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Muscarinic Acetylcholine Receptor M1's Impact on Fear Extinction Learning

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by

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ABSTRACT

Post-Traumatic Stress Disorder (PTSD) is a mental health disorder that can occur following a traumatic event like combat, assault, or disaster. Individuals with PTSD are unable to extinguish fear memories which can become chronic and disabling. However, it remains unclear why some individuals exposed to a traumatic event develop PTSD while others are resilient. Acetylcholine plays a critical role in fear learning, but its role in fear extinction is less well understood. In this investigation, we used a rat model of fear extinction to determine if individual differences in extinction learning are correlated with markers of cholinergic signaling. Cholinergic markers included the M1 muscarinic acetylcholine receptor (M1 m-AChR) and the vesicular acetylcholine transporter (vAChT). These cholinergic markers are strongly expressed in brain regions, such as the amygdala and prefrontal cortex that contribute to the fear extinction circuit. The goal of the present study was to determine whether individual differences in cholinergic signaling in these brain regions could underlie differences in fear extinction. Expression levels of cholinergic markers were measured in amygdala and prefrontal cortex from male Long-Evans rats (N = 13) that had undergone a Pavlovian fear conditioning and extinction paradigm. We found that rats exhibited individual differences in extinction of freezing behavior following twenty presentations of a conditioned auditory stimulus. Six of 13 rats tested failed to extinguish cue-conditioned freezing behavior as defined by a median split in freezing during the last 10 tone presentations. When M1 m-AChR expression in these animals was assessed by Western blot analysis, a significant

correlation was evident between expression level of M1 m-AChR in the amygdala and the freezing behavior during the extinction trials. Expression of M1 m-AChRs in amygdala of animals showing good extinction learning was significantly higher than that in animals resistant to extinction. In contrast, there was no significant correlation between vAChT expression and freezing in either amygdala or prefrontal cortex. These results suggest that low expression of M1 m-AChRs in the amygdala is correlated with deficits in fear extinction, and suggest that therapeutic strategies aimed at enhancing muscarinic signaling in amygdala may enhance fear extinction in animals and perhaps patients with PTSD.

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LIST OF ABBREVIATIONS

ACh ..... Acetylcholine

AChR ..... Acetylcholine Receptor

BA ..... Basal Amygdala

BDNF ..... Brain Derived Neurotrophic Factor

BFc ..... Basal Forebrain Cholinergic

BLA ..... Basolateral Amygdala

BNST ..... Bed Nucleus of the Stria-Terminalis

cAMP ..... Cyclic Adenosine Monophosphate

CeN ..... Central Nucleus of the Amygdala

CNS ..... Central Nervous System

CS ..... Conditioned Stimulus

DAG ..... Diacylglycerol

db ..... Decibels

FDA ..... Federal Drug Administration

HB ..... Homogenization Buffer

Hz ..... Hertz

IEG ..... Immediate Early Gene

IL ..... Infralimbic Cortex

IP ..... Intraperitoneal

IP_3 ..... Inositol Trisphosphate

ITC ..... Intercalated Cells

LA .....Lateral Amygdala

LTP ..... Long Term Potentiation

PAG..... Peri-Aqueductal Grey

PAM.....Positive Allosteric Modulator

PFC ..... Pre-Frontal Cortex

PKA.....Protein Kinase A

PL..... Pre-Limbic Cortex

PNS .....Peripheral Nervous System

PTSD.....Post Traumatic Stress Disorde

M .....Molar

mA..... Milli-Amp

m-AChR.....Muscarinic Acetylcholine Receptor

mm .....Milli-Meters

mM..... Milli-Molar

US ..... Unconditioned Stimulus

VAChT..... Vesicular Acetylcholine Transporter

vmPFC .....Ventro-Medial Pre-Frontal Cortex

CHAPTER 1

INTRODUCTION

1.1 What is "Fear" and How is it Processed?/ Fear Learning

When most individuals describe fear they often associate it with aversive stimuli, negative emotions, and feelings of anxiety, but in its most basic form fear is an evolutionary protective mechanism which provides significant survival instincts to all complex organisms. These survival instincts manifest themselves in a number of ways, and to truly understand their complex roles and behavioral outcomes it is critical to first understand the structures and pathways involved. The general "fear circuit" which is responsible for creating, recalling, and integrating fearful memories is formed by a key forebrain network composed of the frontal cortices, thalamus, hippocampus, and amygdala, with the amygdala being perhaps the most critical of regions. (Falls et al., 1992; Kapp & Pascoe, 1986; Kapp et al., 1984; LeDoux, 1987; Sarter and Markowitsch,1985). As sensory information is received the first structure in this pathway to be activated is the thalamus which receives sensory input and relays the signal to higher order processing centers as well as sends signal encoding a crude representation of the stimuli directly to either the lateral or basao-lateral amygdala (BLA). (LeDoux et al. 1985) This direct projection may only provide a crude awareness of the stimuli to the amygdala, but what it lacks in clarity it makes up for in speed. It is responsible for quickly providing the amygdala with enough information to generally assess threat level so that a more generalized response may be taken in the event or a sudden and imminent

threat when fractions of a second can make the difference between life and death. However, for the most part the amygdala receives very complex and highly processed information which has been relayed from the thalamus through primary association areas before reaching the amygdala. This ensures that the amygdala is receiving a very clear representation of the stimulus before assessing its relevance. This clear representation is very critical because the amygdala is responsible for assessing and assigning emotional context to stimuli and any misjudgment could lead to unbeneficial behavioral responses both in the short and long term. As mentioned previously the amygdala's primary function is evaluating the emotional significance of stimuli, generating an emotional response, and forming an emotional memory correlated with the stimuli for use in interpreting future stimuli. (LeDoux 1994). In order to accomplish this sizeable task the amygdala relies on its bi-directional communication with stimulus association areas. It is key that these areas are not only able to clearly inform the amygdala as to the nature and context of the stimuli, but also that the amygdala is able to relay information back regarding the assessment of the stimuli so that future associative processing may be altered to better interpret similar stimuli. After receiving and emotionally interpreting stimuli the amygdala primarily uses two output pathways to communicate relevance and bring about behavioral reactions. The stria terminalus relays on information which is first processed in the BLA and projected to the bed nucleus of the stria terminalus (BNST). This pathway projects primarily to brainstem regions such as the central and midbrain periaqueductal grey (PAG).(LeDoux, 2000) Its purpose is to elicit behavioral responses to the stimuli and in particular the all too important freezing behavior which is the behavioral focus of this study and many other studies (Fendt and Fanselow, 1999,

Sacchetti et al., 1999a, 1999b). The second primary output pathway of the amygdala is the ventral amygdolofugal pathway which is primarily responsible for the emotional response to a stimulus. This pathway begins in the central nucleus (CeN) of the amygdala which receives projections from many of the same aforementioned association areas as well as the BLA, and it projects to the hypothalamus and brainstem areas where the signal acts or further projects from to illicit an emotional response component. (McDonald et al. 1998, Vertes 2004). Together these structures are responsible for receiving and interpreting environmental stimuli, assigning them emotional content, eliciting an appropriate emotional response, and forming an emotionally based memory of not only the stimuli but also the response and its physiological outcomes all of which we colloquially encapsulate as "fear".

1.2 What is Fear Extinction Learning?

As an evolutionary defense mechanism fear is as ubiquitous as it is powerful, but what is considered fearful stimuli can change throughout the course of life. What was once fear inducing at a given age or time can become unimportant or even pleasurable as we grow and change our surroundings. The process of a stimulus making of this transition from feared to negligible is what is called "fear extinction learning", and it as critical to survival as is its counterpart. When a fearful association is made between an event and stimuli this association a powerful with its level determined by the amount of emotional content. However, when this association becomes reinforced through several pairings of stimuli and aversive outcomes the connection becomes stronger still and the emotional response becomes all the more powerful. This successive reinforcement of pairings makes the neutral stimuli (the CS) become conditioned to elicit an unconditioned

aversive response (the US) prior to or even absent of the actual aversive outcome. Overall this process of fear learning can be described as a form of Pavlovian conditioning, while fear extinction learning includes successive presentations of a conditioned stimulus (CS) in the absence of the unconditioned stimulus (US) provide the training necessary for the CS to no longer elicit the response proper to the US.

1.3 Fear extinction learning and PTSD

In recent years Post-Traumatic Stress Disorder (PTSD) has quickly come to the forefront of psychosocial disorders. This recent surge in awareness is partly due in part to the emerging portion of our nation's population who are returning veterans matriculating into the civilian work force. With this somewhat recent surge in awareness there has been an uptick in research geared to investigate the potential physiologic underpinnings of the disorder. Studies investigating the disorder have revealed that patients suffering from PTSD exhibit hyper-conditioning to fearful stimuli while also showing a resistance to extinguishing these associations. (Pitman 1988, Shin et al. 1999) Colloquially this can be described as a deficit in the ability to reform lasting "safe" associations to normal stimuli over the top of formally heavily engrained fearful associations. PTSD patients have also been reported to show normal with in trial extinction while displaying deficits in their ability to recall this extinction learning at a later time. (Milad et al. 2009) This demonstrates that these patients do not display a learning deficit but rather an inability to recall their formally re-learned associations. This is a very critical piece of data because it shows that people afflicted with this disorder do not lack the ability to overcome their engrained fearful associations, but rather simply need aide in making these "safe" associations more engrained in order to compete with

the fearful ones. Currently this aide is provided through psychotherapy exercises in an attempt to strengthen the relearned safe associations. However, many times this is not enough and patients still remain afflicted with their debilitating symptoms.

1.4 Individual Differences in Fear Extinction Learning

The process of extinguishing fear is a complex one and individuals whether they be rats or humans display different levels of propensity for extinguishing. The American Psychiatric Association affirms that traumatic fearful events can often times lead to development of anxiety disorders such as but not limited to PTSD (A.P.A. 1994). However, approximately 75% of Americans will experience some form of severe trauma in their lifetime, while only approximately 7% of the U.S. population is reported to have an incidence of PTSD (Breslau and Kessler 2001, Kessler 2005). Furthermore, individuals who suffer a severe traumatic event such as deployed combat or victims of terrorist attacks have post exposure PTSD approximations as high as 30% (Wisnivesky et al. 2011). These diverging approximations shows us that PTSD is not as simple as succeeding every kind of trauma for every person to experience it. In fact it also speaks to the very nature of fear learning wherein the more traumatic the event the more filled with emotional content it is and the more heavily engrained in associative memory it becomes. However, even absent of varying levels of trauma there is still observed a large degree of variation in how individuals process traumatic events and there is an expanding body of literary evidence to why this differential processing can lead to PTSD (Holmes and Singewald 2013). Within the human population these individual differences are often accounted for by varying life experiences, previous exposure to extreme stress or trauma, as well as predisposing genetic factors (Caspi et al. 2010). Which is why when PTSD and

individual differences in fear processing are studied rodent models are used which afford a much greater degree of experimental control. Studies examining the neural correlates of these individual differences have activation and recruitment analyses in key brain areas to observe for patterns correlated to behavioral outcomes. These studies have shown that individual rodents who display deficits in fear extinction learning also display hyper-activation of key brain areas such as the ventromedial pre-frontal cortex paralimbic division (Knapska and Maren 2009, Whittle et al. 2010). Furthermore, rats who displayed these same kinds of deficits were also found to have hypo-activation of the prefrontal cortex infralimbic division (Milad and Quirk 2012). Further studies attempted to map the activation and subsequent protein changes in key brain regions relative to individual differences in the ability to extinguish fear is observed as changes in immediate-early gene expression (IEG). They found that IEG activity within the infralimbic cortex was severely reduced within individuals who also displayed deficits in their ability to extinguish fear. (Hefner et al. 2008, Herry and Mons 2004). These studies upon population statistics and physiologic correlates shows that there is a key degree of individual variability that exists within populations which affords a degree of resistance to developing PTSD after traumatic events, and that these individual differences in behavior are carried over in rodents when they are selected from an outbred strain (Bush et al. 2007, Milad and Quirk 2012). Furthermore, these studies focusing on utilizing the rodent model have also reported individual differences in critical brain region activation as well as individual differences in IEG activity which demonstrates a level genetic diversity also at play in controlling the propensity to readily extinguish fear.

1.5 The Process of Fear Extinction Learning

1.5.1 Fear Extinction Learning as Pavlovian Conditioning

In order to accomplish the sizeable task of relearning fearful associations the brain relies upon the process of fear extinction learning. This process is accomplished in three distinct phases: acquisition, consolidation, and retrieval and has been described as early as 1927 to be a special form of inhibition of a conditioned memory requiring protein expression. (Bouton 2004, Bouton et al. 2006, Flood et al. 1977, Harris and Westbrook 1998, Maren 2004, McConnell and Miller 2014, Pavlov 1927). The acquisition phase represents the training phase in which a new "safe" association is formed through repairing or un-pairing of formally fearful stimuli to normally neutral stimuli. The consolidation phase occurs when this new association stored as a memory through changes in protein expression and remodeling of neural networks. This enables the carryover of re-learned associations from one experience or trial to the next through memory and recall without having to be relearned. (Myers and Davis 2007, Pape and Pave 2010, Quirk and Mueller 2010) Overall fear learning and fear extinction learning are forms of basic Pavlovian conditioning where in an US such as imminent mortal danger or any aversive stimuli are paired to a conditioned stimuli CS such as a specific sound, or environment. This conditioning then causes the normally neutral stimuli to elicit the evolutionary appropriate response to the unconditioned stimuli even in its absence. In the context of PTSD this represents the extreme emotional and psychological responses from seemingly harmless everyday occurrences. Once this has occurred the process of fear extinction involves the presentation of the CS in the absence of the US in an effort to no longer cause the CS to elicit the response appropriate to the US. However

fear extinction learning is very much a process and in order to unpair the CS and US responses many unpaired presentations of the CS are required. Often times this pairing of CS to US and subsequent unpaired training occur in distinct settings or "contexts". This is critical because contextual cues provided by overlaps in pairing and un-pairing training contexts can provide associative cues which can cause the re-training to unpair CS and US to be quite difficult due to contextual reinstatement of the pairing (LeDoux 2000).

1.5.2 The Fear Extinction Circuit

1.5.2.1 Anatomy of the Fear Extinction Circuit

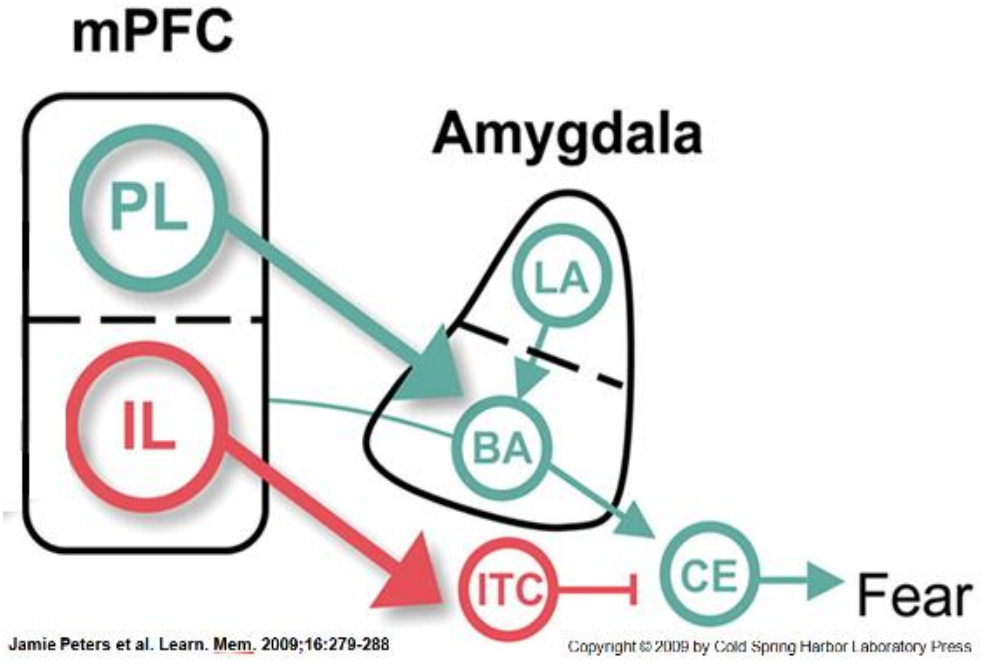

Figure 1.1 Diagram of the fear extinction circuit

Hippocampus

In order to carry out the task of fear extinction learning the brain relies upon 3 main brain regions: the hippocampus, pre-frontal cortex, and the amygdala. Within this circuit the hippocampus is particularly responsible for providing contextually relevant information concerning stimuli and environment to the pre-frontal cortex,

infralimbic (IL) and paralimbic pathways (PL), as well as the basolateral amygdala (Huges and Garcia 2007). This interconnection places it in a prime spot to provide modulation of fear responses. The information conveyed through this circuit is critical because in essence it allows for a contextually based decision on if a given stimuli deserves a fearful response or not, commonly called contextual fear conditioning (Fanselow 2000, Kim and Fanselow 1992, Philips and LeDoux 1992). When a formally fearful stimulus is presented its significance is assessed not only by its strength of pairing to aversive outcome but also through environmental cues which can reinforce or detract from its perceived level of importance. If a stimulus is presented in the presence of environmental cue which associate it with a previous experience then the connection between the stimulus and that event is more readily accessed and the stimulus more easily evokes the response relevant to the previous experience. By the same logic, if these reinforcing environmental cues are absent in the presence of a presented stimulus it stands to reason that the stimuli would be less likely or fail to evoke a fearful response associated with a previous experience, especially after several cue absent presentations of the aversive stimulus. Studies examining the hippocampus within the context of fear extinction have in fact shown that the hippocampus carries out this modulation through contextual gaiting of extinction to specific conditioned stimuli (Bouton et al. 2006, Ji and Maren 2007). This process is known as contextual fear learning and extinction and it is distinct from cued fear learning and extinction as it relies heavily on the contextual information hippocampus to provide the modulation of fear expression rather than a specific CS. This is accomplished through the hippocampus heavy involvement in the process of synaptic and neural plasticity. Furthermore, this hipppocampal dependent

plasticity has been shown to bidirectionally serve to enhance or inhibit fear expression (Huges and Garcia 2007). However, the hippocampus has also been shown to be at work in the cued fear learning and extinction pathway wherein it primarily participates during the retraining or acquisition phase. Studies examining the specific phasic actions of the hippocampus showed that, by blanketly inhibiting the hippocampus through mass GABA activation by the agonist muscimol, that it is most critically involved in the acquisition phase of fear extinction, but not in the consolidation phase (Berlau and McGaugh 2006, Corcoran et al. 2005, Xue et al. 2014). This seems to speak to the idea that the hippocampus is responsible for forming contextually relevant associations during the training portion of fear extinction while remaining quiescent during their recall. This could imply that while the contextual information is initially processed by the hippocampus that it is stored elsewhere for retrieval.

Pre-Frontal Cortex

The PFC and particularly the vmPFC (ventro-medial PFC) have also been shown to play a critical role in the process of fear extinction learning. Anatomical studies have shown that the vmPFC has direct connections to the amygdala through both its IL and PL portions (Hurley et al. 1991, McDonald 1991, 1998) The PL particularly connects bi-directionally to the BLA while the IL connects to the BLA , centra-lateral amygdala (CeL), and intercalated cells (ITC) (McDonald 1998, Vertes 2004). The direct connections of the PL to the BLA serve as a type of signal processing, wherein the PL receives contextually relevant information from the hippocampus and crude awareness signals from other brain regions before forwarding them to the BLA. The BLA in turn processes these signals and returns them as a single transient fear signal to the PL; with

this new more refined signal the PL is then able to respond to the BLA with a sustained fear signal allowing for the sustained activation of BLA neurons (Linnman et al. 2011) In this way the PL acts as a type of processor for interpreting signals from the BLA using them to drive conditioned freezing as well as other fearful association responses. The reciprocal connection that the BLA shares with the PL allows for not only the processing of information, but also allows it to control conditioned fear signaling (Lavioletter et al. 2005, Sortes-Bayon et al. 2010).One study examining this relationship through the use of PL micro-stimulation showed that PL stimulation increased freezing responses and impaired extinction learning (Vidal-Gonzalez et al. 2006). Juxtaposed against the actions of the PL are the actions of the IL. Studies examining the specific role of the IL have shown that with stronger activation of IL neurons during the consolidation phase comes a greater inhibitory effect upon conditioned fear responses, and that thickness of this area is strongly correlated with extinction recall (Hartley et al. 2011, Milad et al. 2005b, Milad et al. 2007). These conditioned fear inhibitions are perhaps due to the IL's connection to the inhibitory areas of the amygdala, CeL and ITC (McDonald 1998, Vertes 2004). Through the activation of these inhibitory areas the IL could exercise its inhibitory effects upon conditioned fear behaviors. Studies examining the IL during fear extinction training showed an increase in activity of IL neurons, indicating their involvement in the extinction learning process (Santini et al. 2008). Similar studies looked at IL activity during fear extinction recall and showed that failure to recall previously re-learned "safe" associations was accompanied by decreased excitement of IL neurons (Burgos-Robels et al. 2007). These findings are consistent with those of other investigators which showed that IL activity is critical for both phases of fear extinction learning (Laurent and

Westbrook 2008, Milad and Quirk 2012, Orsini and Naren 2012, Quirk and Mueller 2008). One study also showed that the IL activation was critical for not only the visible signs of fear extinction learning, but also showed that activity in this area was critical for the development of extinction induced plasticity in the ITC (Amano et al. 2010). Together these two distinct regions of the PFC allow for it to achieve a dual role in control over fear extinction learning, with the PL attenuating or driving fearful responses and the IL inhibiting their physical expression through activation of inhibitory amygdalar structure (Sortes-Bayon and Quirk 2008).

Amygdala

The amygdala represents the perhaps the most critical structure in the fear extinction circuit, and is chiefly viewed as the master control center for fear expression and regulation. Studies examining the amygdala's role in the fear extinction circuit through inactivation by discrete lessoning showed that in the absence of a functioning amygdala conditioned fear acquisition and consolidation cannot occur (Hitchcock and Davis 1986, LeDoux et al. 1984). Further evidence for the amygdala's role has been shown through studies which reported increased amygalar activity during CS/US pairings, as well as studies performed on humans which noted an increase in amygdalar activation during extinction training (Buechel et al. 1999, Gottfried and Dolan 2004, Knight et al. 2004). However, the amygdala does not act as one succinct unit when fulfilling its role in fear expression. Neurobiological investigations into how exactly the amygdala functions in its regulation and expression of fear have shown that the association between CS and US are formed and expressed within different sub-nuclei within the amygdala (Davis 2000, LeDoux 2000, Maren 2005, Sigurdsson et al. 2007).

One such subnucleus is the basolateral amygdala. Studies examining the BLA's role through direct stimulation showed that activation of the BLA alone is capable of associating sensory inputs with conditioned fear responses as well as eliciting fear behaviors (Johansen et al. 2010, Nonaka et al. 2014, Tye et al. 2011, Yiu et al. 2014) . BLA inactivation studies utilizing muscimol have also reported evidence for the BLA's role in fear extinction learning. When the BLA was inactivated during the acquisition phase sever deficits in extinction learning where displayed (Baldi and Bucherelli 2010, Herry et al. 2008, Holmes et al. 2013, Laurent et al 2008, Laurent and Westbrook 2011, Sierra-Mercado 2011).

The BLA is bi-directionally connected with the PL as previously discussed, but this is not the only input to the BLA. The BLA also receives cholinergic input from the basal forebrain cholinergic (BFc) centers as well as the diagonal band of Broca and basal nuclei of the stria terminalus (BNST) (Woolf 1991). The input from the BFc has been reported to facilitate fear memory formation by increasing the signal to noise ratio in the BLA in order to bias synaptic connections in favor of strongly activated cells. BLA neurons which are strongly activated and biased in this manner send out excitatory signals via their projections while quiescent neurons from the same area send out inhibitory signals (Unal et al. 2012). This biasing of signals within the BLA allows for its control over the primary inhibitory areas of the amygdala (CeL/ITC), which represent the other submuceli of the amygdala. The inhibitory areas receive excitatory input from both the IL and BLA which is then conveyed as an inhibitory signal to its downstream effectors such as the hypothalamus and peri-aqueductal grey (McDonald et al. 1998, Vertes 2004). Through these inhibitory connections the CeL and ITC fulfill the role in

fear extinction learning by inhibition of previously established associations of learned fear (Amano et al. 2010, Lin et al. 2003A & B, Royer et al. 1999). Through the use of it distinct sub-nuclei the amygdala acts in a bi-directional role in fear expression by simultaneously facilitating the formation of fearful associations as well as suppressing the expression of previously learned fearful associations.

1.5.2.2 Physiology of the Fear Extinction

Just as there are many regions and sub-regions in play within the fear extinction circuit so are there many neurotransmitters and transmitter systems which have been implicated to be acting within the circuit. Researchers have long been puzzled by which neurotransmitter(s) are in play within this circuit and as such have developed an expansive body of literature which implicates such transmitter systems as: neuropeptide y, brain derived neurotrophic factor (BDNF), serotoninergic, dopaminergic , GABAergic, cholinergic as well as glutamatergic systems. Furthermore, each of these systems has been implicated to act in a slightly different manner to aide in the regulation of fear expression and/or fear extinction learning (Abraham et al. 2014, Bauer 2015, Heldt et al. 2007, Kutlu and Gould 2015, Orsini and Maren 2012, Peters et al. 2010, Tasan et al. 2016). Within this investigation we focused on specifically the cholinergic system due to the extensive amount of evidence showing its direct effects on the BLA as well as learning and memory process which are linked to potent emotional content. Furthermore, many researchers have long implicated and since have shown that cholinergic signaling is critical for attending to and remembering emotionally laden experiences (Hasselmo and Sarter 2011, Hermans et al. 2014, LeDoux 2012, Luchicchi et al. 2014, Sarter et al. 2014). ACh has been shown to strongly modulate performance on BLA dependent tasks

which probe for emotional memory content, as well as having been shown to facilitate memory formation within similar tasks also dependent upon the BLA (Gold 2003, Tinsley et al. 2004, Unal et al. 2015). Research has also shown that there exists a strong correlation between individual variations in ACh release in the BLA during training and the level of recall displayed afterward (McIntyre 2003). However, while these results could hypothetically be mediated by either nicotinic AChRs or muscarinic AChRs there are several more lines of evidence showing that it is mainly the m-AChRs which mediate these observations. Pharmaceutical mediated studies have shown that by acting on these muscarinic type receptors discrepancies in fear extinction learning can produced. One study showed that micro injecting mAChR agonists into the BLA fear extinction learning could be enhanced by activating these receptors. (Boccia et al. 2009). These studies have provided evidence showing that m-AChRs are actively involved in the process of fear extinction, but in order to investigate exactly which receptor subtypes(s) are responsible for the results observed first we must understand more about what exactly a m-AChR is and how they act.

Cholinergic Signaling: m-AChRs

Muscarinic acetylcholine receptors are a class of receptors belonging to the G-protein coupled receptor super-family. These receptors like other muscarinic receptors carry out their actions through second messenger cascades (Caulfield and Birdsall 1998, Wess 1996). There are five subtypes of m-AChRs (M_1 – M_5) which are subdivided into two major classes determined by the type of G-protein to which they couple. M_1, M_3, and M_5 all couple to the G_q/G_{11} class, while M_2 and M_4 couple to the G_i/G_o class. G_q/G_{11} class proteins are responsible for activating secondary messenger cascades which

generate signaling molecules such as DAG and IP_3. These secondary messengers are in turn responsible for modulating the releases of intracellular calcium stores and thereby the activation of protein kinases and protein synthesis. G_i/G_o proteins are primarily responsible for inhibiting the activity of adenylyl cyclase and thereby the production of cAMP. However all subtypes utilizes the same agonist for activation, ACh (Caulfield and Birdsall 1998, Lanzafame et al. 2003, Nathanson 2000, Wess 1996). These two sub-type groupings also differ in their localization within the body with $M_1$and M_5 receptors being primarily expressed in the CNS, and the M_2, M_3, and M_4 receptors being widely expressed throughout the PNS as well as the CNS (Abrams et al. 2006, Caulfield and Birdsall 1998, Wess 1996, Volpicelli and Levey 2004). Perhaps the most important of the m-AChRs in terms of fear expression and fear extinction learning is the M_1 type receptor. This is not simply due to their well established role in general learning and memory processes, but also due to their high degree of localization within primary cells of the BLA (McDonald and Mascagni 2010, Muller et al. 2013, Mrzljak et al. 1993, Yamasaki et al. 2010). Furthermore long term fear extinction has been shown to require PKA activity and protein synthesis, both of which are downstream effectors of G_q/G_{11} signaling cascades (Mueller et al. 2008, Santini et al. 2004)

Muscarinic Acetylcholine Receptor M1's Role in Fear Extinction Learning

Studies attempting to elucidate the role of M1 within the fear extinction circuit have been primarily driven by pharmacologic studies examining the effects of agonist activation or antagonist mediated inactivation. One such study utilizing the muscarinic cholinergic agonist oxotremerine showed that direct infusion into the BLA elicited an enhancement in fear extinction learning (Boccia et al. 2009). Systemic injections of m-

AChR agonist cevimeline also showed similar results, reporting an increase in fear extinction learning recall when given immediately after subject's final cued conditioning training, 24 hours prior to extinction learning testing (Santini et al. 2012). These results were elaborated on through the work of Young and Thomas who showed via M1 knockout mice that this enhancement in extinction learning by cevimeline only occurs when subjects possessed wholly functioning M1 m-AChRs. These results are in agreement with those of other studies utilizing M1 agonists in the same manner (Passani et al. 2001, Vazdarjanova and McGaugh 1999, Young and Thomas 2014). While some work has been utilizing muscarinic agonists much of the studies have focused on the use of antagonists. Studies utilizing the m-AChR antagonist scopolamine found results congruent with those of the utilizing agonists. When scopolamine was used to provide a pharmacologic blockade of m-AChRs researchers reported deficits in many tasks related to fear learning including: contextual fear conditioning and inhibitory avoidance testing (Bang and Brown 2009, Pang et al. 201, Wallenstein and Vago 2001). Similar antagonist studies that looked instead at the effects on fear extinction learning conducted in vivo and ex vivo showed that these effects in the BLA and thusly on extinction learning were brought about by m-AChRs alone, and that other transmitter/receptor systems were not found elicit similar results. (Saunders et al. 2015). Some studies utilized in vivo electrophysiological recordings reported that ACh signaling by way of brief photo-activation of cholinergic terminals in the BLA was sufficient to produce rapid and prolonged changes in network excitability once again supporting the underlying actions of M1 type receptors in this area (Sarter et al. 2014). With m-AChRs role in the circuit and particularly the BLA well established through pharmacologic studies, in combination

with the body of literature describing high degrees of M1 specific localization in this area it is easy to see how alterations in expression and activation of this receptor can lead to expansive consequences in fear extinction learning.

Cholinergic Tone: VAChT

The membrane protein known as vesicular acetylcholine transporter (VAChT) lies within synaptic vesicles of cholinergic neurons, and is responsible for exchanging protons within these vesicles for ACh which resides in the cytoplasm. This exchange of protons for ACh allows VAChT to concentrate ACh within vesicles where it is stored for later release (Nguyen et al. 1998). This concentration of ACh within vesicles is a key component in determining exactly how much ACh is released from the vesicles of cholinergic terminals upon stimulation (Van der Kloot 1991, Prior and Tian 1995, Song et al. 1997, Varoqui and Erickson 1997, Williams 1997, Reimer et al. 1998, Sulzer and Pothos 1999, Kitamoto et al. 2000). Studies examining the exact concentration of ACh that VAChT can achieve in this manner have shown it to be approximately a 100-fold gradient of vesicular ACh to cytoplasmic ACh (Parsons 2000). By localizing ACh into vesicles and enabling its synaptic release VAChT acts as a regulator of ACh availability. Regions which undergo more ACh signaling thusly require a greater amount of VAChT expression, which makes it a good marker for overall number of cholinergic terminals within a given region or cell population.

Hypothesis

Based upon the above information and evidence provided by previous studies, I hypothesize that ***individual differences exist in fear extinction learning, and that these differences are correlated to individual variations in BLA M1 m-AChR expression***. In

order to address this hypothesis I plan to investigate two specific aims. The first is that within a cohort of animals that there are pre-existing individual differences in the degree and manner in which animals process fear extinction learning, and secondly that these individual differences are brought upon by individual differences in BLA M1 m-AChR expression. In order to investigate this first aim I will employ a paradigm of Pavlovian fear conditioning with which to test for individual differences in fear extinction learning processing and expression. This study will be followed by protein expression quantification from the PFC and BLA in order to look for individual differences in expression patterns with which to correlate to behavioral outcomes displayed in the prior experiments. It is our hope that by elucidating the mechanisms underlying observed deficits in fear extinction learning that we will be able to shine light into new treatment avenues for PTSD patients focused on the use of therapeutic compounds which act upon M1 m-ACh receptors.

CHAPTER 2

MATERIALS AND METHODS

Behavioral Testing

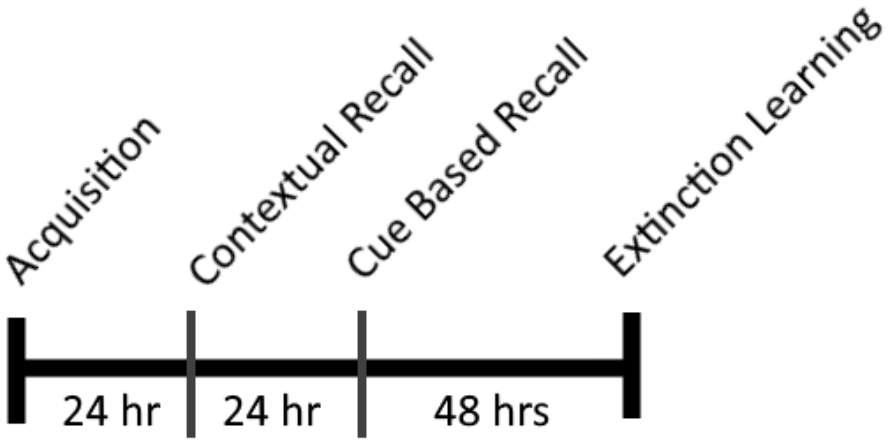

Figure 2.1: Behavioral experiments timeline

Subjects

In order to accomplish fear learning acquisition a cohort of male Long Evans rats (N=13) underwent a three tone shock pairing protocol.

Acquisition

In this protocol animals were placed in a standard shock box scented with 5% ammonia hydroxide. Animals were given a period of 3 minutes to explore before the presentation of the first 20 kHz 85dB tone. The 20 k Hz tone was presented for 10s and co terminated with a 1s shock of 1 mA intensity. One minute after the first shock the second tone shock pairing occurred and similarly the 3rd pairing after the second. During

this trial the Freeze Scan system (Clever Sys Inc., Vienna, VA) was used record and analyzed live video feeds before reporting their time spent immobile as freezing behavior data. This freezing behavior is defined as the subjects remaining completely immobile save for slight head movements for a period of time. The repeated pairing of tone and shock in this experiment is designed to accomplish basic Pavlovian conditioning. Herein the unconditioned stimulus (US) of a 1mA foot shock is paired to the conditioned stimulus of a 20kHz tone for 10s at ~85 dB , and after repeated pairings the rats learn that the CS will proceed the US and begin to display behaviors associated with the US when presented with only the CS. After each trial the enclosure was thoroughly cleaned with 5% ammonium hydroxide to clean the equipment and refresh the contextual scent.

Context Dependent Recall

In order to test for consolidation and contextual based recall of fear memories 24 hours after fear learning, a contextual fear experiment was used, where in rats were once again exposed to the shock box enclosure scented with 5% ammonia hydroxide. However, in this experiment there were no CS or US presentations.

During this time the rats were observed and analyzed for freezing behavior using the Freeze Scan system as previously described. Between each trial the enclosure was thoroughly cleaned and cleaned with 5% ammonium hydroxide.

Cue Based Recall

In this experiment rats were assessed for cue based fear memory retrieval in a novel context 24 hours after their previous test. This was done using the same 20kHz tone (CS), but in a completely different context/environment than the previous

experiments. This new context consisted of a 2.5ft diameter and 2ft high plastic bowl scented with 70% alcohol as well as 20µl of lemon extract added to a 5in x 5 in cloth strip hung from the side of the bowl. By using a new and distinct enclosure which included a distinct scent subject memory retrieval was completely based upon the recall of the CS/US pairing absent of an environmental stimuli associated with their pairing. The rats were placed in this new enclosure and given an initial 3 minute exploratory period, followed by several presentations of the same 20 kHz tone (CS) at 1 minute intervals for a total of 23 minutes and 20 tone presentations. During these trials rats were observed using the Freeze Scan system which analyzed and reported their freezing behaviors. The enclosure was then be thoroughly cleaned with 70% alcohol between each trial to clean it and an additional 20µl of lemon extract was added to the gauze in order to recharge the contextually distinct odor.

Extinction Learning Testing

In this final behavioral experiment rats were tested for their fear extinction learning 48 hours after their cue based trial by assessing for a learned un-pairing of CS and US. In order to test for this fear extinction learning rats were placed into the above described context. After 1minute, 20 tone presentations (20 kHz) were given at 1 minute intervals. During each trial rats were observed by the Freeze Scan system and had their freezing behaviors analyzed and reported. After each trial the enclosure was cleaned thoroughly using 70% alcohol, wiped dry, had fresh bedding added, and had the lemon scent refreshed by adding another 20 µL of extract to the cloth.

Brain Region Punches

Immediately following completion of the extinction learning trial rats were euthanized by immediate live decapitation, and had their brains blocked and flash frozen in dry ice before being stored at -80 C to await slicing and region specific punches. Brain samples from rats were then subjected to cryostat slicing into 1mm thick slices. A list of slices made and regions taken from each via 1mm diameter punches can be seen below with punches from each hemisphere being combined into single samples.

1) Bregma +3.70mm to +2.70mm
    a. PFC - 2x1mm

2) Bregma -2.50mm to -3.50mm
    a. Basolateral amygdala – 2x1mm
    b. Hippocampus – 2x1mm

Tissue Preparation for Western Blot Analysis

Brain region punches were individually homogenized in 600µl of ice cold homogenization buffer (HB = mM: 2 EDTA, 2 EGTA, 20 HEPES, 0.32M sucrose, 1% phosphatase buffer (Sigma Cat#P5726), 1% protease inhibitor (Sigma Cat# P8340). Samples were then spun in a refrigerated microcentrifuge in order to isolate the supernatant ("total membrane") from the pellet ("nuclei"). The 'total membrane" portion of each sample was then centrifuged at 31,000 x g for 30 minutes to isolate the resulting pellet ("crude plasma membrane") from the supernatant ("crude cytosol"). A SS34 rotor in a Sorvall RC5C plus centrifuge was used. The "crude plasma membrane" pellet was then re-suspended in approximately 100µl of PBS. Protein concentration of each sample was then evaluated via a traditional Bradford assay and sample dilutions were then adjusted until each yielded a protein concentration of 1-2.5 µg/µl.

Western Blot Analysis

Ten µl aliquots of membrane fraction brain region samples were separated by SDS/PAGE (4-15% gradient), transferred to polyvinyl difluoride (PVDF) membranes and blocked in TBS plus 0.05% Tween 20 (TBS-T) plus 5% non-fat dry milk for 1 h at room temperature. PVDF membranes were incubated with primary antisera (in TBS-T/5% non-fat dry milk) overnight at 4°C with gentle shaking. Primary antibodies included rabbit polyclonal M1 mAChR (1:1,500; Sigma, M9808), and rabbit polyclonal VAChT (1:2,500; Ab-Cam AB68984). These antibodies were chosen due to their previously proven effectiveness (Bajayo et al. 2012, McDonald and Mascagni 2010, Mullet et al. 2013, Ricard and Gudas 2013, Smith et al 2015, Vetreno et al. 2014). Following overnight incubation membranes were washed 3x/10 min with TBS-T and incubated with peroxidase-labeled, species-specific secondary antibodies. PVDF membranes were washed 3x/10min with TBS-T and developed using enhanced chemiluminescence reagents (Pierce ECL; Thermo Scientific, #32106) as described by manufacturer. Normalization for protein loading was performed using a mouse monoclonal primary antibody selective for β-actin (1:20,000; Vector, #). Analysis was completed by using a scanner and the IMGJ computer program which quantifies each lane/samples protein of interest optical density normalized to that lane's β-actin band optical density.

Statistical Analysis

Given the variation in cued fear extinction, for statistical analysis animals were divided into low or high responder groups based on a median split of the cue conditioned freezing during the last 10 minutes of the cued extinction trial. Data from the high/low

group divisions was then compared one to another for the remainder of this investigation using a t-test or ANOVA with repeated measures (high versus low freezing behavior over time) with post-hoc analysis to detect specific group differences. Correlation analysis was used to compare freezing behaviors during extinction to levels of cholinergic markers. Significance was set at $\alpha = .05$ / $p = .05$.

CHAPTER 3

RESULTS

Behavioral Trials

When rats were split into high and low responder groups by post hoc analysis based on median split of freezing behaviors (median = 32.66% freezing) within the last 10 tone presentations of the cue based recall trial, 6 were categorized as high responders and 7 as low responders. (*Fig 3.1)* We then used this group split to analyze the data from the behavioral experiment, protein quantification, as well as correlation analyses.

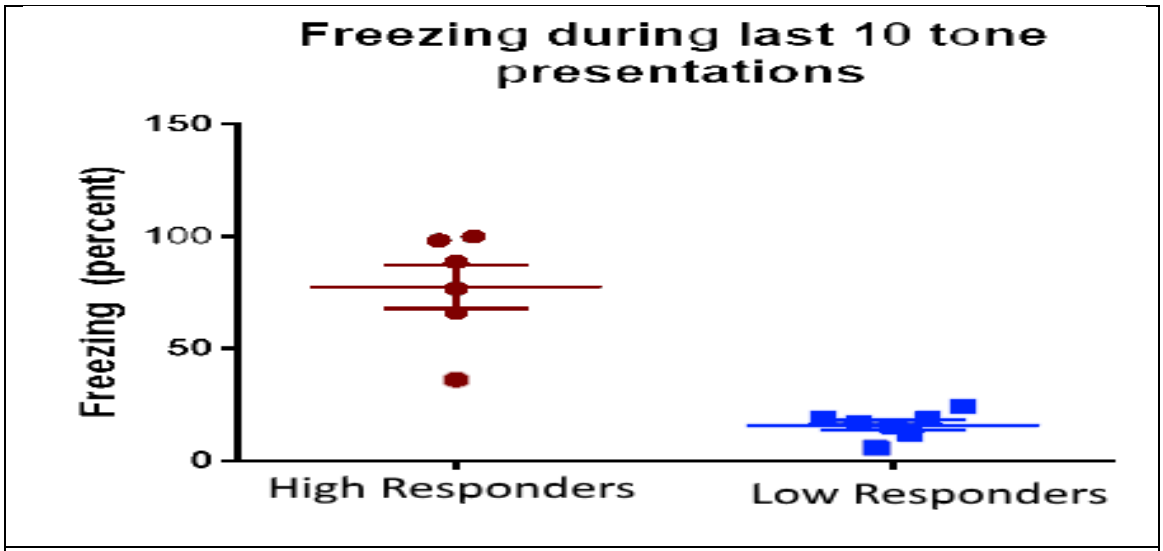

Figure 3.1 Each rat's average percent time spent immobile over the last 10 presented tones of the cue based experiment. Rats were categorized into two distinctly responding groups, and classified as either displaying high (median freezing < displayed freezing) or low (median freezing > displayed freezing) freezing responders. This division of freezing behaviors denoted by the color of the rats bar, with red indicating high responders and blue indicating low responders.

When the data from the conditioned freezing acquisition trial were assessed in this manner the results showed that both groups displayed similar fear acquisition as measured by their comparable levels of percent freezing for the duration of the conditioning trial (*Fig 3.2.)*.

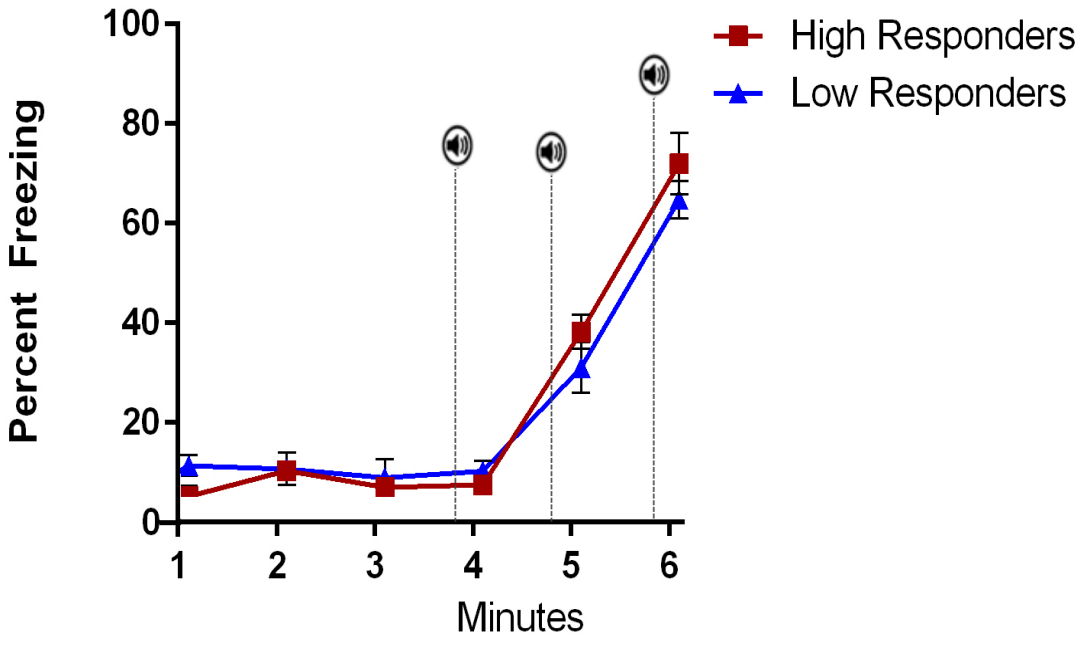

Figure 3.2 Acquisition of conditioned freezing, where the rats are learning the association between tone and foot shock. The first three minutes are left as an exploratory period to assess unconditioned freezing. The dramatic increase in freezing behavior between minutes 3 and 6 demonstrates both high and low responding groups quickly learn to associate the tone with the proceeding shock. Furthermore little to no differences in forming fearful associative memories exists between these two groups, which show that all animals display the same relative propensity for fear learning.

The ANOVA indicated there were no differences in conditioned fear acquisition between high and low groups ($F(1, 11) = 0.5548$, $P = 0.4720$) although there was a main effect of time ($F(6, 66) = 72.53$, $P < 0.0001$) and an interaction ($F(6, 66) = 2.927$, $P = 0.0136$) The next behavioral trial to be assessed was the context dependent recall trial

wherein no stimuli were presented. Once again rats from both groups performed very similarly, displaying levels of freezing behavior that were comparable both overall and for each individual time point indicating no differences in their contextually based recall between high and low groups *(Fig 3.3)*. The ANOVA analysis demonstrated no affect of group (high vs low) ($F(1, 11) = 0.0005889$, $P = 0.9811$) or significant interaction ($F(7, 77) = 0.8286$, $P = 0.5668$). However, there was a main affect by time ($F(7, 77) = 3.084$, $P = 0.0064$).

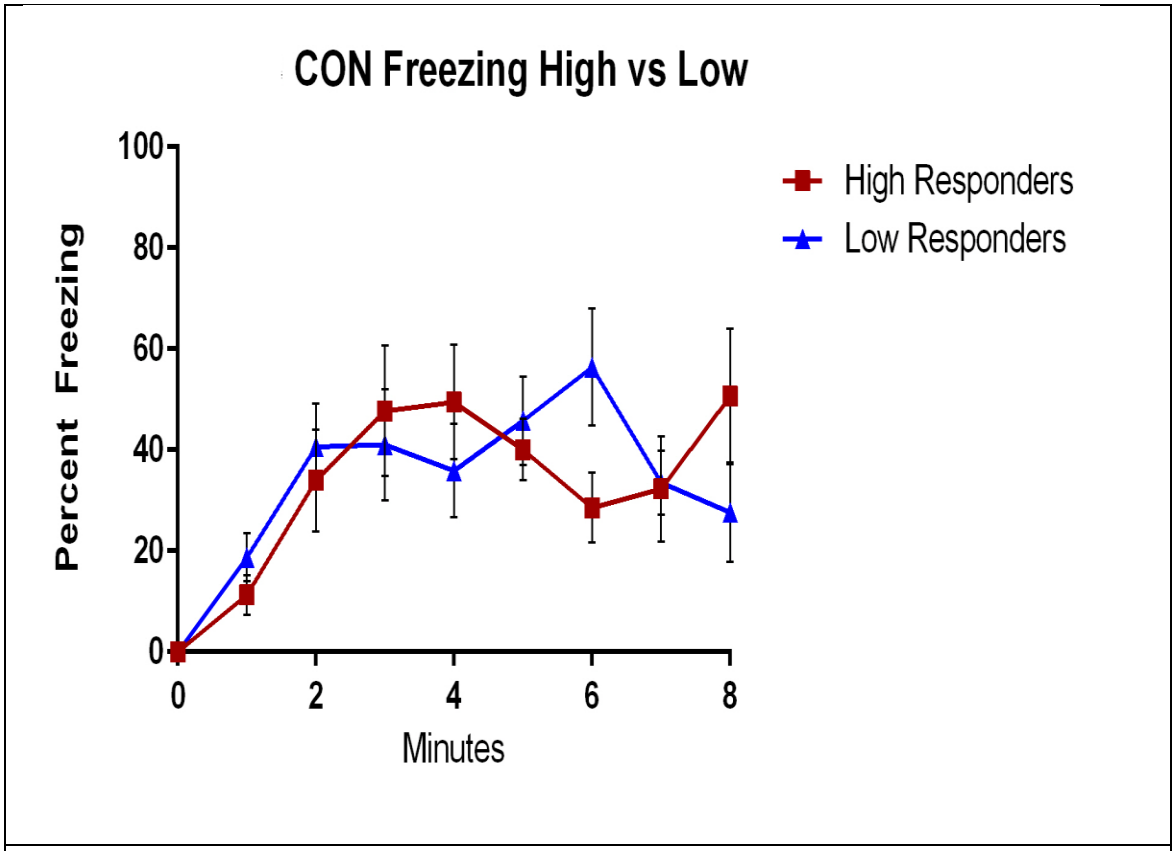

Figure 3.3 In this contextually based recall trial the exactly same enclosure is used to test for recall of the US based upon contextual recall and completely absent of cues. High and low responding groups display freezing behavior at very similar levels through the trial. This indicates that despite their categorization, all rats exhibit the same level of contextually based recall ability.

When the results from the cue only based fear extinction recall trial were assessed in this manner it was found that the two groups performed quite differently.

The low responder group showed a significant reduction in freezing starting at the 8th minute, while the high responder group did not display nearly as great a reduction in freezing behaviors and also did not show a steady decline until the 18th minute. This was supported by statistical analysis, with significant differences between high and low groups ($F(1, 11) = 38.36$, $P < 0.0001$) a main effect of time ($F(22, 242) = 15.72$, $P < 0.0001$), and a significant interaction ($F(22, 242) = 2.358$, $P = 0.0008$). Furthermore, freezing behavior displayed at minutes 8-23 were shown to be significantly different via a post-hoc t-test (see Fig 3.4). This demonstrates a deficit in fear extinction learning in the high responder group as compared to the low responders. *(Fig 3.4)*

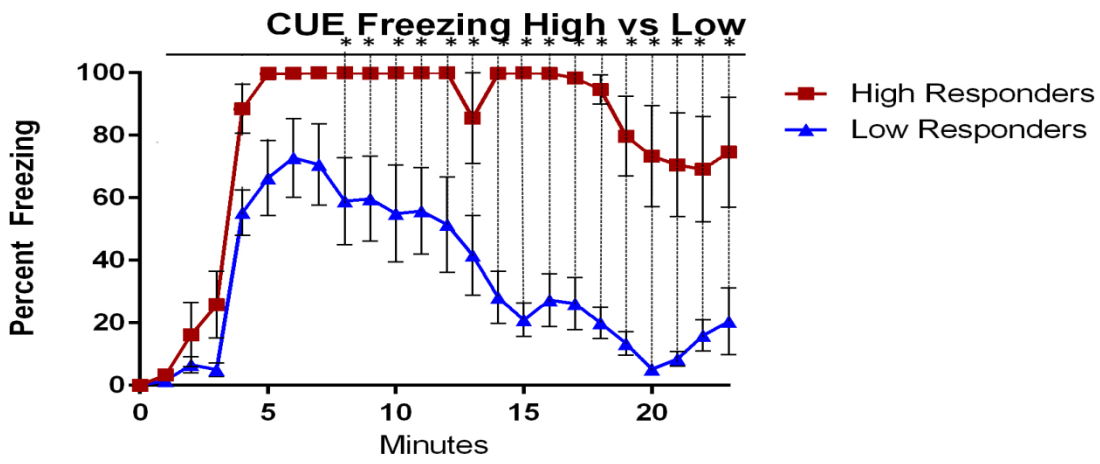

Figure 3.4 This graph illustrates freezing behaviors over the course of the cue based behavioral trial. In this trial rats were tested for their recall of cued fear response in a completely new enclosure. Specific details about the alterations to the enclosure can be seen in the methods and materials design section. After the first few tone presentations the low responders can begin to show extinction learning as a reduction in freezing in response to the tone. However, the high responding group can be seen to exhibit sustained freezing behavior until almost the end of the trial and even then the decrease in percent freezing is quite minimal in comparison to the low responding group.

The fear extinction learning trial was assessed in the same manner and reported quite a distinct difference between the two groups. *(Fig 3.5)* The low responder group showed a dramatic decrease in freezing behavior after the 3rd minute and sustained a very low level of freezing behaviors in response to tones for the remainder of the trial. However, the high responder group did not display a significant sustained decrease in percent freezing until after the 17th minute. ANOVA analysis demonstrated a significant affect by group (high vs low) ($F(1, 11) = 19.86$, $P = 0.0010$), a main effect by time ($F(20, 220) = 6.306$, $P < 0.0001$) as well as a significant interaction ($F(20, 220) = 2.672$, $P = 0.0002$). Furthermore when single bins of time were assessed for significant differences by a post hoc t-test minutes 2, 4-11 and 15-17 were found to be significantly different. This disparity indicates a sign of differences between the two groups.

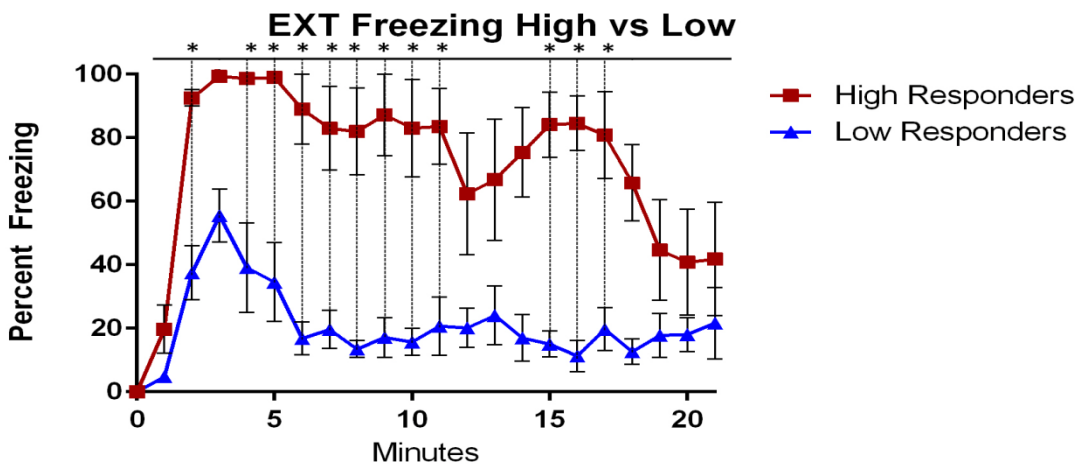

Figure 3.5 Graph of extinction learning shows that the high responder group displays high freeing in the first few tones, compared to the low responding group, while the low trait group has already begun to re-associate the tone as a neutral stimulus shown through their level of percent freezing. However, the high trait group can be seen to display fear extinction learning similar to the low group by the end of the trial. This data shows that the high trait group is still very capable of fear extinction learning, but that they display a deficit somewhere in the learning process which requires a much longer time course with more reinforcements in order to learn.

Western Blot Analysis

When protein expression in the BLA was assessed via western blot analysis two markers were analyzed. BLA m-AChR M1 as well as VAChT expression was found to be significantly different between the two groups. BLA m-AChR M1 high vs low t-test low (t=2.372, df=11, P= 0.0370). Low responders showed an approximate two fold higher expression level of m-AChR M1 as compared to their high responder counterparts. *(Fig 3.6)*

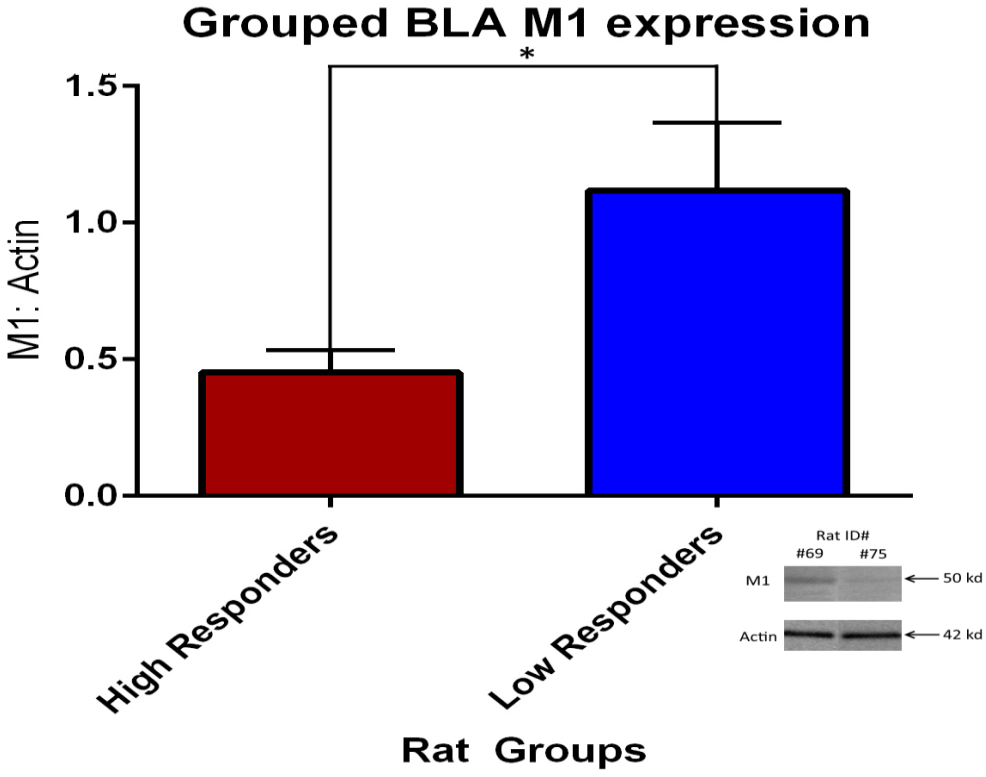

Figure 3.6 This graph depicts the high/low responder grouped expression levels of m-AChR M1 in the BLA with an example image of the western blot. As can be seen the two groups displayed significantly different levels of M1 expression with low responders displaying an average expression of two fold higher than that of the high responders. BLA m-AChR M1 high vs low (t=2.372, df=11, P= 0.0370).

VAChT expression also showed the same approximate difference of two fold the expression level in the low compared to high responder groupings, but no significant difference (t=1.274 df=11, P= 0.2290). *(Fig 3.7)*

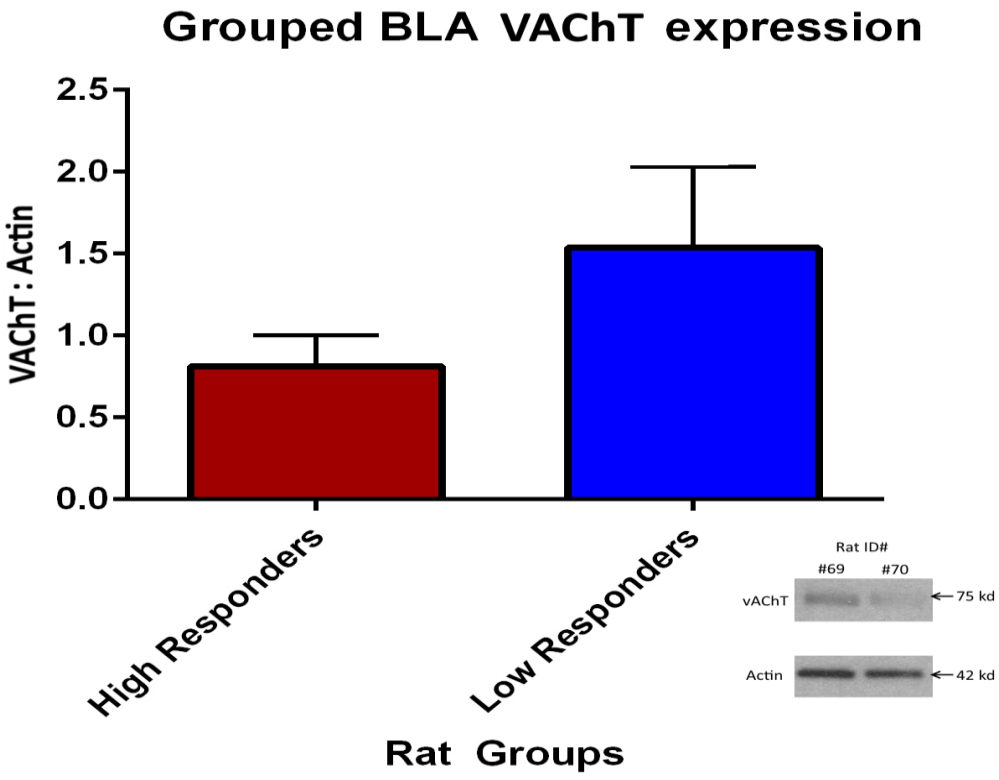

Figure 3.7 This graph depicts the high/low responder grouped expression levels of VAChT in the BLA with an example image of the western blot. As can be seen the two groups did not display significantly different levels of VAChT expression. (t=1.274 df=11, P= 0.2290).

VAChT expression was also analyzed in the PFC, and it was found that the groups once again displayed non-significantly different levels of VAChT as measured by a t-test. VAChT high vs low (t=1.183 df=11, P= 0.2619). Furthermore, the same approximate 2:1 ratio for low: high responding groups was found to exist. *(Fig 3.8)*

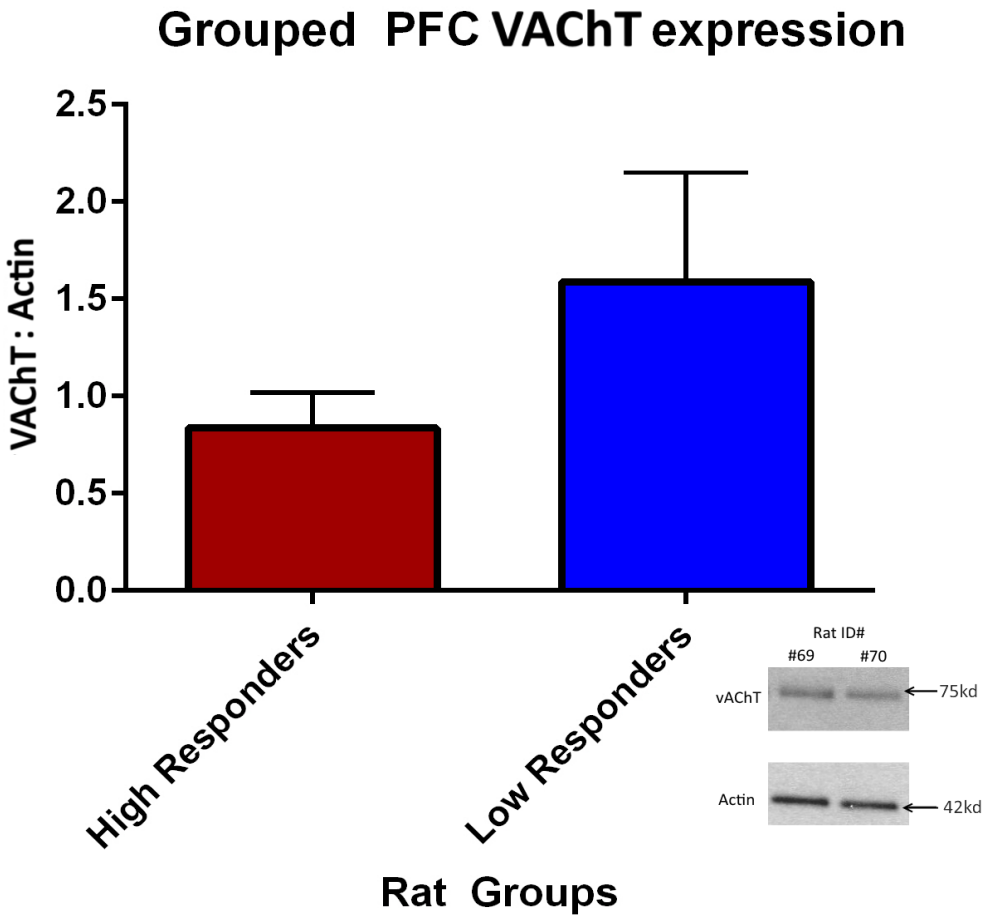

Figure 3.8 This graph depicts the high/low responder grouped expression levels of VAChT in the PFC with an example image of the western blot. As can be seen the two groups did not display significantly different levels of VAChT expression. (t=1.183 df=11, P= 0.2619).

Statistical Analysis

When subjects' behavioral data over the first 5 tones of extinction learning testing were compared to their cholinergic marker expression data some very interesting correlations were apparent. Comparing behavior and protein expression in this manner within the BLA yielded a significant correlation between M1 expression and percent freezing during the recall of extinction. *(Fig 2.9)*

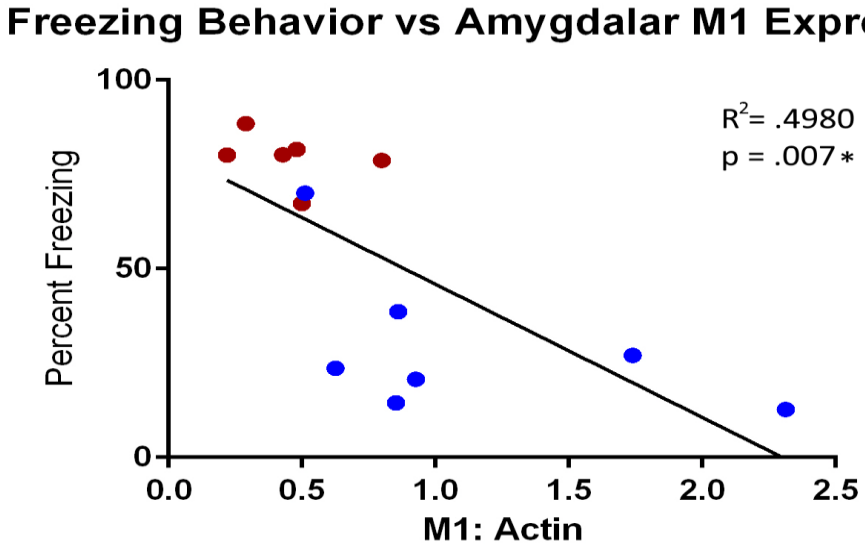

Figure 3.9 This graph depicts the correlation analysis of the first 5 tones of the extinction learning trial to the level of M1 expression in the BLA.

However, when vAChT expression within the BLA was similarly compared no significant correlation was found. *(Fig 2.10)*

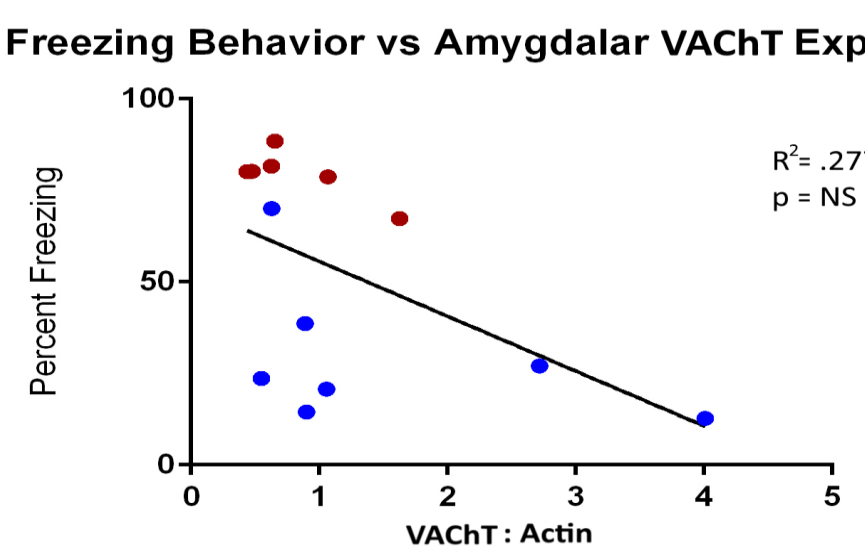

Figure 3.10 This graph depicts the correlation analysis of the first 5 tones of the extinction learning trial to the level of vAChT expression in the BLA.

Furthermore, when vAChT within the PFC were compared to behavior there comparisons also failed to produce significant correlations. *(Fig 2.11)*

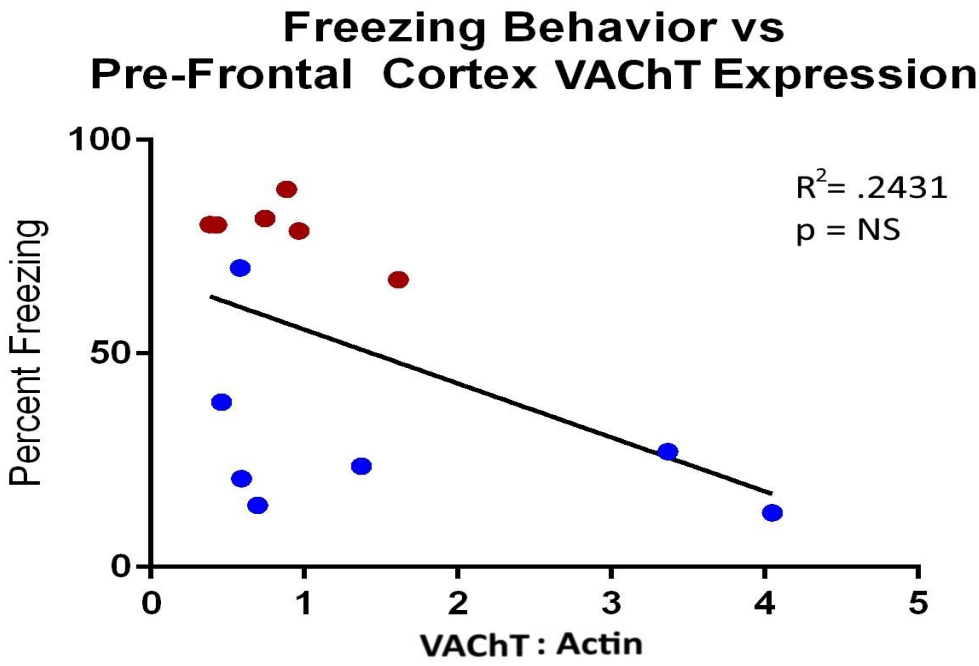

Figure 3.11 This graph depicts the correlation analysis of the first 5 tones of the extinction learning trial to the level of vAChT expression in the PFC.

CHAPTER 4

CONCLUSION

The data provided in this study has demonstrated that individual differences in fear extinction learning do exist within cohorts of animals, and that these differences are specific to fear extinction learning while fear acquisition remains similar. When the results of the acquisition trial are observed it can be seen that both groups of animals displayed relatively equal levels of percent freezing behaviors at the same time points in response to tone/shock presentations. These data indicate that all animals within the cohort are displaying relatively the same propensity to learn fearful associations. Similarly when the context based trial results are assessed they show us that the two groups performed very comparably when assessed only for fear memory recall based upon contextual cues only. This suggests that these animals have very comparable hippocampal function in the context of fear learning in the cue based recall trial However, the low responders relatively quickly extinguish their previous fearful association between tone and shock while the high responders take quite a while longer to display the reduction in freezing behaviors which accompany this form of learning. These data indicate that high responder animals still display the ability for fear extinction learning, but that a deficit exists requiring a greater length of time and number of cue presentations in order to learn new associations during extinction. The fear extinction

trial is where the greatest discrepancy between high and low responder groups can be seen. When low responders are placed into the extinction enclosure and subjected to tone presentations the reduced amount of freezing suggests they remember the previously re-learned associations and display a steep decline in freezing behaviors within the first 10 tones of the trial. However, high responder individuals show no such evidence of recalling their small degree of extinction learning demonstrated in the previous trial. Instead they seem to not remember their previous extinction experiences at all, and instead seem to show that they are once again attempting to re-learn their fearful associations just as in the cue based trial. This observation has also been made in human studies focused on PTSD patients. When a cohort of PTSD patients was assessed similarly for fear extinction learning deficits it was found that they displayed the ability to condition and demonstrate within trial extinction, but failed to exhibit normal levels of fear extinction recall akin to what was observed in this study (Milad et al. 2009, Rougemont-Bucking et al. 2011) This is indicated by their prolonged time spent displaying freezing behaviors in response to the tone presentations. Furthermore, when the western blot analyses is observed animals that displayed deficits in fear extinction learning also displayed reduced expression of M1 m-AChR within the BLA.

Perhaps the most interesting and striking observations though are the correlation analyses. When protein expression within the aforementioned brain regions was analyzed against freezing behaviors over the first 5 tones of the extinction learning trial, only one marker was found to have a significant correlation with behavior. The expression levels of M1 mAChR within the amygdala were found to significantly correlate with rapid memory retrieval of fear extinction learning while VAChT in either the BLA or PFC was

not. Taken together these correlation analyses tell us that low levels of M1 expression within the BLA seems to be correlated with individual differences in fear extinction learning displayed by the high responders, and that robust expression of M1 in the BLA could be linked to intact and fully functional fear extinction learning. It is interesting to note that when low and high responder animals alike have their VAChT expression profile compared to their M1 expression profiles that there exists an approximate two fold expression of VAChT compared to M1. This preserved ratio among all individuals also seems to indicate that the variations in VAChT are co-variates in response to the amount of cholinergic tone needed to activate a given level of expression of M1 m-ACh receptors.

These results are in agreement with the previously reported pharmacologic studies focusing on M1 within fear extinction. Within these studies researchers found that when M1 agonists were applied to the BLA via cannula guided micro injections post extinction training that enhancements in fear extinction consolidation were witnessed (Boccia et al. 2009). Furthermore, a study examining the direct stimulation of muscarinic receptors in the amygdala reported an increase in Pavlovian conditioning consolidation (Vazdarjanova and McGaugh, 1999, Passani et al. 2001). Conversely, when the BLA was inhibition inactivated by way of targeted mucimol injection deficits were witnessed in fear extinction learning. Not only are the results observed in this study in agreement with data reported in pharmacologic studies, but also with the data of studies focusing on acetylcholine signaling within the BLA.

Photoactivation or inhibition of cholinergic terminals within the BLA was shown to alter fear extinction acquisition as well as extinction consolidation, once again demonstrating that mAChRs within the BLA are well established as key role players in the process of fear extinction learning (Jiang et al. 2016).

Studies done on human subjects have also shown similarities to observations made upon rodent subjects. PTSD patients display stronger activation and therefore higher levels of downstream cholinergic tone, of the vm-PFC is accompanied by more strongly inhibited conditioned memories which lead credence to this study's findings (Milad et al. 2007). Furthermore, a study by performed on human subjects suffering from PTSD showed that these patients also displayed hypo-activation within the vm-PFC when compared to control subjects, which is akin to the reduction in cholinergic terminals observed in the high responder group (Milad et al. 2009b). Further studies aimed at investigating this apparent congruency between human and rodent fear extinction circuits and outcomes reported that there is an extremely high level of similarities (Schiller and Delgado 2012).

While this observation has provided meaningful insight into the mechanisms and outcomes of individual differences in fear extinction learning there still remain certain problems and limitations within. A potential limitation within this study exists within the less than exact sample procurement method that is brain region punches. When brains are punched for specific regions and sub regions it is near impossible to tell whether that bunch completely encompasses the whole region desired or if it is only region enriched and displays some amount of crossover of tissue sampled from adjoining regions. While brain region punching is an established technique it still bears mentioning that this

method may display region overflow. Western blot analysis as a technique also comes with its own problems and limitations. For instance western blot analysis is only a semi-quantitative method of measuring protein expression as it relies upon actin expression normalization to produce quantified expression data as a ratio. Furthermore, this method is in essence an estimation of expression and does not accurately say Within this study we also encountered a limitation due to anti-body specificity. While many antibodies are sold by various companies claiming to be highly specific and easy to use we in fact found the opposite in the context of M1 specifically. After much experimentation and research we finally settled on the antibody used in this study, but it still needed a fair amount of time investment to improve the resolution and specificity of our western blot analysis.

There are many observations and valuable insights left to glean from fear extinction learning research, and future investigations will be needed to further elucidate the entire intertwined story that is fear extinction learning expression. The next step in looking at protein expression in this type of context is to increase the resolution power of this analysis by utilizing more advanced techniques. One example of such a technique would be to utilize immunohistochemistry to better quantify region specific protein expression. Such a technique would in increase the anatomical resolution power of quantification while simultaneously relieving much of the problems with antibody specificity, because many of the antibodies available which label for M1 m-AChR have a larger literature backing up the quality of staining using immunohistochemistry (Bajayo et al. 2012, McDonald and Mascagni 2010, Mullet et al. 2013, Ricard and Gudas 2013, Smith et al 2015, Vetreno et al. 2014). The downside of this approach is the reduced ability to quantify changes in expression. Another step forward into understanding the

underpinnings of fear extinction learning would be to utilize electrophysiology to determine if the receptors we are analyzing are actually functional and intact receptors and investigating the exact outcome of the changes in cholinergic tone observed in both human PTSD patients and rodent subjects, and while some research has been done to this end much still remains. Pharmaceutical modulation of fear extinction learning is the last piece of the puzzle in understanding how we may transition these research findings into therapeutics. While many studies including ones aforementioned in the beginning of this exposé have looked at the effects of different compounds on fear extinction learning within the rodent Pavlovian conditioning paradigm, research on humans subjects utilizing such compounds remains to be investigated.

The data and conclusions reported herein may not be a complete surprise considering the wide body of literature that exists which back up M1's role in many learning and memory processes across several brain regions. However, it is uniquely insightful when examined from the view point of potential PTSD therapeutics. If indeed reduced M1 expression in the BLA is responsible for the types of fear extinction learning deficits that are witnessed in PTSD patients as these data seem to tell us then this would pave the way for a wide area of pharmacologic agents to be used to enhance the effectiveness of psychotherapy and thusly the treatment of PTSD. However, producing new pharmaceutical compounds is an extremely lengthy process which requires huge monetary and work investments. The beauty of utilizing the M1 receptor as a therapeutic target in the treatment of PTSD is that agents which act on this receptor in an agonist and PAM like fashion already exist and have been FDA approved for use in treating Alzheimer's disease and other cognitive disorders.

While rebranding their use for PTSD treatment may still require some investigation, the lengthy process of drug discovery and safety verification for use on human subjects has already taken place eliminating the delay in production and expediting its availability as a treatment option for those afflicted with PTSD.

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