THE ROLE OF BASAL FOREBRAIN CHOLINERGIC PROJECTIONS TO THE ANTERIOR CINGULATE CORTEX IN CUED AND CONTEXTUAL FEAR CONDITIONED SUPPRESSION PARADIGMS

by

Caroline Lawless

A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Master of Science in Neuroscience

Fall 2017

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Approved: _____
Amy L. Griffin, Ph.D.
Professor in charge of thesis on behalf of the Advisory Committee

Approved: _____
Robert F. Simons, Ph.D.
Chair of the Department of Psychological and Brain Sciences

Approved: _____
George H. Watson, Ph.D.
Dean of the College of Arts and Sciences

Approved: _____
Ann L. Ardis, Ph.D.
Senior Vice Provost for Graduate and Professional Education

ACKNOWLEDGMENTS

I would like to covey my deep appreciation for the support and guidance of the PBS faculty and graduate students throughout my Master's experience at the University of Delaware. I would like to thank Dr. Amy Griffin specifically for her emotional and intellectual support not only throughout my graduate education, but also during my undergraduate studies.

Additionally, I would like to thank all of my undergraduates in the Knox lab for assisting me with scoring data as well as performing experiments. Without their emotional support and diligence in working on my project, I would not be where I am today. Finally, I would like to thank my thesis committee, Dr. Anna Klinsova and Dr. Mark Stanton for supporting me throughout my defense and for their valuable feedback.

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ABSTRACT

Basal forebrain corticopetal cholinergic neurons are critical for contextual and cued fear memory in the conditioned suppression paradigm, but neural mechanisms that alter these neurons in fear memory remain unknown. Interestingly, basal forebrain cholinergic lesions have no effect on behavioral performance in commonly-studied fear conditioning paradigms like Pavlovian conditioned freezing or fear-potentiated startle, yet impair fear memory in the conditioned suppression paradigm. Many studies conducted have experimented with lesions of cell bodies of corticopetal cholinergic neurons in the nucleus basalis magnocellularis (NBM), but there is a void in the literature defining which specific projections may be responsible for their discrepant role in different fear memory paradigms. The basal forebrain projects to the anterior cingulate cortex (ACC), a subregion of the medial prefrontal cortex. The ACC is a well-established portion of the fear circuit across all fear conditioning paradigms and has a clear role in decision-making in the conditioned suppression paradigm. Given the role in choice conflict that the ACC plays in operant tasks involved in the conditioned suppression paradigm, it is plausible that it may be a region that allows basal forebrain cholinergic neurons to alter a fear memory in the conditioned suppression paradigm. The goal of this study is to examine the specific roles that basal forebrain cholinergic projections to the ACC play in fear memory, specifically in the conditioned suppression paradigm. These lesions may target specific cholinergic input to the ACC from the NBM in the basal forebrain and this may isolate a specific fear circuit involved in fear memory in the conditioned suppression paradigm. Data have

suggested that ACC lesioned animals demonstrate less fear-conditioned suppression over sham animals, but further experiments and cohorts of animals are required. If ACC cholinergic lesions are shown to produce deficits in fear memory in the conditioned suppression paradigm, it may suggest that the presence of the appetitive task, which only occurs in the conditioned suppression paradigm and not in any of the other commonly studied fear paradigms, may be able to elicit changes in functional connectivity to incorporate this projection from the NBM to the ACC to the fear circuit. Discrepancies in fear memory between fear conditioning paradigms demand to be addressed because assumptions about functional connectivity across different paradigms are assumed to be similar in the literature. If the notion of paradigm-dependent functional connectivity presented here is true, deductions about this functional connectivity may only be made in the context of one fear paradigm and may not necessarily be applicable across paradigms. In other words, to say that Pavlovian fear conditioning and fear-potentiated startle are indicative of the broad neurobiology of fear memory would only be looking at a fraction of the reality behind how fear circuitry operates. In order to further the literature to propose holistic circuits, molecular processes and constructs that apply to all fear memory regardless of protocol or paradigm, it is necessary to investigate neural involvement across alternative fear paradigms, like conditioned suppression. This study supports the novel idea that neural circuitry that supports fear can expand with new learning tasks or events and therefore, may be more susceptible to change than previously considered, but future studies are required.

Chapter 1

INTRODUCTION

1.1 The Role of Cholinergic Projections in the Fear Circuit

The septohippocampal and corticopetal branches of the basal forebrain cholinergic system have each been implicated in fear and anxiety-like states. Cell bodies belonging to the septohippocampal branch are located in the diagonal bands of Broca and medial septum; the ones within the corticopetal branch are located in the nucleus basalis mangocellularis (NBM) (Mesulam et al. 1983; Zaborszky et al. 1999). While these branches have unmistakably different origins in the brain, it should also be noted that many studies assert functional independence between the two. Two particular studies demonstrated that specific cholinergic lesions in each pathway were able to impair different aspects of conditioned stimulus processing (Baxter, Bucci, Holland & Gallagher 1999; Knox & Berntson 2006). Therefore, we can infer that individual cholinergic targeting of either of these branches will allow us to interpret behavioral outcomes for both branches separately.

Previous studies examining corticopetal basal forebrain cholinergic lesions in the NBM have demonstrated effects in the cued and contextual fear memory in the conditioned suppression paradigm, but interestingly enough, these lesions do not have any effect in other fear paradigms that are predominantly used to study fear memory and circuitry in prolonged anxiety states in animals (Conner 2003; Frick et all. 2004; Schauz & Koch 1999; Knox & Berntson 2006; Knox & Berntson 2008; Stowell et al., 2000; Knox & Keller 2015; Baysinger et al. 2012). This discrepancy in circuitry

between studies using different fear conditioning paradigms in fear memory poses an interesting question; why do these cholinergic lesions, or their projections, in the conditioned suppression paradigm elicit alternative behavioral, lesion-specific effects compared to other paradigms? Examples of these alternative fear conditioning paradigms used in the literature include Pavlovian conditioned freezing and fear-potentiated startle. In both of these paradigms, subjects demonstrate behavior indicative of fear learning when a conditioned stimulus (CS) is paired with an unconditioned stimulus (UCS). Unlike these two paradigms that are commonly studied and equated with each other in the literature, subjects in the conditioned suppression paradigm will acquire a fear memory and will change their operant performance based on the salience of that fear memory. That operant task in the context of this study requires the animal to push a retractable lever for a food reward. The presence of this appetitive task may allow for changes in functional connectivity to occur to incorporate different parts of the brain that are not involved in the fear circuit in other paradigms. Additionally, older studies have reported significant differences in neurobiological mechanisms involved in conditioned suppression and conditioned freezing, which suggests that it is plausible that mechanisms of fear memory may be different in the conditioned suppression paradigm (Amorapanth et al. 1999; Killcross et al. 1997).

The neurobiological mechanisms underlying differences in lesion-induced behavior remain unknown, yet seem to point us in the direction of one specific target of many from the NBM: the anterior cingulate cortex (ACC). The ACC is specifically involved in planning and choice-oriented tasks, attention selection and contextual processing (Baddeley 1996; Brown & Bowman 2002; Fuster 2000; Miller 2000),

which are required in the operant task specific to fear conditioned suppression but not other fear paradigms. Additionally, in previous studies the ACC has been shown to be necessary for expression and development of cued (Bissiere et al. 2008) and contextual fear memory (Einarsson & Nader 2012; Frankland et al. 2004). Conveniently, the NBM cholinergic projections from the basal forebrain to the ACC are able to be easily targeted due to the fact that they are the only presently known group of neurons in the basal forebrain that express p75 receptors (Frick, Kim and Baxter 2004; Heckers et al. 1994). This will allow for a retrograde lesion of a specific NBM cholinergic projection that may be critical to understanding any underlying mechanisms of the fear circuit. By investigating the effects of cholinergic lesions in the ACC in the presence of appetitive task learning in the context of the conditioned suppression paradigm, we hope to further understand the mechanisms and circuitry behind the differences observed in fear memory between paradigms.

Chapter 2

MATERIALS AND METHODS

2.1 Experimental Overview

All subjects in each of the experiments completed various schedules of operant training, fear conditioning and context exposures. Each of these behavioral manipulations occurred within a MedPC operant training box and AnyMaze scored freezing behavior, which will be explained in further detail below. Once behavioral criteria were reached in each group of sham or lesion animals, stereotaxic surgery was performed to produce a cholinergic lesion in the ACC. After sacrifices, brains were extracted for acetylcholinesterase (AChE) and choline acetyltransferase (ChAT) staining to verify cholinergic lesions in the ACC. Only animals with observable cholinergic lesions were taken into account for behavioral analysis.

2.2 Subjects

In each of the experiments mentioned below, male Sprague Dawley rats at post-natal day 53-56 were housed in pairs until after their respective lesion or sham surgery. A total of five animals were used for cued fear conditioned suppression, and a total of ten animals were used for contextual fear conditioned suppression. All subjects were housed in the same colony room under a 12 hour light-dark cycle. Animals were organized into cohorts of six, with equal sham and ACC lesions in each cohort of animals. All protocols used in the following experiments were in accordance with NIH

regulations approved by the University of Delaware's Institutional Animal Care and Use Committee.

2.3 Operant Protocols

All training and testing behavior before and after surgical procedures was conducted during the light phase between 12:00 to 15:00. The first three days after each cohort's arrival served as an acclimation period with exposure to 10 pellets of sucrose daily (used in the operant conditioning task) in their home cage, 23.5 grams of food per day and *ad libtum* access to water. Food restriction was required for both of the following experiments because operant task performance was based around a food reward. All subjects in each respective experiment- contextual or cued fear conditioned suppression- underwent various schedules of operant training, fear conditioning and context exposure.

2.3.1 Operant Training

Operant training in both cued and contextual experiments consisted of a minimum 12-hour period of food deprivation before each testing or training session was conducted, which occurred in a distinct context. The operant training conditioned the rat to depress a lever three times for a sucrose reward within a 15 second time period over the course of 20 trials. All training programs commenced with a three-minute baseline period and inter-trial interval of 20 seconds between each trial. Training was always conducted in one of two different contexts: Context A included a red light, citrus scent, absence of Plexiglas floor insert (leaving just plain metal grate), and Context B included a white light, vinegar scent, and clear Plexiglas insert present on the floor of the box. Training began with an untimed, FR1 schedule (one response

warrants one reward), and then progressed to an untimed FR3 schedule (three responses warrant one reward). Both FR1 and FR3 training programs ran for a total of one hour. Criterion for the FR1 and FR3 programs was set to 60 responses and 100 responses per hour, respectively. After each untimed trial had been successfully completed, the animal moved on to a timed trial schedule, namely an FR3-30 program. In these timed trials, the animal had a 30 second window to make a correct response (push the lever 3 times before it retracts) in order to receive a reward. There were a total of twenty trials per training session. When each animal produced successful responses for 16/20 trials (80% response rate) with each response under 15 seconds, they were said to be "at criterion". Animals were trained on this FR3-30 schedule but were held to a standard to perform at an FR3-15 criterion. Another program, FR3-15, follows the same timed protocol and criteria above, and was used only after an animal had undergone 4 successful FR3-30 training sessions. There were a minimum of one but maximum of two training sessions conducted per day with each session scheduled more than 3 hours apart from the previous one. If two training sessions were conducted in one day, they both occurred in the same training program (FR1, FR3, FR3-30 or FR3-15), irrespective of previous performance that day. Once an animal displayed clear maintenance of the task (two or more consecutive correct trials in each context of an 80% accuracy rate), they were referred to as "at or above criteria" and were ready for surgical lesion or sham procedures.

2.3.2 Fear Conditioning and Operant Suppression Testing

The fear conditioning program occurred in Context A, after the animal had reached criteria for the FR3-15 training program twice in each context after the animal's recovery from their respective lesion or sham surgery. The contextual fear

conditioned suppression experiment began with a fear conditioning program that included a three-minute baseline period, followed by three non-stimulus (NS) trials and then ten consecutive, 20-second fear conditioning (FC) trials. Fear conditioning trials consisted of a one second in duration, 0.5 mA foot shock delivered through the grate floor of the MedPC box after the retractable lever had been available for a maximum of 30 seconds for the rat to perform a FR3-30 trial. In between each trial, there was a 20-second inter-trial interval (ITI) when the lever was retracted and the animal did not have the opportunity to respond. Retraction of the lever preceding the shock served as a discrete cue for the footshock, instead of a more commonly thought of signal, like a tone. Conditioned suppression was measured by the time it took each animal to respond to the FR3-30 trial after each bar pressing opportunity was presented. Freezing was measured for each animal in all behavioral testing of operant suppression. Program error was accounted for by personally watching for true freezing behavior and not freezing due to reward-related anticipatory behaviors. Context-dependent fear was measured 24 hours after in both fear and neutral contexts as well. The subjects performed a series of 10 consecutive NS trials with an ITI of 20 seconds separating each trial. Animals usually take longer amounts of time to perform the FR3-30 trial, which we observed as a measure of operant suppression.

The cued fear conditioned suppression experiment adhered to all protocols previously mentioned in the contextual fear conditioned suppression experiment, except for the fear conditioning program used and the addition of a cued fear testing program. After the three baseline trials in the cued fear conditioning program, there was a period of time lasting ten trials when the rat was able to respond as trained previously, but presented with a 30 second, 2 kHz tone (CS) throughout the duration

of the FR3-30 trial that co-terminated with a 0.5 mA footshock. Each CS presentation was separated by a 20 second ITI. Additionally, in the cued fear testing program, the subject was presented with three baseline FR3-30 trials, then ten consecutive CS and ten "no stimulus" (NS) trials with a 20 second ITI in between each. The response time was measured with each trial as a measure of conditioned suppression and was completed in both the fear and alternative context.

After context exposure in each respective experiment, rats were placed under general anesthesia using 5% isoflurane in air prior to euthanasia and were sacrificed by rapid decapitation. Brains will be extracted for immunohistological testing (AChE and ChAT staining) to ensure the presence of a true lesion.

2.4 Surgical Procedures

For each of the experiments, cholinergic input was removed from the anterior cingulate cortex (ACC) with infusions of 0.22 µg/µL 192 IgG-saporin. The toxin used was a basal forebrain cholinergic specific lesion, obtained from Advanced Targeting Systems Inc. The toxin is composed of a monoclonal antibody, 192 IgG, linked to saporin. It is found specifically in NGF receptor-positive cholinergic neurons in the basal forebrain, and allows for precise targeting of p75 receptors found only on these specific basal forebrain cholinergic neurons. This cholinergic toxin at the above concentration has been seen to produce selective basal forebrain cholinergic neuronal loss **(**Baxter et al., 1997; Conner et al., 2003; Frick et al., 2004; Knox and Berntson, 2006) to reach its maximal effect after fourteen days post-infusion (Book et al., 1992). Sham surgeries used the same volume of an infusion of cold 0.2M phosphate buffered saline (PBS), which was the vehicle for the toxin. The toxin was transported in a

retrograde fashion from the site of infusion to the cell body originating in the NBM. The toxin was infused directly into the ACC at the following coordinates: AP +2.7; ML +/- .7; DV -2.0 (Paxinos & Watson 1998).

All surgical procedures took place after animals were injected subcutaneously with xylazine and anesthetized using 5% isoflurane in air. Animals were then fitted into a Kopf stereotaxic apparatus and anesthesia was maintained using 2% isoflurane in air. After the location of Bregma was measured, the toxin was infused from a 5 uL Hamilton syringe with a 26-gauge needle directly into the ACC at the following coordinates: AP +2.7; ML +/- .7; DV -2.0. The single infusion took place over the course of 30 seconds, and the needle was left in the injection site for a minimum of two minutes to allow for proper toxin dispersion before it was removed.

2.5 Histological Analysis

Coronal sections of 40 μm thickness were taken through the regions containing the ACC and the NBM from each subject. Two kinds of staining were performed for all subjects: ACC sections were stained for the presence of acetylcholinesterase, and all NBM sections were stained for qualitative measurements of choline acetyltransferase. All subjects were verified by acetylcholinesterase (AChE) staining in order to quantify cholinergic fiber losses among sham and lesion animals. Histological procedures were based on a previous paper (Tago et al., 1986) and modified slightly according to lab protocols. ACC slides were incubated in a 4% paraformaldehyde solution for ninety minutes, then submerged in a mixture of 20 mg of acetylthiocholine iodide, 448 mg sodium citrate, 100 mg cupric sulfate, and 65.6 mg potassium ferricyanide dissolved in 0.1 M tris-maleate buffer (TMB). After forty-

five minutes, slides were rinsed in TMB and then stained for a maximum of ten minutes in a diaminobenzidine (DAB) solution. After staining, sections were dehydrated in various concentrations of ethanol and then fixed in a boat containing xylene overnight.

Choline acetyltransferase (ChAT) staining was performed on coronal NBM sections for cholinergic fiber loss from the NBM cell bodies to the ACC. ChAT protocol was adopted from Millipore. All sections were incubated in 4% paraformaldehyde for two hours. After incubation of TritonX-100 for 30 minutes, slides were incubated in a blocking solution of 1.5% goat serum (Vector Labs) in .1M TBS, then immersed in primary antibody rabbit ChAT at a concentration of 1:500 via coverslip and were allowed to incubate overnight. The next day, slides were washed and immersed in a goat anti-rabbit IgG secondary antibody solution, concentrated at for an hour, and then incubated in an ABC reagent (Vector Labs) as per the manufacturer's instructions for an hour via coverslip. After, slides were incubated in a DAB solution and then dehydrated in various ethanol washes and immersed in xylene for five hours before being permanently coverslipped for visual analysis with DPX.

2.6 Data Analysis

All subjects were verified by acetylcholinesterase (AChE) staining in order to quantify cholinergic fiber losses among sham and lesion animals and scored via ImageJ software. All pictures of stained sections used for quantitative analysis were imaged via microscope 2.5X lens. ChAT images used for qualitative analysis were imaged at 15X magnification. Images for AChE stained sections were taken of the ACC, while images for ChAT staining were taken of the NBM. AChE fiber loss was quantified by comparing optical density of the ACC area to the density of white matter

from the corpus callosum (Figure 1). After these values were normalized compared to sham animals, they were analyzed with a t-test (lesion vs. sham).

AnyMaze software (Stoelting Inc., Kiel WI) was utilized to score freezing from the beginning to the end of each of the 20 trials per session, averaging each CS presentation with its corresponding ISI. Behavioral results were assessed using Student's t-test. A p-value cutoff of less than .05 was used to measure significance. Suppression was also measured using the same statistical design.

Chapter 3

RESULTS

3.1 Cholinergic Lesions in the ACC

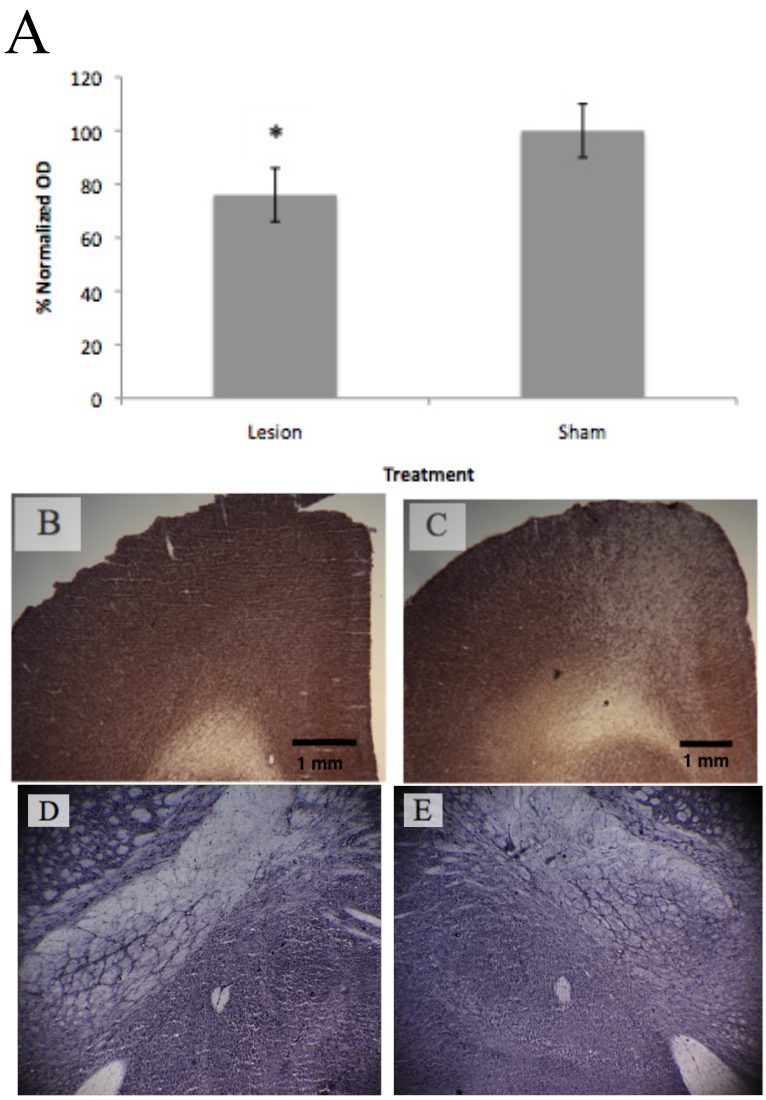
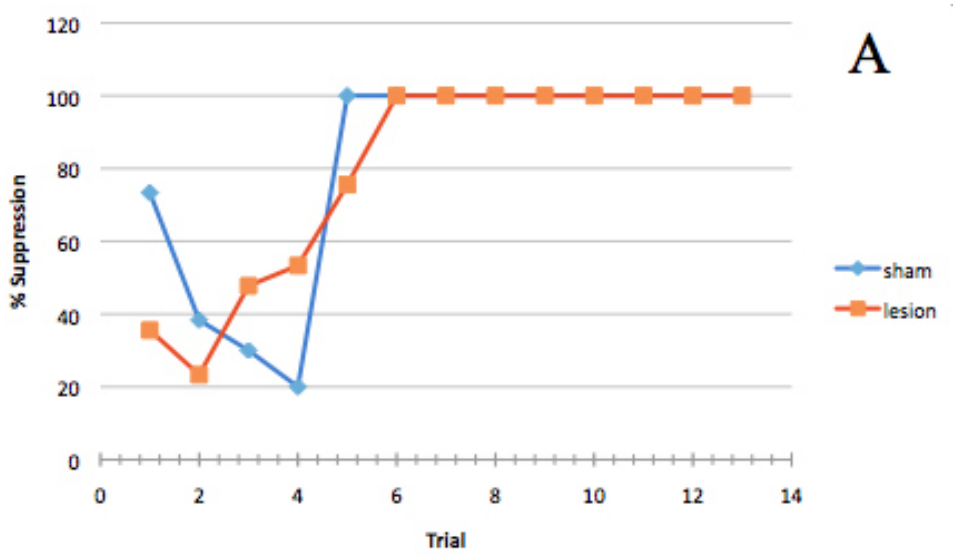


Figure 1. AChE and ChAT data for lesions placed in the anterior cingulate cortex. A) Graphed data of Student's t-test displaying significant cholinergic loss in the ACC. B) Imaging of the ACC of a sham animal and C) imaging of an ACC lesion animal. D) Imaged sham and E) lesion NBM after ChAT staining. All pictures for quantitative analysis were imaged at a 2.5X magnification.

Five animals in total, three lesion and two sham, were used to measure cued fear conditioned suppression. The AChE staining verified the presence of the cholinergic lesion [t(14)= 2.58, p-value = 0.022]. By these findings, it is very likely that the surgical lesion or sham procedures were effective in targeting the ACC specifically.

3.2 Cued Fear Conditioned Suppression



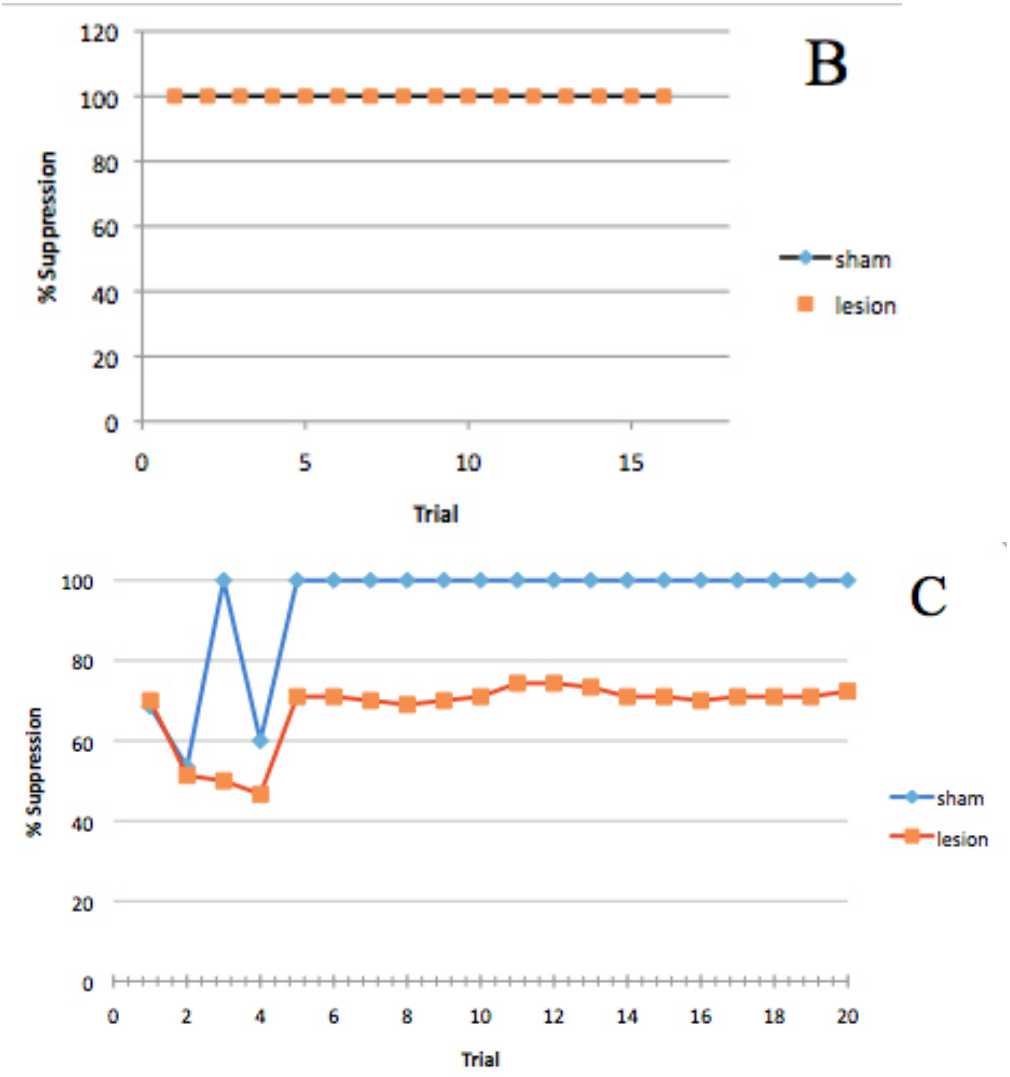


Figure 2. Cued Fear Conditioned Suppression behavioral performance. A) Graphed data average lesion and sham suppression for three baseline trials then ten presentations of the CS accompanied by a 0.5 mA footshock. B) Fear context and CS presentation without a shock 24 hours after fear conditioning. C) Alternative, non-fear context, 24 hours after fear context exposure.

Behavioral results for animals with ACC cholinergic lesions in cued fear conditioned suppression. No significant differences were found between lesion and sham during fear conditioning [F (4) = .16, p-value = .6917] or during the representation of the CS 24 hours later, when all five lesion and sham animals

displayed 100% suppression in a group x trial design [F(4) = .92, p-value = .5388]. However, significant differences in suppression were discovered between lesion and sham animals during alternate context testing 48 hours after fear conditioning [F(4) = 9.33, p-value = .0034], indicating that shams demonstrated increased context generalization than lesion animals.

3.2.1 Cued Fear Freezing

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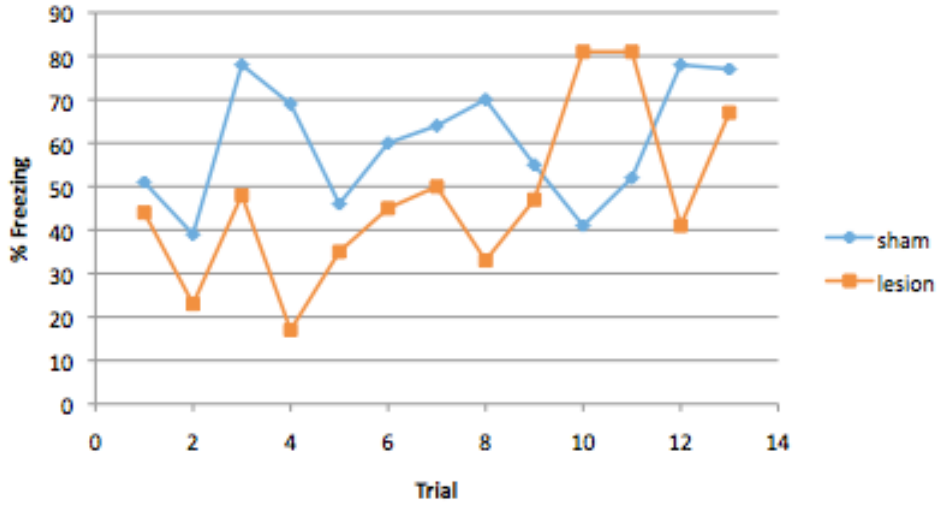

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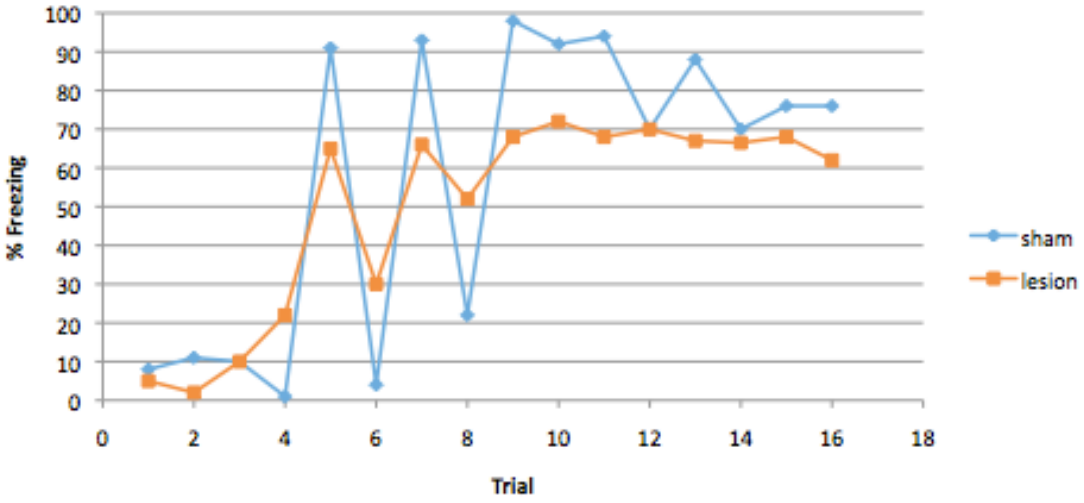
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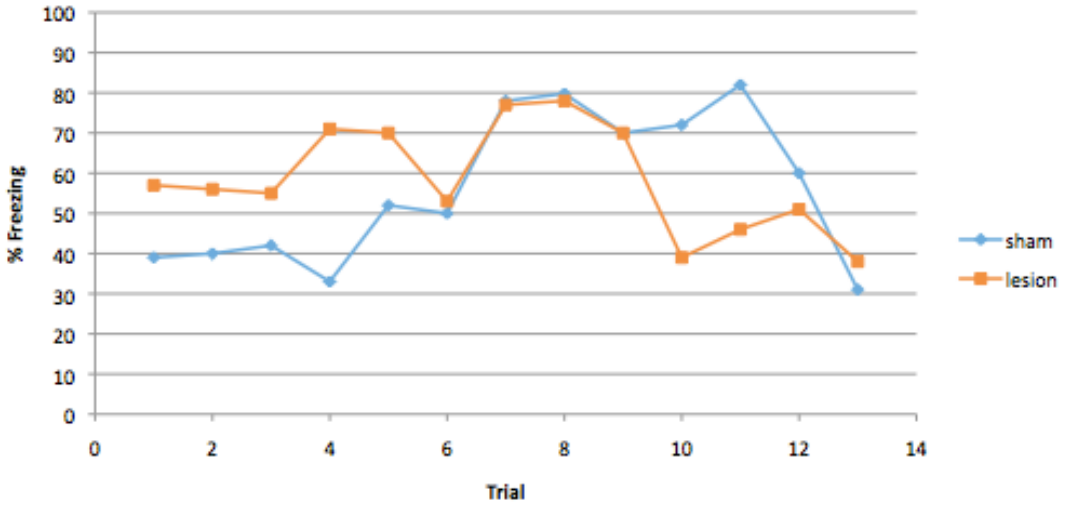
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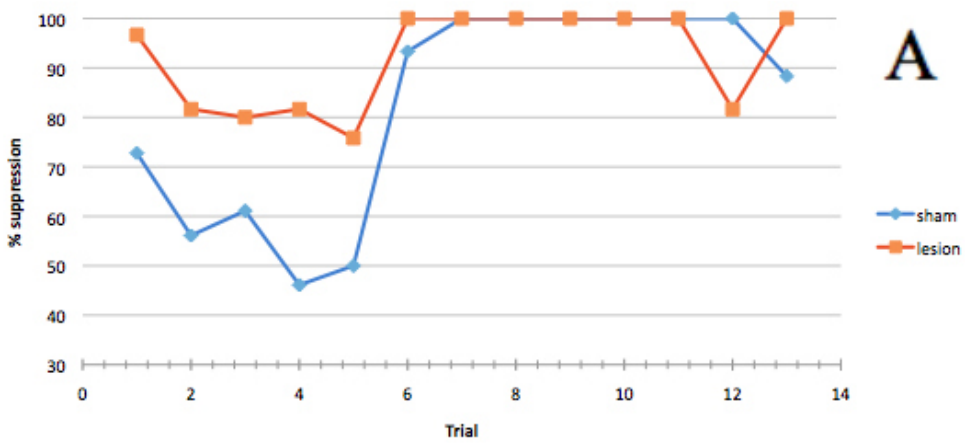
Figure 3. Measures of freezing in the cued fear conditioned suppression behavioral paradigm. A) Graphed data average lesion and sham suppression for three baseline trials then ten presentations of the CS accompanied by a 0.5 mA footshock. B) Three baseline trials, three alternative noise trials, then ten presentations of the CS without a footshock, 24 hours after fear conditioning. C) Alternative, non-fear context, 24 hours after fear context exposure.

Freezing results for animals with ACC cholinergic lesions in cued fear conditioned suppression. No significant differences were found between lesion and sham during fear conditioning [F(4) = .76, p-value = 0.3878]. No significant differences were discovered for freezing behavior during cued fear testing with representation of the CS [F(4) = 0.1, p-value =.7561] or in context testing [F(4) = .42, p-value = .9872].

There were no identifiable statistical differences between shams and lesions in freezing for cued fear or fear context testing. No significant differences between groups were found among all three behavioral manipulations in the cued fear conditioned suppression paradigm.

3.3 Contextual Fear Conditioned Suppression



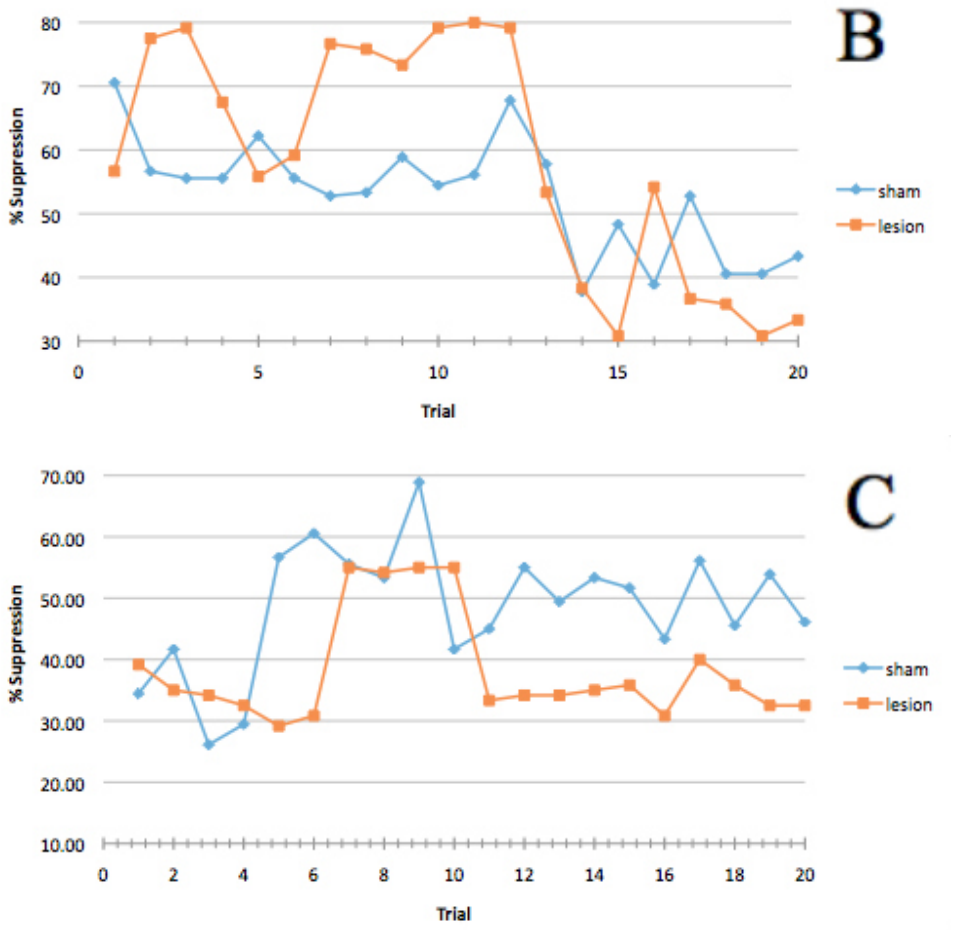


Figure 4. Measures of contextual fear conditioned suppression behavioral performance. A) Graphed data average lesion and sham suppression for three baseline trials then ten presentations of the CS accompanied by a 0.5 mA footshock. B) Fear context testing 24 hours after fear conditioning. C) Alternative, non-fear context, 24 hours after fear context exposure.

For contextual fear conditioned suppression, four out of five designated lesion animals were confirmed to have successful lesions. Therefore, four lesion and six sham animals were tested for contextual fear conditioned suppression for a total of ten subjects. Two subjects did not learn the operant task presented in an adequate amount of time and therefore, were removed from the study without conducting any further surgical procedures. Contextual fear conditioning was not found to be significant [F(9)

= .71, p-value = .399]. Fear context testing was not found to be significant between sham and lesion groups [F(9) =.25, p-value = 0.995], and alternative context testing was found to show no statistical differences between lesion and sham animals [F(9)= 2.28, p-value = .1328].

3.3.1 Contextual Fear Freezing

Animals displayed fear memory not only via operant suppression, but also freezing. There were statistically significant differences found in behavioral testing, found in all three behavioral procedures. Overall, lesion animals had lower freezing and lower operant suppression scores when compared to shams. Significance was reached in fear conditioning [F(9) =5.5, p-value = 0.0209] between lesion and sham animals.

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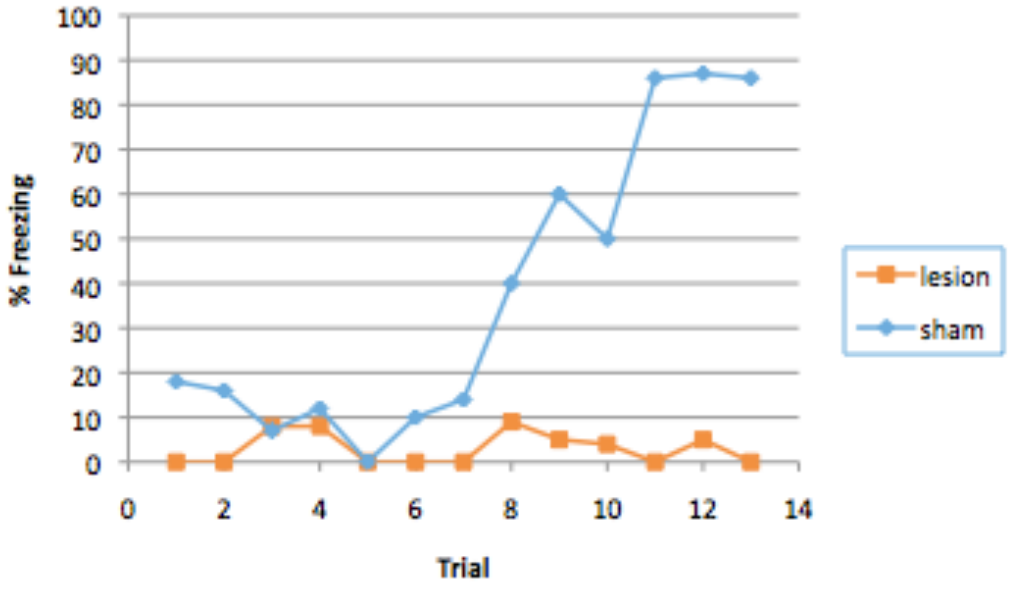

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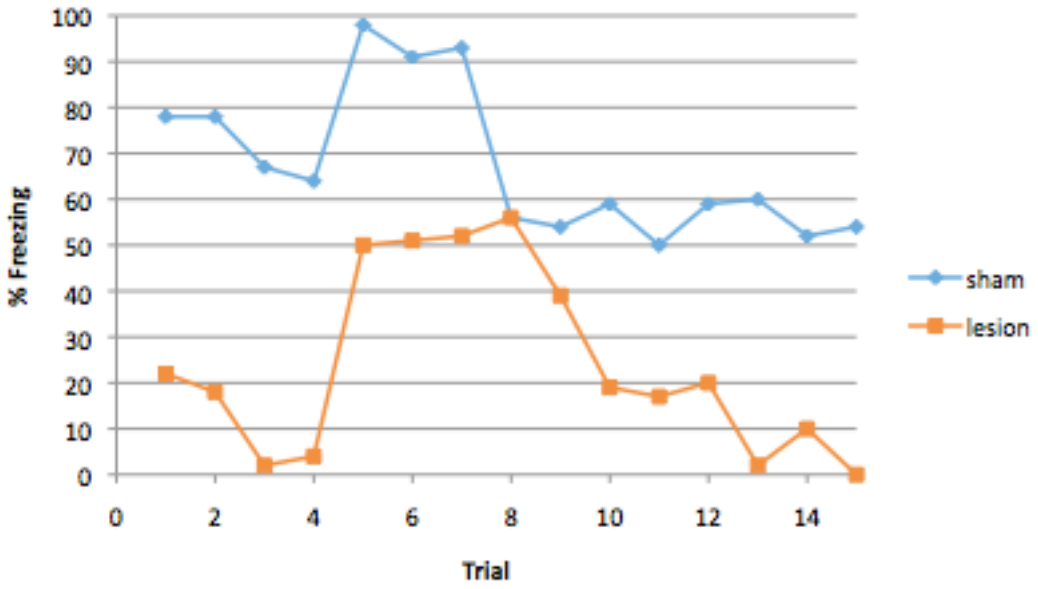
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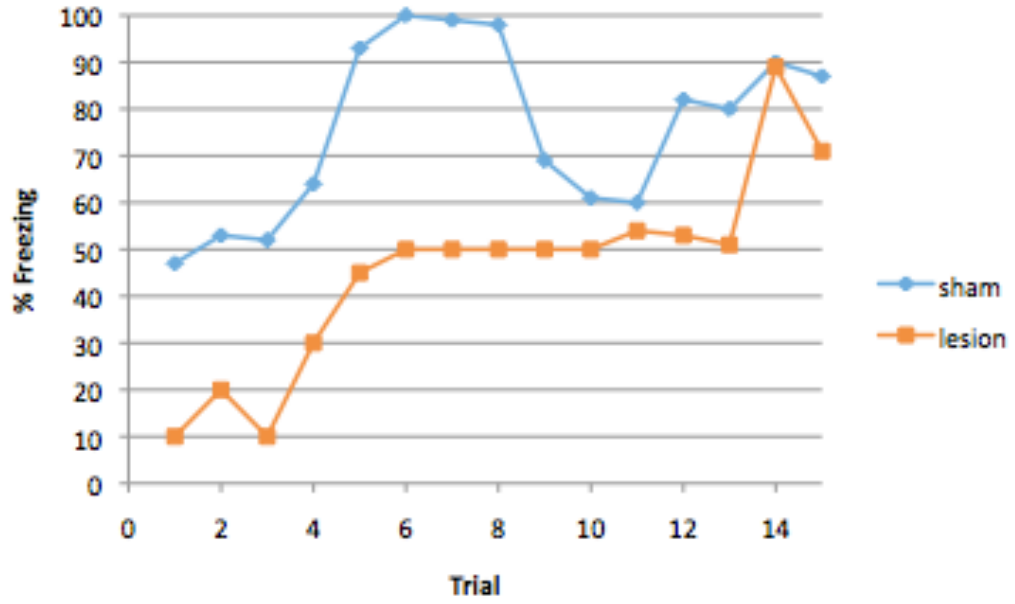
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Figure 5. Measures of contextual fear conditioned freezing behavior. A) Graphed data average lesion and sham suppression for three baseline trials then ten presentations of a 0.5 mA footshock. B) Fear context testing 24 hours after fear conditioning. C) Alternative, non-fear context, 48 hours after fear conditioning.

Chapter 4

DISCUSSION

4.1 Limitations

While the data presented seems to suggest ACC lesion animals demonstrating increased levels of contextual fear conditioned suppression, therefore supporting the initial hypothesis of this experiment, more animals will be required to run further statistical analysis. Presently, there have been a total of five animals utilized for cued fear conditioned suppression analysis, as described above, and a total of ten animals utilized for contextual fear conditioned suppression. More experimental design adjustments will be required to accurately address any phenomena involved in the high levels of baseline freezing seen throughout cued fear conditioned suppression in this study. This may include but is not limited to experimenting with varying the length ISIs, ITIs, volume, pitch, duration and quality of the conditioned stimulus tone, and adding or adjusting context presentation within the cued conditioned suppression design. Due to the current cued design having a relatively loud and aggressive tone, which could theoretically be inducing a premature startle response in these animals after fear conditioning, the conditioned stimulus should be reevaluated and its specifics (ex: pitch, rise and fall, volume, duration) mirrored after similar experiments used in other conditioned suppression paradigms in the fear conditioning literature. These multiple variables worth investigating have proved to be more time consuming

than the duration of a one-year project; however, they are imperative in addressing effects of these lesions in cued fear memory. Additionally, given that this baseline freezing and fearful behavior have been seen in preliminary data from another, non-conditioned suppression paradigm and a previous conditioned suppression paradigm study that took place in the lab, it is clear that more time and funding is necessary to elucidate any true phenomena behind high levels of baseline freezing after fear conditioning in the cued fear conditioned suppression paradigm.

Additionally, a third study would be necessary to investigate the effects of ACC lesion and sham animals in cued and contextual fear conditioned suppression. The study design above should be used, but there should be a significantly larger time interval between when the animal is fear conditioned and tested for cued or contextual fear conditioned suppression to truly evaluate the salience of the fear memory between lesion and sham animals. The basal forebrain cholinergic toxin that was used in this study, 192 IgG-saporin, reaches its maximal effect at fourteen days post-infusion. Research has shown that lesion effects with this specific toxin last approximately sixty days, so the timing and execution of this follow-up study may prove to be challenging.

Additional experiments to investigate the mechanism behind any functional connectivity-related changes should be conducted as well. To ensure that the discrepancies in performance in fear conditioned suppression are due to changes in functional connectivity involving an operant task, it is necessary to demonstrate that fear memory in the conditioned suppression paradigm doesn't require cholinergic input to the ACC if the operant training does not occur before the fear memory. Ideally, the amount of conditioned suppression will not change between sham and lesion animals if fear conditioning occurs before the learning of the operant task,

which would imply that either a circuit-level interaction or some type of change in functional connectivity occurs due to the presence of an operant task. This could be conducted by initially lesioning animals, then fear conditioned to Context B, then trained in Context A and further contextually tested for discrepancies between lesion and sham animals. In another future study, instead of a cholinergic lesion, two bilateral guide cannulas should be surgically placed and varying dosages of infusions of the muscarinic antagonist, scopolamine, or nicotinic antagonist, mecamylamine, should be infused just prior to fear conditioning. These antagonists should ideally interfere with the animal's ability to retain a fear memory that is specific to fear-conditioned suppression. If this is shown to be true, this may lead us to believe these receptor agonists will activate these receptors in the ACC, which could enhance or exacerbate fear memory. The muscarinic agonist, carbachol, and the nicotinic agonist, nicotine could be used in varying dosages to observe various effects on fear memory within the conditioned suppression paradigm.

It should also be recognized that the ACC in the mammalian brain is critical for many other phenomena related to learning and memory, pain and cognitive processes outside of the cholinergic system. This region has multiple different kinds of fibers that terminate from many different types of projections, which allows for a much more complex picture of the fear circuit than we may account for. While the literature has yet to understand the exact functional circuitry behind each of these different types of projections, the variety in the types and quantity of these projections seems to imply that the ACC has a much more pronounced role in fear memory than previously considered. It should be noted that this study attempts to address only a small avenue

to unraveling the neural substrates that are involved in fear memory in one of four predominant fear conditioning paradigms used in fear literature.

4.2 Broader Implications

While the duration of this study has been short in length, the preliminary findings seem to raise more questions than they answer. Based on the data outlined previously in addition to previous cholinergic fear circuit studies, it seems that differences in circuitry between paradigms do exist. This could suggest any one of three things. First, fear conditioning paradigms may have fundamentally different circuitry and cannot be superimposed to reference circuitry deduced from other behavioral fear conditioning paradigms, like Pavlovian conditioned freezing or fear potentiated startle. This would have drastic effects on fear circuitry literature, because this would suggest that alternative results might arise from conclusions involving circuitry drawn from Pavlovian fear conditioning experiments. These findings would suggest that these experiments must be replicable in alternative fear conditioning paradigms or must be studied only within the confines of the specific paradigm in order to deduce valid circuitry. Secondly, a severe limitation of this study is rooted in data that could be collected by using the same training and lesion protocol, but in the absence of any cued or contextual fear conditioning. Although it may seem as if this study has controlled for any ACC lesion-based effects on a bar pressing task by retraining of animals after surgery, yet before fear conditioning, in order to be thorough, more animals should be tested by initially lesioning basal forebrain projections to the ACC, and then testing for behavior discrepancies in learning and memory or task acquisition between sham and lesion animals.

Thirdly, differences in circuitry could suggest that one common fear circuit exists between paradigms, but that basal forebrain cholinergic projections may undergo changes in functional connectivity to incorporate new areas of the brain to the fear circuit based on previous experience (say, an operant task involved in conditioned suppression). The mechanism behind this notion of changing functional connectivity remains unknown; however, it is plausible to guess that long term potentiation (LTP) and long term depression (LTD) could be involved within AMPA or NDMA receptors on cholinergic projections. The study previously outlined involving muscarinic and nicotinic agonism and antagonism attempts to address this.

These directions will further support the rationale behind this thesis project; there are noticeable behavioral differences between fear conditioning paradigms that are commonly used in fear memory literature. Discrepancies between paradigms may allow for broadening our understanding of fear memory and in turn, reevaluate how we study anxiety and fear related disorders, like posttraumatic stress disorder (PTSD) or specific phobia in animal and potentially human models. A greater understanding of neural circuitry behind fear memory could point future studies or psychological and pharmacological treatments of these disorders in a novel direction. These directions could include investigating circuitry and behavioral-related risk factors that predispose individuals to become more susceptible to any one of many crippling mental illnesses. Additionally, many cholinergic drugs are used currently in medicine, which provides an opportunity to change how many fear-related mental disorders may be approached from a clinical standpoint. Continued research on dissecting functional differences in neural circuitry are required for more effective treatment and understanding of excessive fear related disorders.

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Appendix A

APPROVAL FOR THE USE OF ANIMAL SUBJECTS

UNIVERSITY OF DELAWARE | Office of Laboratory Animal Medicine

Life Science Research Facility
79 E. Delaware Avenue
Newark, DE 19711
Phone: 302-831-2616
Fax: 302-831-0154

To: Office of Graduate and Professional Education

From: Gwen Talham, DVM,

Subject: IACUC approval for Caroline Lawless

Date: 4/17/2017

Caroline Lawless was approved by the IACUC to work with animals on Dayan Knox's protocol #1304 "Role of basal forebrain cholinergic neurons in fear and extinction memory", protocol #1293 "Expansion of the fear circuit in the rat brain", and protocol #1264 "Identifying a novel psychological process that can be targeted to treat excessive fear in PTSD in male and female rats". Please contact me at 831-2980 at gtalham@udel.edu with any additional questions.

Sincerely,

Gwen Talham, DVM, MS, DACLAM

Director, Animal Care Program