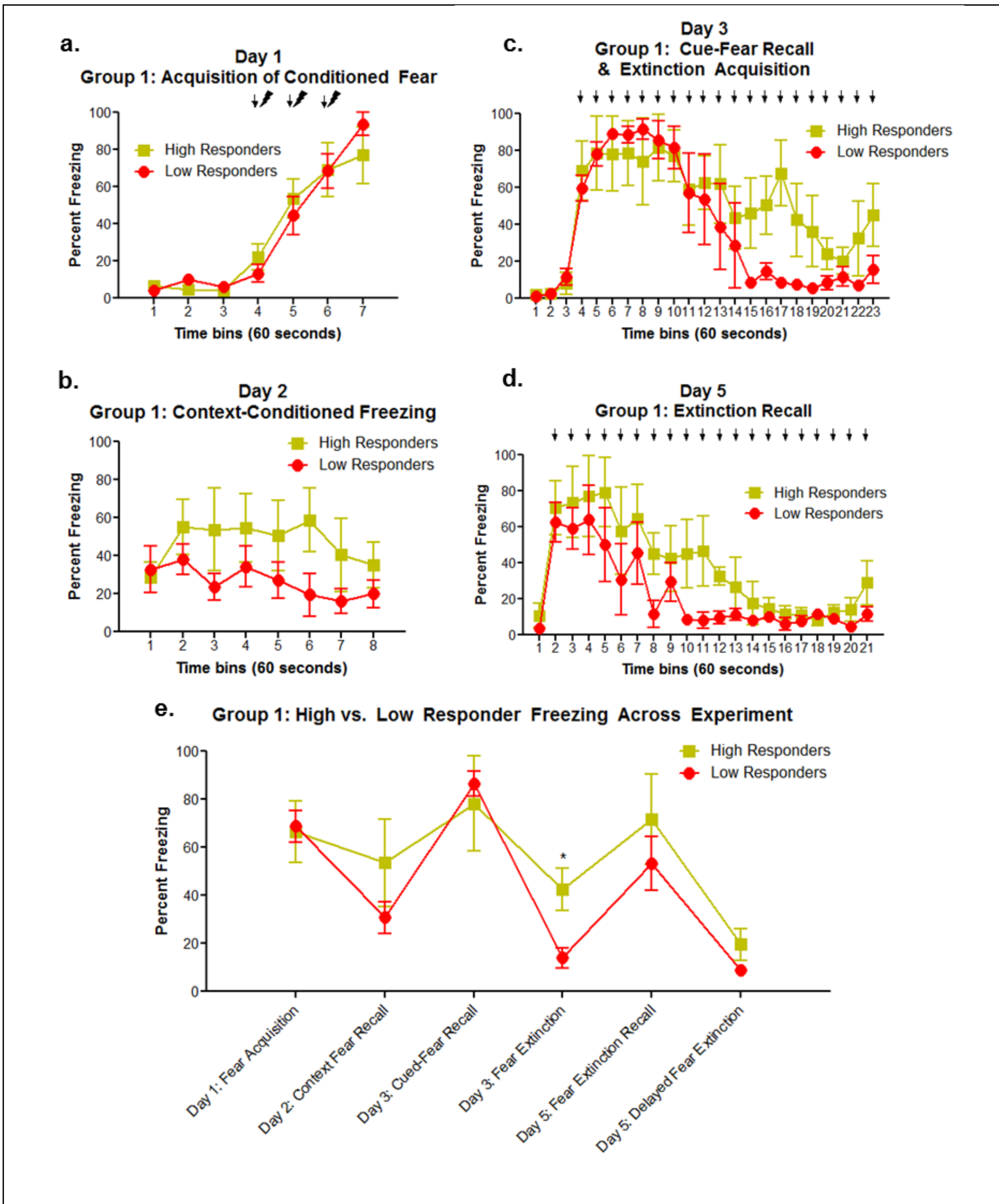

Figure 3.1: Group 1- Grouped Differences in Freezing Behavior During Fear and Extinction Paradigm. Group 1 rats were divided into high and low responder groups based on a median split of the mean percent freezing during the last 10 tones of day 3, c (median= 21.77%). There we no grouped statistical differences during any phase. Graph e breaks up the behavior paradigm into the learning segments outlined in Table 3.1 and shows that high and low responder groups show a statistical difference during day 3 cued fear extinction. Arows indicate tone presentation, lightninig bolts indicate shock presentation, asterisks indicate statistical significance (*=p<0.05).

2. Group 2 Behavior Results:

Although there were observed individual differences, all rats in group 2 acquired fear on day 1 of the behavior paradigm and did not show high vs low grouped differences ($F[1,10]=2.46$; $p=0.148$) (Figure 3.2.a, e). Group 2 then shows context recall and within session context extinction on day 2, which shows grouped differences between high and low responders, with high responders showing better context recall than the low responders ($F[1,10]=8.40$; $p=0.0159$) (Figure 3.2.b, e). Grouped differences between high and low responders on day 3 cue-conditioned freezing and extinction learning was found to be significant ($F[1,10]=14.47$; $p=0.0035$) (Figure 3.2.c). Fear recall and extinction learning can be broken into 2 different phases: the first few tone exposures (tones 1-5, minutes 5-9) which indicate cue-conditioned freezing in response to experiencing the CS in a new context, and the last 10 CS/tone presentations when the rats were undergoing within-session extinction learning, learning that the CS is no longer associated with the US. All animals in group 2 showed good cue-condition freezing, indicating both high and low responders were able to recall the CS-associated fear memory ($F[1,10]=4.61$; $p=0.0572$) (Figure 3.2.c, e). The last 10 tone presentations of cue exposure, indicating fear extinction learning, show stark differences in group 2 split between high and low responders, indicating low responders underwent cued fear extinction learning ($F[1,10]=17.58$; $p=0.0018$) (Figure 3.2.c, e). Animals were grouped into high vs low responder groups based on the median split of freezing during this behavioral stage (median value = 34.27%).Grouped differences between high and low responders on day 5 extinction recall was found to be significant ($F[1,10]=5.22$; $p=0.0454$) (Figure 3.2.d). Extinction recall, on day 5, can also be broken into 2 different phases: the first few

minutes of tone exposure (tones 1-5, minutes 2-6) which indicate recall of the previously learned extinction memory, and the last 10 tone presentations indicate within session extinction learning for those individuals who had not yet undergone extinction learning, or extinction learning reinforcement. Group 2 shows very clear distinctions between high and low responders extinction recall, where high responders demonstrated a recovery of the fear memory and subsequent freezing response and low responders demonstrated recall of the fear extinction memory ($F[1,10]=13.23$; $p=0.0046$) (Figure 3.2.d, e). Finally, both high and low responders undergo within session extinction learning ($F[1,10]=0.32$; $p=0.585$) (Figure 3.2.d, 3.2.e). Group 2 rats demonstrate clear grouped differences in the extinction processes, including acquisition and recall. This test does not indicate if recall differences are due to individual differences in ability to recall extinction memory or differences in ability to consolidate the extinction memory.

It is questionable as to if group 1 and group 2 rats can be combined. One reason is that that group 2 did not undergo ferret odor exposure prior to fear conditioning. Zoladz and Diamond’s 2016 review examined the effect predator-based psychological stress has on animal models used to study PTSD. The PTSD model examined equated predator exposure to “an inescapable, life-threatening experience”, a classified PTSD-inducing experiences. However, they found that predator exposure alone did not produce PTSD-like symptoms and that this may not produce symptoms translatable to humans (Zoladz and Diamond, 2016). Considering behavioral inconsistencies observed in group 1 and as of yet inconclusive results exploring the implications of ferret odor exposure on fear acquisition and fear extinction learning, group 1 was not included in imaging or correlation analysis.

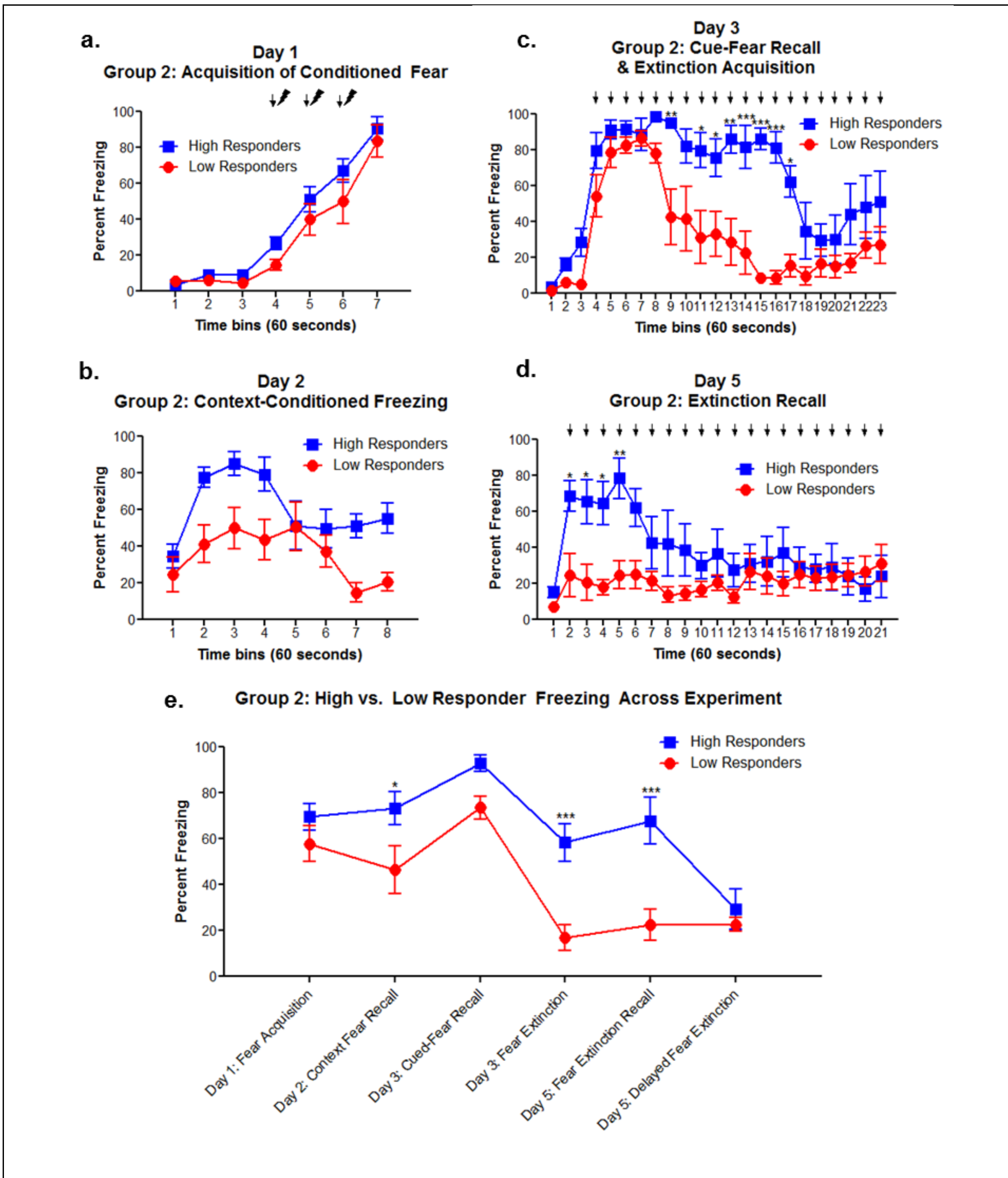

Figure 3.2: Group 2- Grouped Differences in Freezing Behavior During Fear and Extinction Paradigm. Group 2 rats were divided into high and low responder groups based on a median split of the mean percent freezing during the last 10 tone presentations of cued-fear recall & extincion acquisition, c (median= 34.27%). High vs low responder groups showed strong statistical differences during extinction acquisition (c, e) and extinction recall (d, e). Graph e breaks up the behavior paradigm into the learning segments outlined in Table 3.1. Arows indicate tone presentation, lightninig bolts indicate shock presentation, asterisks indicate statistical significance (*=p<0.05; **=p<0.01; ***=p<0.001).

B. Fluorescent Imaging Results and Analysis

M1 mAChR labeling and distribution observed in this study was similar to that observed in McDonald and Mascagni (2010). Dense M1 mAChR cell body labeling is evident in many temporal lobe structures imaged, including the LA, BLA, BLV, and the periform cortex (Pir), where cell body labeling is absent in BMA and CEA (Figure 3.3.a, b, A.1). The M1 mAChR positive cells labeled in Figures 3.3 and 3.4 have the morphology of pyramidal-like neurons, with a somewhat triangular cell body and between 1 and 3 apparent projections emanating from the apex and base of the cell, evident in high magnification images (Figure 3.4.a, b) (Sah et al., 2003). The labeled neurons also appear to have a random organization, opposed to the parallel organization seen in the Pir (Figure 3.3.a, b), which is typical of the cortex and hippocampus (Sah et al., 2003). Consistent with the cell body labeling seen in McDonald and Mascagni, this labeling pattern indicates that the M1 mAChR labeling observed and analyzed consists largely of postsynaptic receptors contained within the cell body of pyramidal neurons (2010). While labeling was observed in a number of regions, the BLA consistently had the densest M1 mAChR cell body labeling; this is illustrated in Figure 3.3. d, showing a higher average M1 mAChR expression in the BLA than in the CEA, when averaging left and right amygdala from each animal. Figure 3.3.c illustrates that left and right amygdala differs on average by no more than 5 units (on scale of 0-255). This indicates that M1 mAChR amygdalar expression does not vary from left and right in each animal, and the averaging of these values does not alter analysis. However, variation from animal to animal can be drastic, as is evident in intensity differences between images 3.3.a and b.

Labeling in the BLA was primarily cell body labeling, which can be clearly seen in the higher power image showing M1 mAChR + DAPI labeling (Figure 3.4. a, b). It has been reported that approximately 85% of neurons in the basolateral nuclear complex (BLC) of the amygdala (consisting of the LA, BLA, and BM) are positive for calcium/calmodulin protein kinase II (CaMK), a known marker of pyramidal neurons, and almost all CaMK positive/pyramidal neurons in the BLC are also M1 mAChR positive cells (McDonald, 1992; McDonald and Mascagni 2010). However, when examining M1 mAChR + DAPI labeled tissue, it appears that a far lower percentage than 85% of BLC neurons are M1 mAChR positive (Figure 3.4.a, b). This could be explained by examining the other types of cells in the BLC, such as inhibitory neurons, including those imaged in Figure 3.4.c, parvalbumin (PV) interneurons. A more detailed analysis of cell counts in M1 mAChR and DAPI labeled images is required to clarify this idea. Figure 3.4.a and b also illustrate a variation in extent of M1 mAChR cell body labeling. These images are 40X Z-series images collected in the center of the BLA. Image a appears to have fewer M1 mAChR positive cells compared to image b. Similar background levels indicate this is not an artifact of tissue processing or imaging, but rather could be indicative of the idea that individuals have different levels of M1 mAChR positive cells in the BLA. This, too, requires further analysis for clarification.

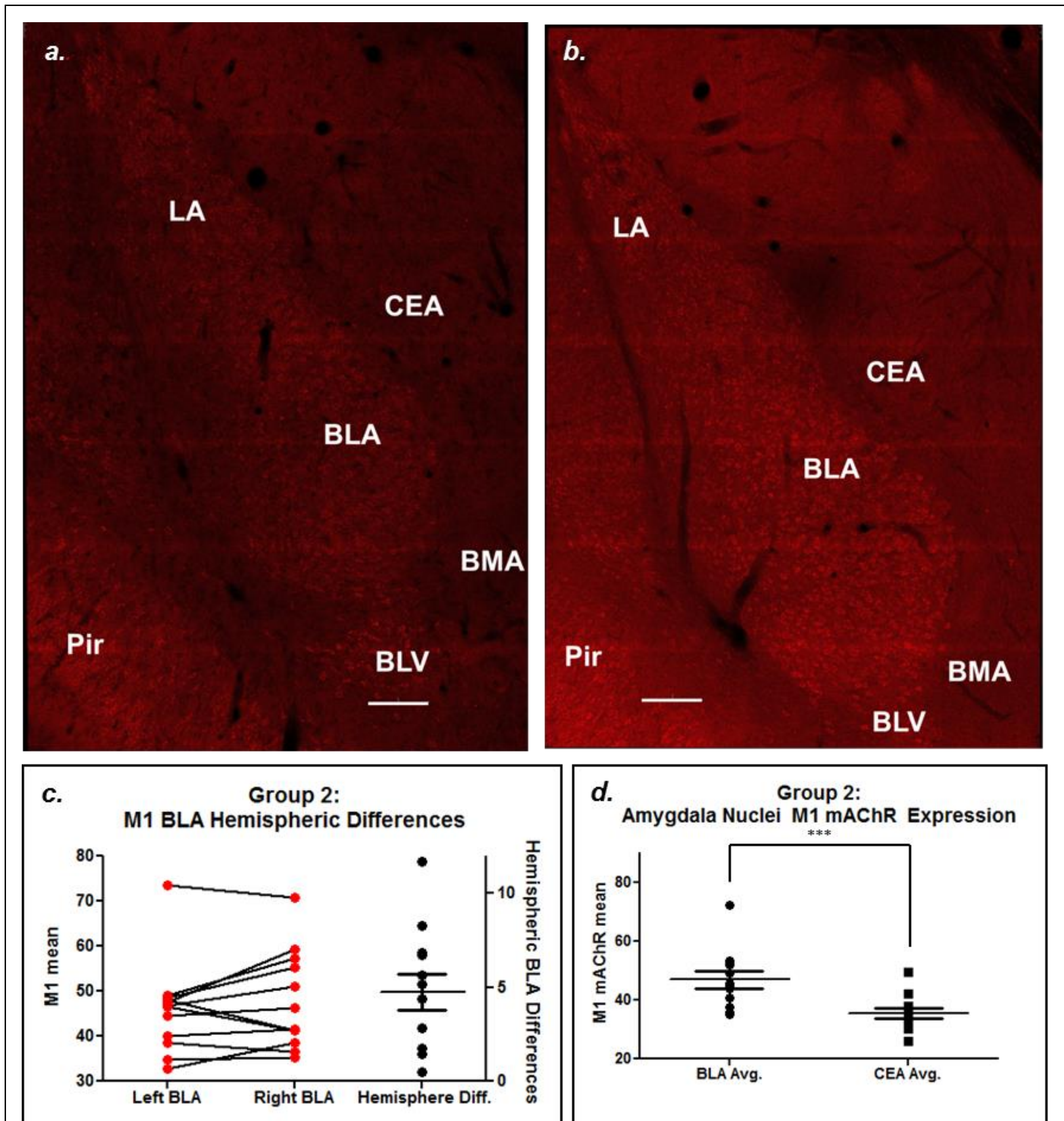

Figure 3.3: M1 mAChR Immunofluorescence. Image a has *low BLA M1 mAChR expression*. Image b has *high BLA M1 mAChR expression*. Image a and b are approximately Bregma -2.16mm (Paxinos and Watson, 2008); scale bars are 200µm. Graph c column 1 and 2 illustrate the measured differences between M1 mAChR protein expression levels in the different hemispheres (left/right designation arbitrary). The 3rd column, illustrating hemisphere differences, was generated by taking the absolute value of the M1 mAChR left value minus the M1 mAChR right value. This indicates that measurable left versus right differences are relatively small and can thus be averaged for analysis. Graph d shows that BLA M1 mAChR expression levels are significantly higher than that observed in the CEA ($t(DF)=11$, $p<0.0001$).

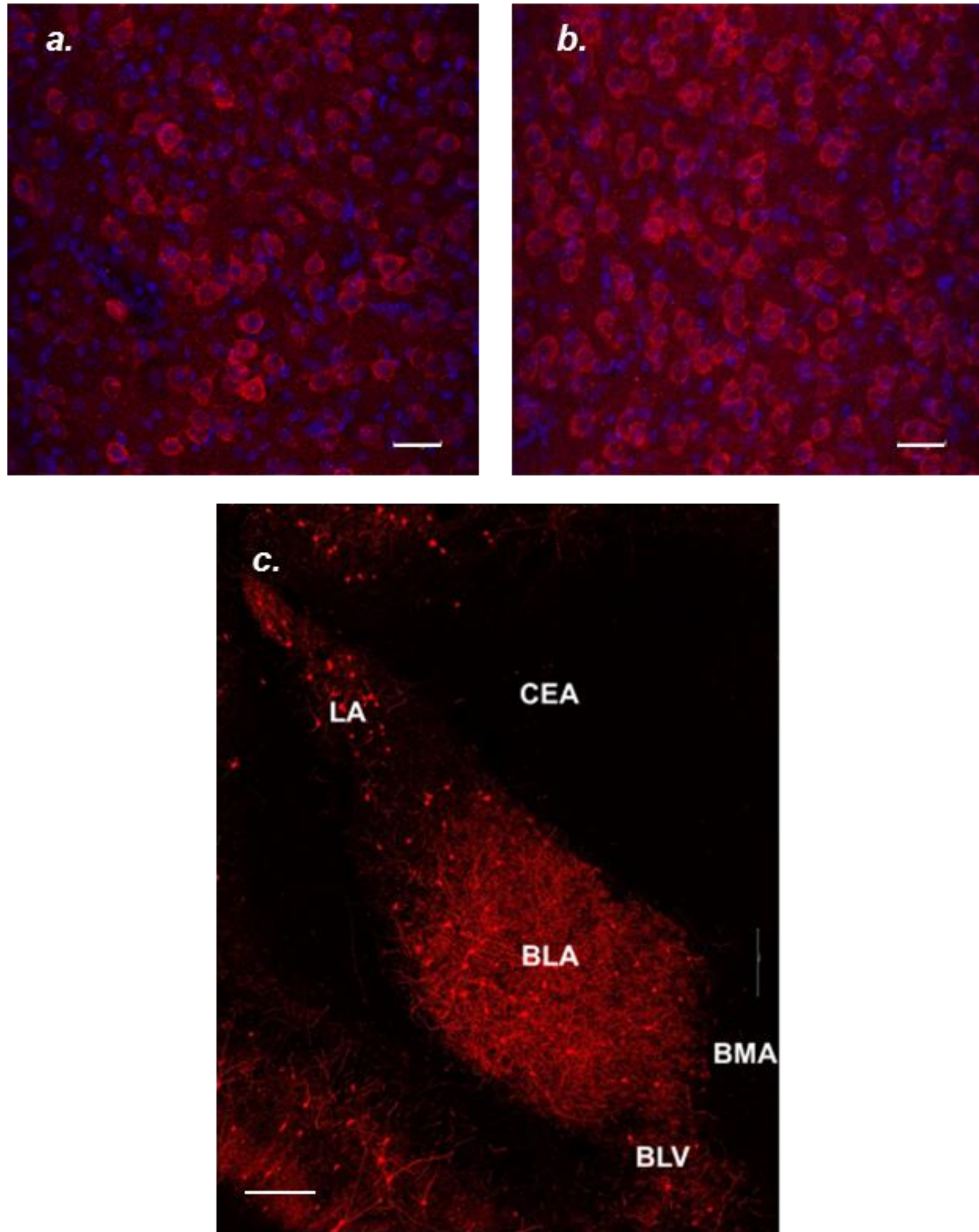

Figure 3.4: Basolateral Amygdala M1 mAChR+ Cell Density. Image a and b are images of BLA collected at 40X on the confocal, red is M1 mAChR, blue is DAPI. Image a has far fewer M1 mAChR+ cells than image b, while they both have similar background levels. Image a and b scale bar 10μm. Image c is a gridded confocal image of a rat amygdala labeled for parvalbumin (PV), illustrating that the amygdala has a relatively dense PV interneuron population, which helps to explain the density of M1 mAChR negative cells seen in images a and b (cells that are DAPI labeled but not M1 mAChR labeled). Image c scale bar 200μm.

Tissue sections for M1 mAChR labeling were selected based on McDonald and Mascagni's analysis of density of M1 mAChR labeling from rostral to caudal amygdala, with the most robust M1 mAChR immunoreactivity seen in the anterior divisions of the basolateral amygdala (2010). Tissue sections labeled and imaged ranged from Bregma -2.05mm to Bregma -2.30mm, according to Paxinos and Watson, The Rat Brain in Stereotaxic Coordinates (2008), all of which contained BLA seen to be densely labeled with M1 mAChR in McDonald and Mascagni (2010). The CEA showed some neuropil labeling and an absence of cell body labeling across all animals (Appendix A, Figure A.1). We felt confident normalizing BLA M1 mAChR intensity to CEA M1 mAChR intensity due to McDonald and Mascagni's assertion that the majority of differences between amygdalar nuclei was due to cell body labeling, not neuropil labeling (2010).

One issue that was observed upon image collection and analysis was that overall intensity varied, not just in the amygdala, but the entire image. This begs the question as to if variations are due to overall artefactual intensity or legitimate changes in receptor expression. This issue was the motivation behind using the histogram mean BLA value divided by the mean CEA value. Control sections with no primary antibody labeling were imaged and analyzed but generate no measureable autofluorescence. Additionally, images were collected in an arbitrary cortical region, the barrel field of the somatosensory cortex (S1BF) (Figure A.2). The ratio comparing intensity of M1 mAChR labeling seen in the BLA of a high M1 mAChR expresser and a low expresser compared to the ratio comparing intensity of labeling see in the S1BF of the same high (Figure A.2.a) and low (Figure A.2.b) expresser shows that differences observed in M1 mAChR expression are not uniform across an entire tissue section (Figure A.2.c). Where high and low M1

mAChR expressers appear to have a uniform brightness or dimness in amygdalar images, this analysis shows that the changes observed in the BLA are greater than those seen across an entire tissue section and are thus not due to differences in perfusion or tissue processing. More controls will need to be imaged in order to further prove this finding and validate this technique and the correlation findings.

While some structures can be differentiated by examining M1 mAChR labeling alone, AChE labeling was conducted to cleanly and reliably identify temporal lobe nuclei. AChE was one of the labels used in Paxinos and Watson (2008) to differentiate between brain regions due to its clearly defined expression pattern. Amygdala AChE expression has been observed to be some of the densest in the brain, which allowed for clean distinguishing of amygdalar nuclei (Ben-Ari et al. 1997; Girgis 1980). This is useful in this study for amygdalar nuclei separation.

AChE expression in this study closely matches that previously observed; dense expression in the BLA, clear distinction from the LA, which has significantly lower expression, and even lesser amounts seen in the CEA (Figure 3.5.a, b, c), illustrated between the BLA and CEA in figure 3.5.e, where average BLA expression is almost double that of CEA. Only neuropil AChE labeling is visible in the BLA and LA, as is reported in studies which utilize an AChE stain, versus AChE antibody labeling used here (Ben-Ari et al. 1997; Girgis 1980). Images 3.5.a and b illustrate the drastic differences in AChE labeling observed from animal to animal, where figure 3.5.c is an enlarged version of figure 3.5.b that has been brightened 3-fold. To generate AChE expression values for analysis, left and right amygdalar values were averaged. However, figure 3.5.d illustrates that the average left vs right AChE differences was more than double that observed in M1

mAChR image analysis. Image 3.5.f is a confocal image showing the most drastic left vs right disparities. This begs the question as to if AChE functionality varies between left and right amygdala in each animal. Further analysis is required to resolve these issues.

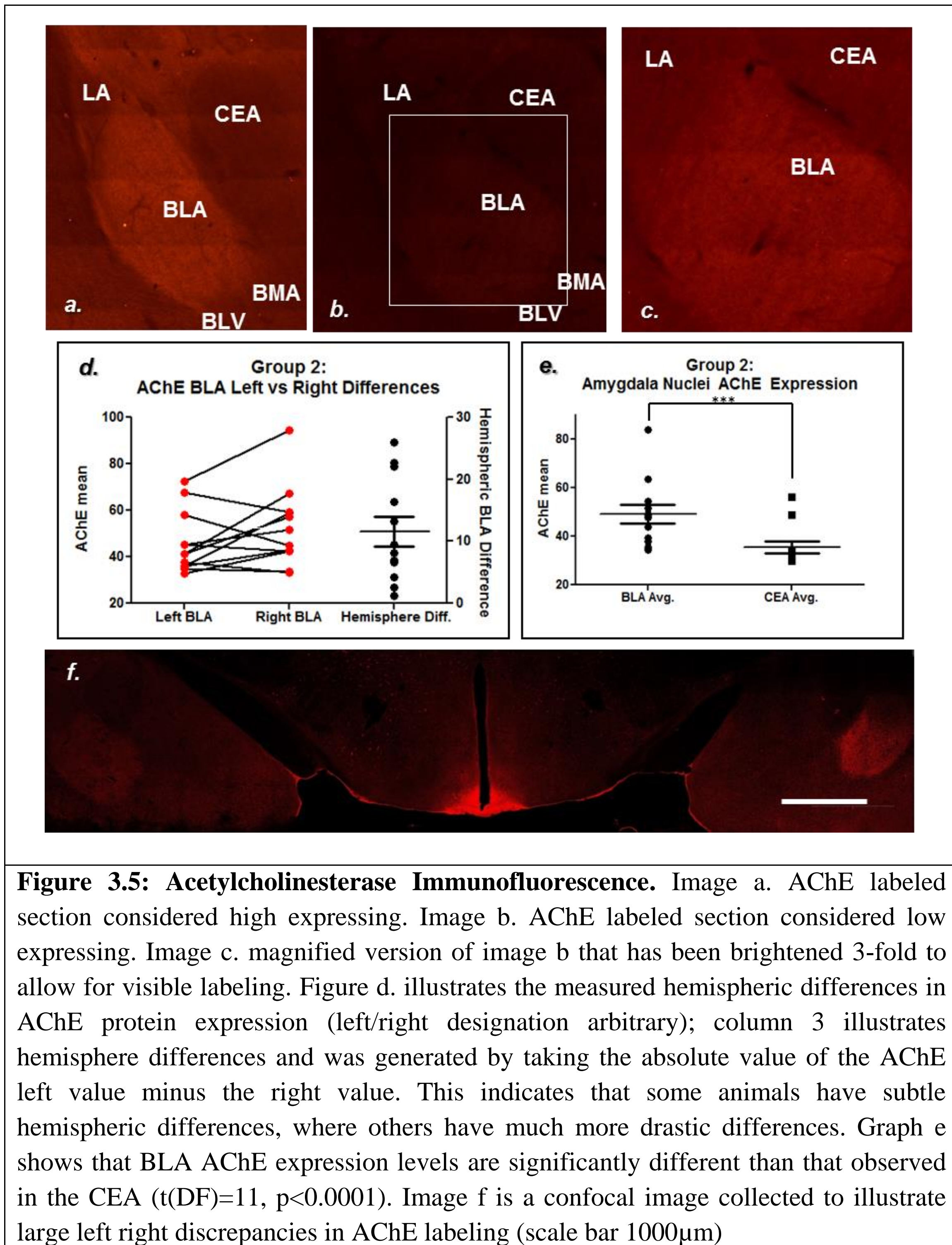

Figure 3.5: Acetylcholinesterase Immunofluorescence. Image a. AChE labeled section considered high expressing. Image b. AChE labeled section considered low expressing. Image c. magnified version of image b that has been brightened 3-fold to allow for visible labeling. Figure d. illustrates the measured hemispheric differences in AChE protein expression (left/right designation arbitrary); column 3 illustrates hemisphere differences and was generated by taking the absolute value of the AChE left value minus the right value. This indicates that some animals have subtle hemispheric differences, where others have much more drastic differences. Graph e shows that BLA AChE expression levels are significantly different than that observed in the CEA (t(DF)=11, $p<0.0001$). Image f is a confocal image collected to illustrate large left right discrepancies in AChE labeling (scale bar 1000μm)

Correlation analysis between AChE and M1 mAChR was conducted to examine if there was a correlation between these two protein's expressions of these two proteins in the BLA or if the different protein levels observed existed independent of overall cholinergic influence. No statistical significance was observed between the two proteins expression level (F[1,10]=1.194; p>0.1) (FUGURE 3.6). This finding suggests that if significant correlations are observed, they are not due to increased cholinergic proteins, as was suggested in early studies (Power et al., 2003b; Gold, 2003).

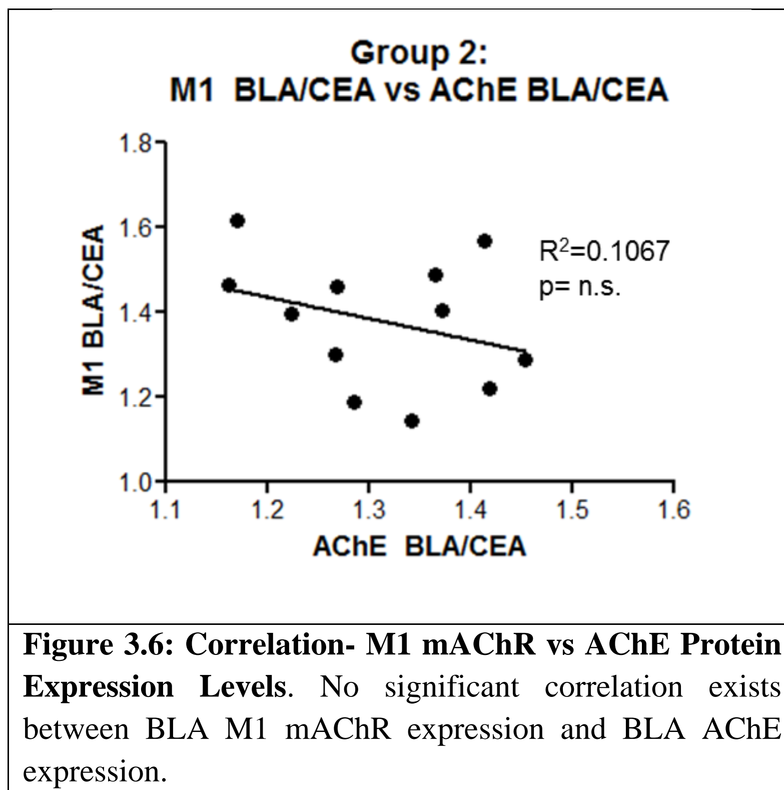

Figure 3.6: Correlation- M1 mAChR vs AChE Protein Expression Levels. No significant correlation exists between BLA M1 mAChR expression and BLA AChE expression.

C. Correlation between Behavior and Image Analysis

Correlation analysis was conducted to compare the extent of protein expression (M1 mAChR or AChE) to the stages of fear and extinction learning by examining levels

of measured freezing behavior in specific time bins, outlined in TABLE 3.1 (group 2 only). These time bins were also used to generate figure 3.1.e and 3.2.e.

Table 3.1: Freezing behavior representing stages of fear learning used for imaging correlation analysis

Learning processes	***Stage of behavior paradigm***	***Time bins used for analysis of learning processes***	***Correlation analysis***
Fear Acquisition	Day 1: Acquisition of Conditioned Fear	Average of last 3 minutes	M1 BLA/CEA: p=0.0251 AChE BLA/CEA: p>0.05; n.s.
Context Fear Recall	Day 2: Context-Conditioned Freezing	Average of minutes 2-5	M1 BLA/CEA: p>0.05; n.s. AChE BLA/CEA: p>0.05; n.s.
Cued-Fear Recall	Day 3: Cued-Fear Recall & Extinction Acquisition	Average of tones 2-6 (minutes 5-9)	M1 BLA/CEA: p>0.05; n.s. AChE BLA/CEA: p>0.05; n.s.
Extinction Acquisition	Day 3: Cued-Fear Recall & Extinction Acquisition	Average of tones 10-19 (minutes 13-22)	M1 BLA/CEA: p>0.05; n.s. AChE BLA/CEA: p>0.05; n.s.
Extinction Recall	Day 5: Extinction Recall	Average of tones 1-5 (minutes 2-6)	M1 BLA/CEA: p=0.0230 AChE BLA/CEA: p>0.05; n.s.
Delayed Extinction Acquisition	Day 5 Extinction Recall	Average of tones 10-19 (minutes 11-21)	M1 BLA/CEA: p>0.05; n.s. AChE BLA/CEA: p>0.05; n.s.

1. M1 mAChR behavior correlation results

The histogram mean values showing average pixel intensity collected and used for individual rat image analysis of M1 mAChR labeled tissue can be found in TABLE A.1. Correlation analysis between M1 mAChR expression and *fear acquisition*, represented by the average percent freezing in the last 3 minutes of day 1, was found to

be statistically significant (F[1,10]=6.929; p=0.0251) (Figure 3.7.a.). Correlation analysis between M1 mAChR expression and *contextual-fear recall*, represented by the average percent freezing during minutes 2-5 of day 2, was found not to be statistically significant (F[1,10]=0.2724; p=0.6131) (Appendix A, Figure A.3.a). Correlation analysis between M1 mAChR expression and *cued-fear recall*, represented by the average percent freezing during minutes 5-9, capturing behavior after the first tone presentation of day 3, was found not to be statistically significant (F[1,10]=0.040; p=0.8455) (Figure 3.7.b).

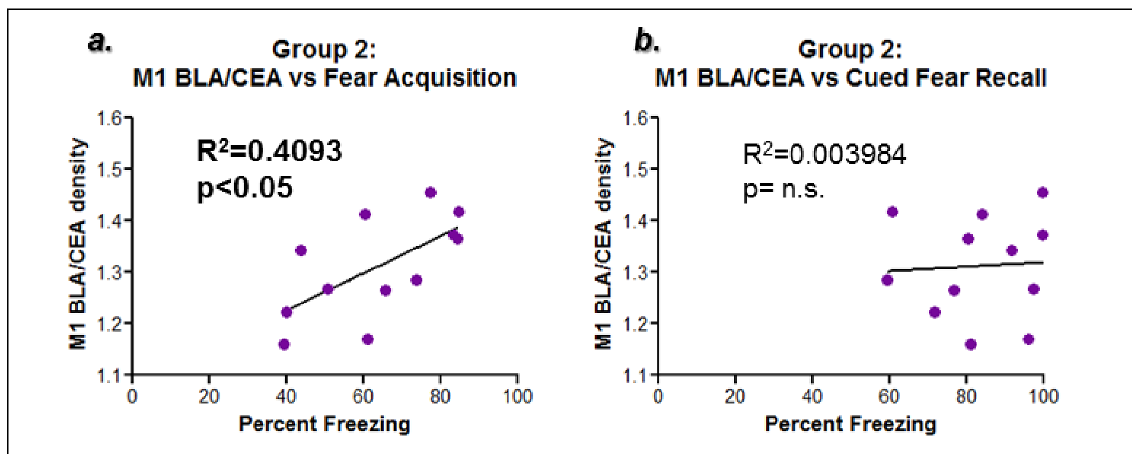

Figure 3.7: Correlation- M1 mAChR expression levels vs fear learning processes. Figures a and b examine the correlation between M1 mAChR BLA expression levels and different aspects of the fear learning process, including fear acquisition, a, measured by the average percent freezing during last 3 minutes of day 1, and cued fear recall (or consolidation), b, measured by the average percent freezing during minutes 2-5 of day 3. No statistical significance was found between M1 mAChR expression and fear recall, b. Statistical significance was found between fear acquisition and M1 mAChR BLA expression, a.

This analysis captures the aspects involved in fear memory, including contextual and cued fear memory acquisition, consolidation, and recall. The significant correlation observed between M1 mAChR expression and fear acquisition would indicate that this proteins expression level positively correlates with the rat's ability to acquire fear. This indicates that PL projections from the PFC to the BLA synapse onto M1 mAChR+

pyramidal neurons in the BLA, allowing for inhibition of M-current which allows for synaptic plasticity to occur. A number of studies discussed previously have found similar results, reinforcing this finding (Rudy, 1996; Young et al., 1995; Feiro and Gould, 2005; Jiang et al., 2016; Fornari et al., 2000; for review see Wilson and Fadel, 2017).

The non-significant correlation observed between contextual- and cued-fear recall seems to indicate that M1 mAChR are not functioning in either consolidation or recall of the fear memory. However, several studies would disagree with this finding. A recent study by Patricio et al., found that M1 mAChR are important in the recall of contextual-fear memories (2017). Similarly, Young and Thomas found that specific M1 mAChR activation increases the consolidation of fear memories (2014). Young, Bohenek, and Fanselow, however, found that administration of a mAChR inhibitor actually increased consolidation of the fear memory (1995). Further studies are necessary to elucidate the precise function and involvement of M1 mAChR in the fear learning process.

Correlation analysis between M1 mAChR expression and *extinction acquisition*, represented by the average percent freezing during minutes 13-22, capturing behavior through the last 10 tone presentations of day 3, was found not to be statistically significant ($F[1,10]=2.367$; $p=0.155$) (Figure 3.8.a). Correlation analysis between M1 mAChR expression and *extinction recall*, represented by the average percent freezing during the first 5 minutes after the first tone presentation (minutes 2-6) of day 5, was found to be statistically significant ($F[1,10]=7.198$; $p=0.0230$) (Figure 3.8.b). Correlation analysis between M1 mAChR expression and *delayed extinction acquisition*, represented by the average percent freezing during minutes 10-19, capturing behavior through the last

10 tone presentations of day 5, was found not to be statistically significant ($F[1,10]=0.5613$; $p=0.4710$) (Appendix A, Figure A.3.b).

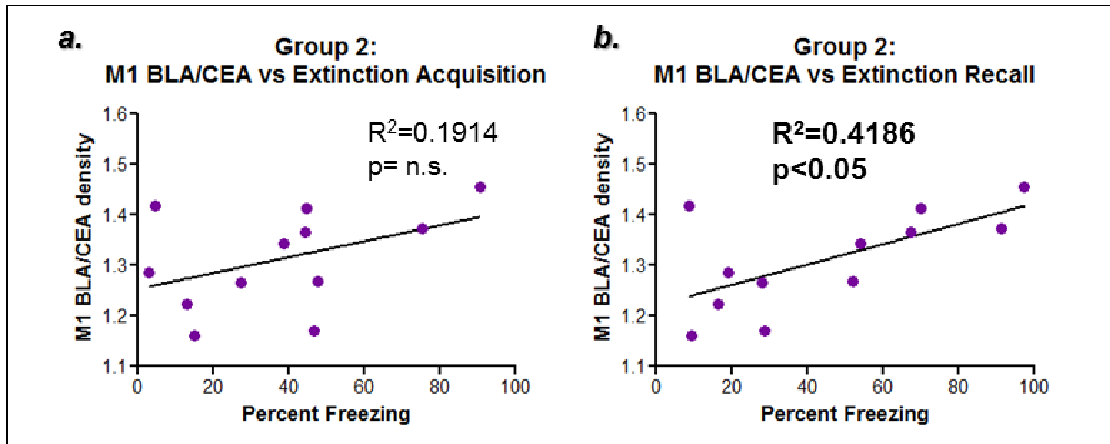

Figure 3.8: Correlation- M1 mAChR expression levels vs the extinction learning processes. Figures 3.8.a and b examine the correlation between BLA M1 mAChR expression levels and different aspects of the extinction learning process, including extinction acquisition, a, measured by the average percent freezing during tones 10-19 on day 3, and extinction recall (or consolidation), b, measured by the average percent freezing during tones 1-5 on day 5. A trend was observed between extinction acquisition and M1 mAChR expression, a, where a statistically significant correlation was observed between extinction recall and BLA M1 mAChR expression.

This analysis captures the aspects involved in cued-fear extinction memory, including acquisition, consolidation, and recall. Contextual-fear extinction was not thoroughly analyzed due to the nature of the behavior paradigm and focus of this study. The non-significant correlation observed during extinction acquisition would seem to indicate that M1 mAChR are not functioning in the rat's ability to acquire the extinction memory, where they were seen to function in fear memory acquisition. The significant positive correlation observed between M1 mAChR expression and percent freezing during re-exposure to the previously extinguished fear memory indicates that higher M1 mAChR protein expression indicates a deficit in rat's ability to recall the extinction

memory. This study does not allow for the differentiation between consolidation and recall so this finding could, likewise, be indicating that high M1 mAChR protein expression prevents consolidation of extinction memories. This finding is the opposite of the original hypothesis, which stated that higher M1 mAChR expression was expected to improve extinction acquisition.

This finding also contradicts previous research conducted in the Mott and Wilson labs by Joshua McElroy, a study which found that animals with higher BLA M1 mAChR protein expression were better able to undergo extinction learning (McElroy, 2016). The current study's finding could be due to the solidity of the previously acquired fear memory, which is enhanced by high levels of M1 mAChR protein expression in the BLA, and that more extinction training is required to allow for proper extinction memory recall in the high responding rats. Delayed extinction acquisition, measuring the within session extinction that occurs on the second round of CS exposure, could allow individuals to better acquire the extinction memory. Delayed extinction acquisition was found not to be correlated to M1 mAChR expression. This is to be expected due to the poor correlation seen between M1 expression and the initial extinction acquisition.

Few studies have examined mAChR function in the various phases of cued-fear extinction memory, one of the reasons this study is very important. Those that have studied this process have found M1 mAChR function to be important for extinction memory processes. While not addressing cued-extinction memory, Boccia et al. found that a general mAChR agonist improved the rat's ability to undergo contextual extinction consolidation, specifically (2009). Santini et al. found that mAChR are important for both extinction memory acquisition and consolidation (2012). Zelikowsky et al. found that

mAChR inhibition impaired rat's ability to extinguish conditioned fear (2013). Schroeder and Packard showed that mAChR agonists improve ability to extinguish amphetamine-induced place preference (2004). These findings, along with those in the present study, paint a confusing picture of M1 mAChR involvement in fear extinction. As it currently stands, it seems safe to say that mAChR are, at the very least, important in extinction learning. My data, along with previous work, allow for the solidification of the hypothesis that M1 mAChR are highly functional in the BLA's role in extinction acquisition and extinction memory consolidation. The precise function and if up or down regulation of M1 mAChR would be beneficial in extinction learning, however, is somewhat more confusing. Previous literature outlined above would seem to indicate that more mAChR functionality would mean better extinction consolidation, although not specifically speaking to which receptor subtype. This study found that higher M1 mAChR expression, specifically, indicates worse extinction consolidation.

2. AChE behavior correlation results

The histogram mean values showing average pixel intensity collected and used for individual rat image analysis of AChE labeled tissue can be found in TABLE A.2. Correlation analysis between AChE expression and *fear acquisition*, represented by the average percent freezing in the last 3 minutes of day 1, was found not to be statistically significant ($F[1,10]=0.1831$; $p=0.6778$) (Figure 3.9.a). Correlation analysis between AChE expression and *contextual-fear recall*, represented by the average percent freezing during minutes 2-5 of day 2, was found not to be statistically significant ($F[1,10]=3.260$; $p=0.1011$) (appendix A, Figure A.4.a). Correlation analysis between AChE expression and *cued-fear recall*, represented by the average percent freezing during minutes 5-9,

capturing behavior after the first tone presentation of day 3, was found not to be statistically significant (F[1,10]=1.716; p=0.2195) (Figure 3.9.b).

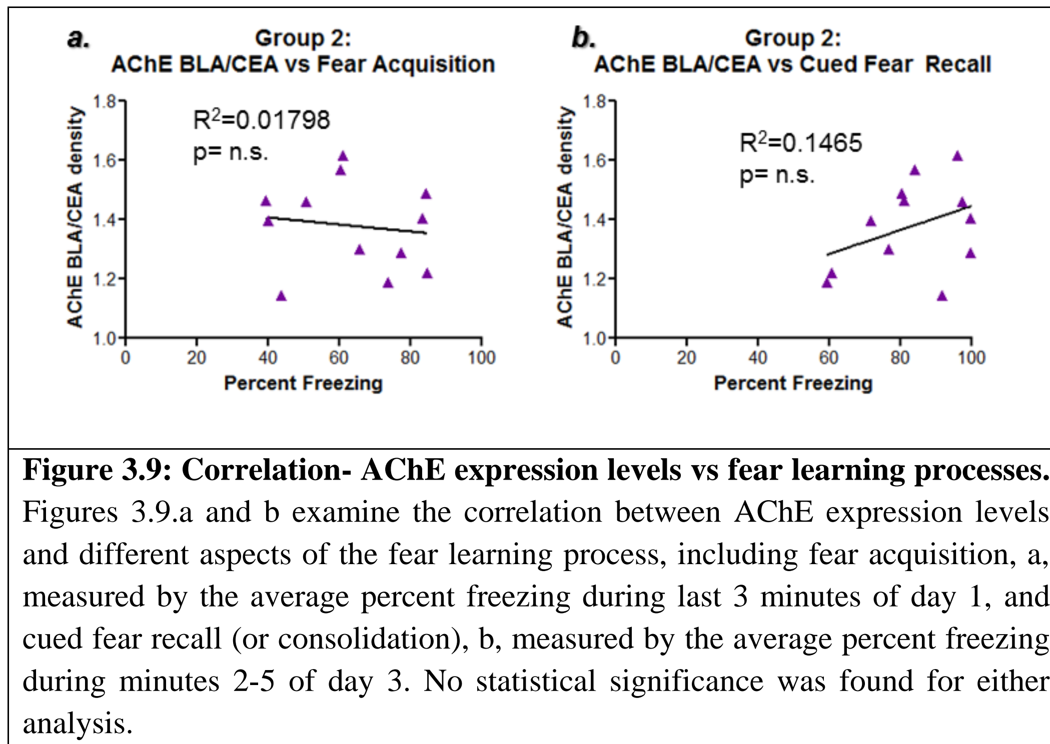

Figure 3.9: Correlation- AChE expression levels vs fear learning processes. Figures 3.9.a and b examine the correlation between AChE expression levels and different aspects of the fear learning process, including fear acquisition, a, measured by the average percent freezing during last 3 minutes of day 1, and cued fear recall (or consolidation), b, measured by the average percent freezing during minutes 2-5 of day 3. No statistical significance was found for either analysis.

Correlation analysis between AChE expression and *extinction acquisition*, represented by the average percent freezing during minutes 13-22, capturing behavior through the last 10 tone presentations of day 3, was found not to be statistically significant (F[1,10]=0.6590; p=0.4358) (Figure 3.10.a). Correlation analysis between AChE expression and *extinction recall*, represented by the average percent freezing during the first 5 minutes after the first tone presentation (minutes 2-6) of day 5, was not found to be statistically significant (F[1,10]=0.1703 ; p=0.6885) (Figure 3.10.b). Correlation analysis between AChE expression and *delayed extinction acquisition*, represented by the average percent freezing during minutes 10-19, capturing behavior

through the last 10 tone presentations of day 5, was found not to be statistically significant (F[1,10]=0.5613; p=0.5914) (appendix A, Figure A.4.b).

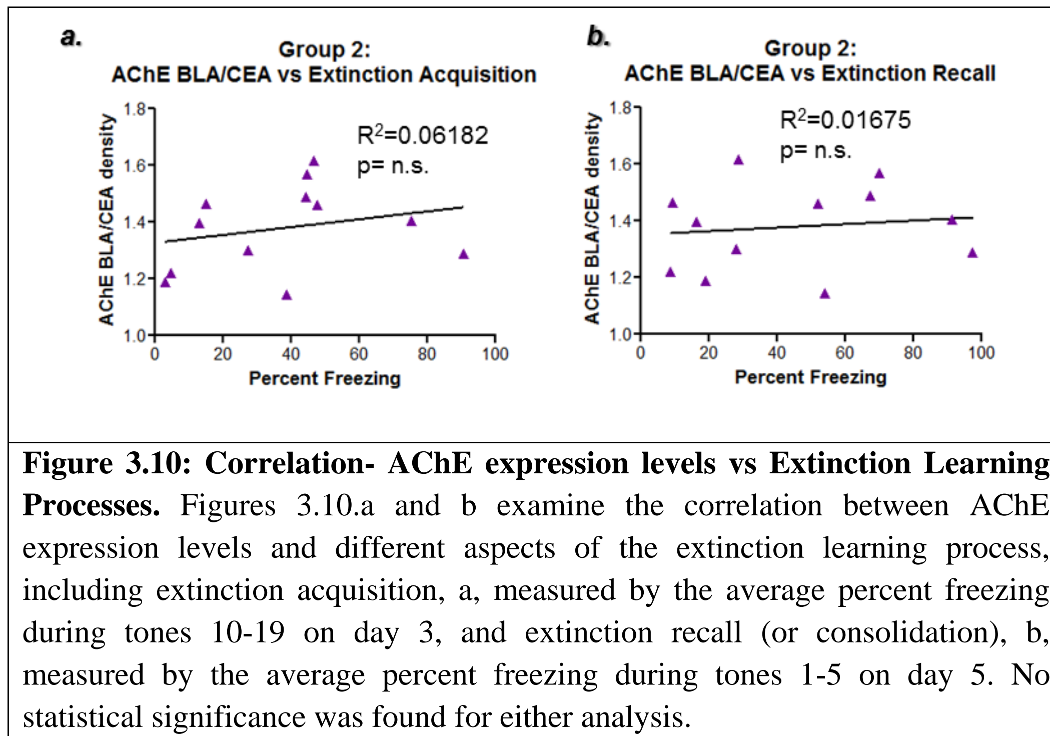

Figure 3.10: Correlation- AChE expression levels vs Extinction Learning Processes. Figures 3.10.a and b examine the correlation between AChE expression levels and different aspects of the extinction learning process, including extinction acquisition, a, measured by the average percent freezing during tones 10-19 on day 3, and extinction recall (or consolidation), b, measured by the average percent freezing during tones 1-5 on day 5. No statistical significance was found for either analysis.

While studies have examined the role of AChE in the fear learning process, many of those studies have done so using acetylcholinesterase inhibitors or have been studies examining nicotinic receptor functioning, not AChE. This can be done because AChE inhibitors reverse nicotine withdrawal effects (Wilson and Fadel, 2017). Understanding what roles all aspects of the cholinergic system play in fear and extinction learning and memory is necessary if treatments for fear related disorders are to be developed surrounding the cholinergic system. However, the results presented in this study would lead us to conclude that AChE protein expression does not directly dictate one's ability to acquire, consolidate, or recall fear or extinction memories.

Chapter 4: Conclusions

The observable deviation of group 2 rats into two different responder groups, high and low responders was similar to that observed by Sharko et al. (2016) and McElroy (2015). Similar to these two studies, the results of this study show that rats demonstrate observable and quantifiable individual differences that go beyond behavioral differences. While grouped differences cannot be seen in ability to *acquire fear*, when comparing individual differences in this ability to protein expression, substantial individual differences can be observed, and these differences positively correlate to M1 mAChR protein expression. These data seem to indicate that an animal's ability to acquire cued-fear is dependent upon M1 mAChR expression, with better fear acquisition correlating to higher M1 mAChR BLA expression.

Statistically significant differences were observed between high and low responder groups in ability to *acquire cued-extinction* memory due to repeated CS exposure during the second half of the day 3 trial. When examining correlation between individual differences in extinction acquisition and M1 mAChR expression, a trend is visible but no statistical differences were observed. A significant correlation *was* observed between M1 mAChR expression level and rats ability to *recall cued-extinction memory* during the first 5 tone presentations on day 5. This behavior paradigm does not indicate if this difference is due to M1 mAChR function in extinction memory consolidation or recall, where previous literature seems to indicate mAChR are

functioning in extinction consolidation (Boccia et al., 2009; Santini et al., 2012; Schroder and Packard, 2004; Zelikowsky et al., 2013).

The several significant correlative findings discussed implicate M1 mAChR in the initial fear acquisition and the consolidation of extinction memory. Together, these two findings seem to indicate that those individuals that had better initial fear acquisition, potentially caused by higher M1 mAChR expression levels, had worse extinction recall or consolidation. These data allow for two different interpretations: M1 mAChR function in directly inhibiting the extinction learning pathway (IL pathway), or that M1 mAChR in the BLA primarily function in strengthening the fear learning pathway (PL pathway) which inhibit extinction by making the strongly formed fear memory difficult to overcome. If M1 mAChR are directly functioning in inhibition of the IL pathway, giving animals an M1 mAChR antagonist during extinction learning processes would theoretically result in improved extinction learning, and a M1 mAChR agonist would inhibit extinction learning. Seeing that the opposite has been observed, where mAChR antagonists impair extinction and mAChR agonists enhance extinction, this interpretation of these data is unlikely to be correct (Boccia et al., 2009; Santini et al., 2012; Schroeder and Packard, 2004; Zelikowsky et al., 2013). However, the interpretation could be true that M1 mAChR are primarily functioning in the strengthening of fear learning pathway, creating a stronger fear memory than that of individuals with less dense BLA M1 mAChR expression. This interpretation is consistent with previous studies that show that mAChR agonists improve fear learning and antagonists impair fear learning (Feiro and Gould, 2005; Fornari et al., 2000; Jiang et al., 2016; Power et al., 2003a; Rudy, 1996; Vazdarjanova and McGaugh, 1999; Young et al., 1995; Wilson and Fadel, 2017).

Furthermore, this interpretation does not discount a role for M1 mAChR in fear extinction, for beyond-physiological activation or inhibition of M1 mAChR in the BLA during fear extinction learning processes could still be effecting ability to undergo extinction acquisition, consolidation, or recall.

No significant correlations were observed between AChE protein expression and fear learning or extinction phases or between extent of M1 mAChR expression. This seems to indicate that overall cholinergic protein expression is not the driver of individual ability to learn or extinguish fears.

Few studies thoroughly examine fear learning and even fewer examine fear extinction. In studies that do examine these processes, there is such a myriad of behavioral paradigms utilized, comparing any two studies can challenging. Attempting to understand the mechanisms of not only fear and emotion, but all aspects of behavior, is a relatively new aspect of neuroscience, which is itself a relatively new and unexplored field. Nevertheless, it is crucial. Understanding the mechanisms behind fear and extinction learning, a primal and complex behavioral system, would aid in our understanding of a variety of emotional behaviors and disorders, including other trauma and stressor related disorders, such as stress and adjustment disorders. The ability to utilize animals in neuroscience allows us to deeply examine neurological underpinnings that would be impossible to investigate in humans. It is important to remember that the goal of animal studies is not to cure the animal's diseases or disorders and that our work must be more or less directly translatable to humans. To our great fortune, the rodent fear circuit has been shown to be homologous to that observed in humans (for review, see Milad and Quirk, 2012). As such, drug treatments administered to rodents can be

expected to have similar effects in human trials. This study along with previous fear and extinction learning studies would seem to encourage the use of M1 mAChR positive allosteric modulators (PAM) in the treatment of PTSD. PAMs reversibly bind to allosteric receptor sites, causing conformation changes that result in an increase in receptor cooperativity and increasing binding of its neurotransmitter, such as ACh in the case of mAChR (Jakubik and El-Fakahany, 2010). A survivor of abuse or veteran of war seeking treatment for PTSD would be encouraged to undergo exposure therapy. During therapy sessions, individual identifiable triggers would be presented, terminating with the PAM binding M1 mAChR in the amygdala. Administration timing would be critical, seeing that the consolidation process occurs for a limited period of time. The drug would need to be given in time for it to pass the blood-brain barrier, enter the amygdala, and react with receptors at the beginning of the consolidation process. This should, in theory, allow for improved consolidation of the newly acquired extinction memory. Rodent trials should give similar results; PAM treatment immediately following extinction acquisition should cause all individuals to have a low percent freezing during extinction memory recall. Such treatment given before fear acquisition would be expected to have a similar result, seeing that preventing strong fear memory formation could cause a weaker PL fear pathway and allow for improved extinction. However, this would also not be translationally useful seeing that one would have to know when an event would be occurring which would cause them to develop PTSD, allowing them to take the drug immediately before the event occurs, or would have to take the drug chronically, which could be damaging, expensive, and, if nothing else, excessive.

The necessity for better understanding and treatment of PTSD is very evident, with the yearly prevalence in America being 3.5% and combat-veteran prevalence being higher than 13% (Kessler et al., 2005; Tamelian and Jaycox, 2008; for review see Wilson and Reagan 2016). This study uniquely adds to the growing body of literature implicating components of the cholinergic system in the process of fear learning and fear extinction, allowing us to inch closer to a mechanistic understanding and useful treatment for PTSD.

Chapter 5: Future Directions

Additional image collection is necessary for the solidification of the results of this study. The difficulty with which metabotropic receptors are labeled and imaged made high power confocal image collection a necessity, as opposed to widefield microscopy used for AChE labeled tissue imaging. Seeing the considerable amount of time M1 mAChR image collection takes and the cost of collection, it was first necessary to determine if significant, meaningful correlations existed between behavior and protein expression. Now that such correlation has been established, it is pertinent to continue image collection and generate amygdalar images from no fewer than 3 tissue sections for each animal in group 2. Additionally, proper controls from each animal must be collected. This would consist of image collection of a second region unassociated with the described behavioral process, as presented and described in figure A.2. It is our belief that further image collection will strengthen the protein-behavioral correlations observed. In addition, continued EVOS image collection of AChE labeled tissue, bringing theimage collection up to at least 3 sections per animal, is also valuable for elucidating any correlations. Additionally, analysis of hemispheric differences in AChE expression within each animal could prove to be a more valuable means for evaluating the protein's role in the fear and extinction learning processes.

In addition to this, expanding the present study to examining other muscarinic receptors would be of great value. In a recent publication out of the Mott and McDonald

labs, M2 mAChR were implicated in the modulation of cholinergic terminals within the BLA (Fajardo-Serrano et al., 2017). Examining M2 mAChR expression and the PV interneuron population in behaved tissue could give valuable insight into how the interneuron population within the BLA relates to fear learning and extinction processes. Seeing that many behavioral studies utilize scopolamine, a non-selective mAChR antagonist, it would be useful to examine not only M2 mAChR but also M3, M4, and M5 mAChR in order to clarify which of the mAChR are the main contributors in BLA function and regulation of fear learning and extinction.

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Appendix A: Supplemental Data and Figures

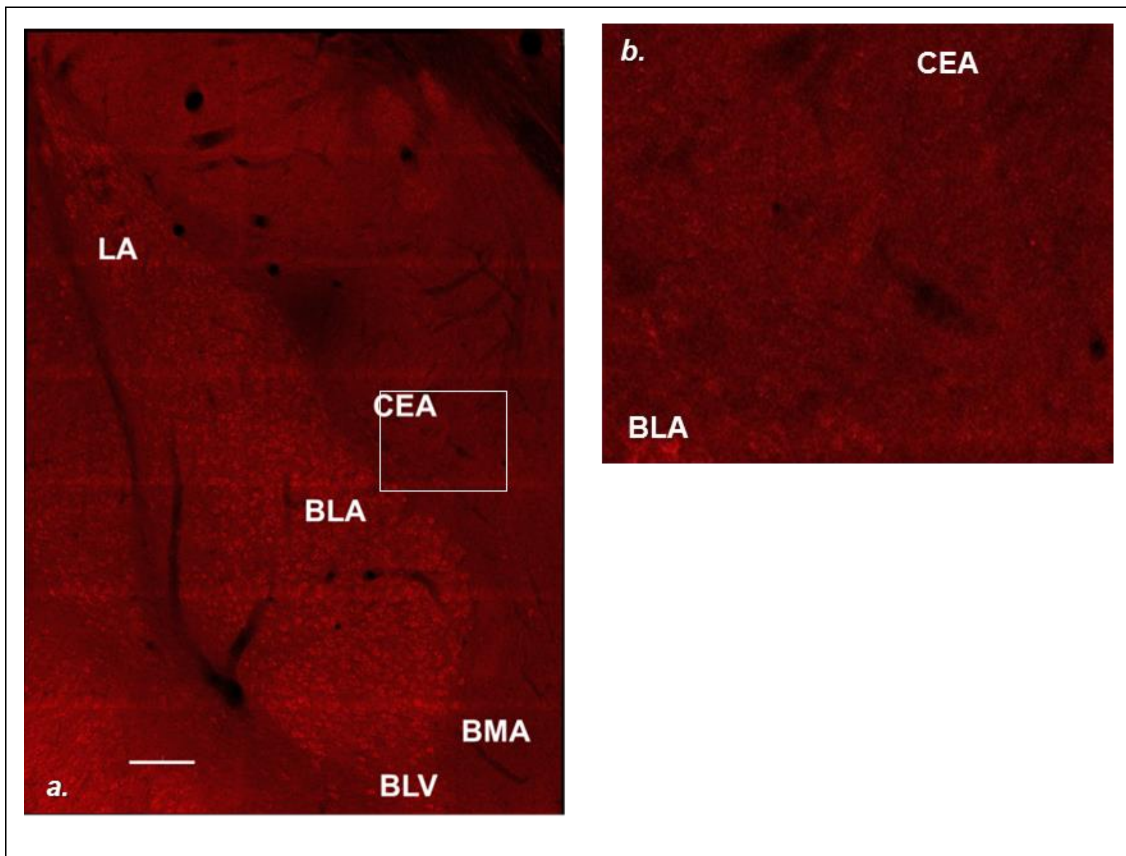

Figure A.1. M1 mAChR CEA expression. Image b is a high magnification image of M1 mAChR labeled CEA, outlined by the white box in image a, showing only neuropil labeling (no cell body labeling) in this region. Image a scale bar 200μm.

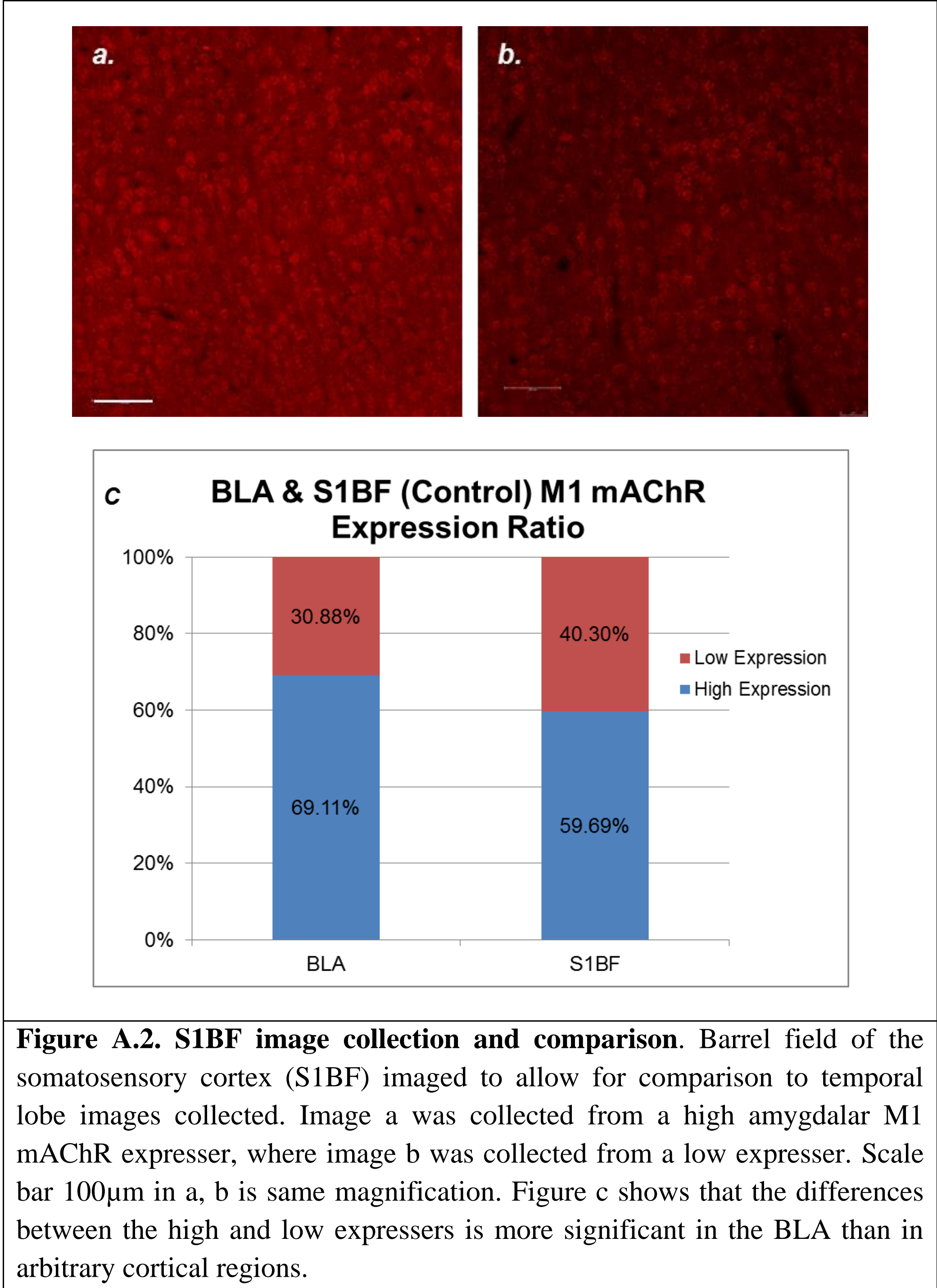

Figure A.2. S1BF image collection and comparison. Barrel field of the somatosensory cortex (S1BF) imaged to allow for comparison to temporal lobe images collected. Image a was collected from a high amygdalar M1 mAChR expresser, where image b was collected from a low expresser. Scale bar 100µm in a, b is same magnification. Figure c shows that the differences between the high and low expressers is more significant in the BLA than in arbitrary cortical regions.

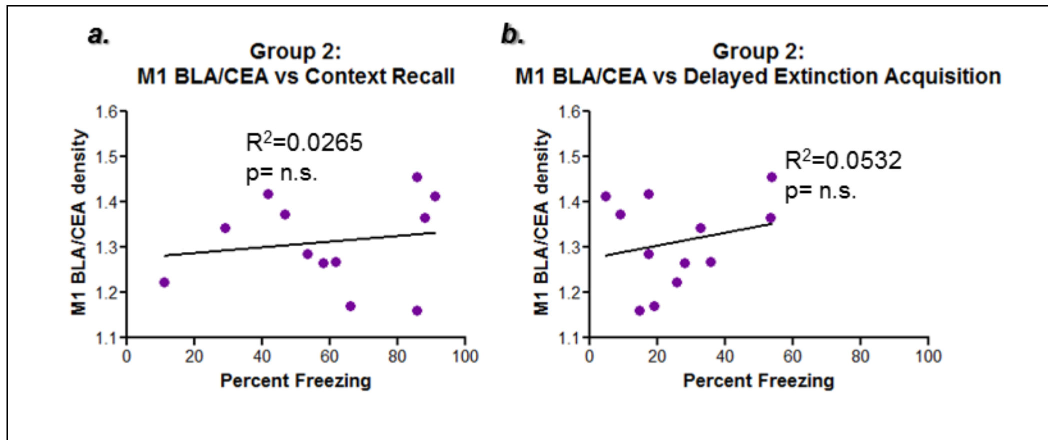

Figure A.3: Correlation- M1 mAChR expression levels vs contextual fear recall and delayed extinction acquisition. Figures a and b examine the correlation between M1 mAChR BLA expression levels and different aspects of the fear learning and extinction process. In figure a, context recall values were generated by the average percent freezing during minutes 2-5 of day 2. No statistical significance was found between M1 mAChR expression and context recall. In figure b, delayed extinction acquisition values were generated by the average percent freezing during minutes 11-21 of day 5. No statistical significance was found between M1 mAChR BLA expression and delayed extinction acquisition.

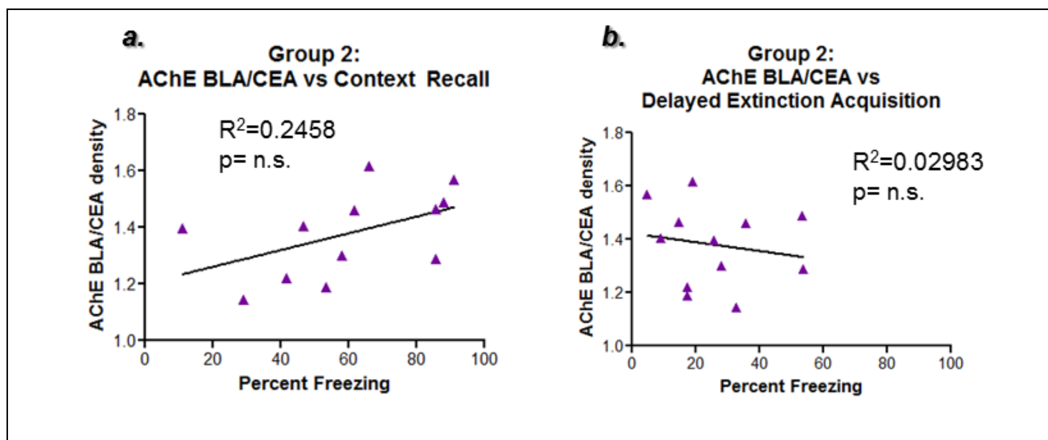

Figure A.4: Correlation- AChE expression levels vs contextual fear recall and delayed extinction acquisition. Figures a and b examine the correlation between AChE BLA expression levels and different aspects of the fear learning and extinction process. In figure a, context recall values were generated by the average percent freezing during minutes 2-5 of day 2. No statistical significance was found between AChE expression and context recall. In figure b, delayed extinction acquisition values were generated by the average percent freezing during minutes 11-21 of day 5. No statistical significance was found between AChE BLA expression and delayed extinction acquisition.

Table A.1: The histogram average pixel intensity of M1 mAChR labeled tissue with hemispheres averaged

Designated Rat Number	*Averaged M1 intensity: entire image*	*Averaged M1 intensity: LA + BLA*	*Averaged M1 intensity: BLA*	*Averaged M1 intensity: CEA*	*Averaged M1 intensity: BLA/CEA*
186	41.59	49.47	51.88	38.00	*1.36*
187	38.19	43.03	43.94	34.69	*1.27*
188	30.02	33.67	35.68	26.00	*1.37*
189	47.50	52.13	53.09	37.43	*1.42*
190	29.83	33.29	35.04	30.17	*1.16*
191	37.78	42.94	45.51	33.90	*1.34*
192	48.01	52.01	53.46	42.14	*1.27*
193	32.20	35.28	37.54	32.11	*1.17*
194	59.73	69.78	72.11	49.59	*1.45*
195	33.49	40.01	40.77	33.31	*1.22*
196	40.84	44.75	48.95	34.62	*1.41*
197	39.91	42.89	44.68	34.76	*1.28*

Table A.2: The histogram average pixel intensity of AChE labeled tissue with hemispheres averaged

Designated Rat Number	*Averaged AChE intensity: entire image*	*Averaged AChE intensity: LA + BLA*	*Averaged AChE intensity: BLA*	*Averaged AChE intensity: CEA*	*Averaged AChE intensity: BLA/CEA*
186	69.47	76.53	83.60	56.12	*1.49*
187	55.80	61.09	63.94	49.69	*1.29*
188	39.50	45.28	48.50	34.55	*1.40*
189	33.91	36.27	37.73	30.89	*1.22*
190	40.12	46.13	49.24	33.64	*1.46*
191	31.70	32.78	34.89	30.14	*1.16*
192	37.41	43.62	47.52	32.80	*1.45*
193	39.79	49.65	54.34	33.65	*1.61*
194	32.32	36.94	39.38	30.56	*1.29*
195	36.29	41.42	43.94	31.41	*1.40*
196	35.79	46.77	51.43	32.78	*1.57*
197	32.20	34.11	35.45	29.84	*1.19*