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Role of Recent and Remote Context Exposures on Incubation of Fear Memories

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Abstract

Studying learning and memory through the methods of Pavlovian fear conditioning has been a topic of behavioral neuroscience research for decades. Anxiety disorders such as post-traumatic stress disorder can be modeled in rodents through fear conditioning. This study took on a different approach to study the learning and recall of a fear memory by using a within subjects group. This group was tested at both a recent and remote interval. The recent timepoint was three days after conditioning and the remote timepoint was thirty-one days after conditioning. The timepoints are used to observe the effect the passage of time has on a fear memory, and multiple tests are used to model exposures someone with PTSD might experience. The time spent between acquisition and testing and the number of tests did have an effect on the percent freezing of each group. Remotely tested animals had higher freezing levels than the recently tested group. The group tested twice had some animals increasing in freezing and others decreasing in freezing at the later test point. To investigate the neural correlates of these differences c-Fos was stained for in the Basolateral amygdala complex to signify neuronal activation, and GAD-65 for neuronal inhibition.

Introduction

Approximately 25% of individuals diagnosed with post-traumatic stress disorder (PTSD) will not display symptoms for at least six months following a traumatic event; a qualifier for delayed onset PTSD (DSM V, 2013). In a specific case during the aftermath of the Lebanon War, a small percent of veterans took years to develop PTSD symptoms (Soloman et al., 1989). While many studies have been done on PTSD, few have attempted to understand the behavioral and neural mechanisms involved in delayed onset PTSD (Carty, 2006).

In humans and rodents it has been well demonstrated that after an association between a conditional stimulus (CS) and unconditional stimulus (US), presenting the CS without the US can decrease the response (Bouton, 2004; Inda et al, 2011; Pavlov, 1927). This phenomenon is known as extinction and patients with PTSD do not extinguish their fear successfully even after being presented with the CS multiple times. Another area of interest when studying extinction is incubation, where over time the conditional response (CR) elicited from the CS increases (Tsuda et al., 2015). One of the first studies involving incubation in rats was McAllister and McAllister (1967), which showed over the span of hours, an increase in freezing to the CS. Most incubation studies only focus on the first 24 hours after the subject receives the footshock, and those that have tested across weeks have shown various results about whether incubation occurs across remote time points (Poulos et al., *submitted*; Pickens et al., 2013; Gale et al,. 2004).

Extinction is not forgetting the association between the US and CS, but a competition between the associated memory being expressed and new inhibitory memory being expressed (Bouton, 2006). The associated memory can be inhibited to allow the extinction memory to come forward. There are different proposed mechanisms and structures involved in the inhibition of a fear memory (Gisutino & Maren, 2015). One such mechanism is the inhibitory

neurotransmitter γ-aminobutyric acid (GABA) released by intercalated neurons within the basolateral amygdala complex (BLA) (Rea et al., 2009). Because GABA is important for the normal functioning of the brain it is always present and it is difficult to see significant changes in GABA levels using florescent immunohistochemistry (Wang et al., 2014). That is why glutamic acid decarboxylase (GAD) can be stained to determine changes in GABA. GAD is an enzyme that synthesizes GABA throughout the brain (Wang et al., 2014). There are two isoforms of GAD in the adult rodent brain, GAD-65 and GAD-67. GAD-65 is an isoform of GAD that synthesizes GABA in response to an event. GAD-67 is required for the metabolic synthesis of GABA (Wang et al., 2014).

The importance of these two types of GAD have been shown using knock-out mice, where either the gene encodes for GAD-65 or GAD-67 is deleted from the mouse. While GAD-67 knockout mice do survive long enough to be born, they die of seizures. (Wang et al., 2014; Muller et al., 2015). GAD-65 knockout mice alternatively do not die from seizures, but do have behavioral deficits in extinction of fear memories. There is specifically a decrease in GABA in the amygdala, suggesting the importance of reactionary GAD-65 producing GABA in an area important in the expression of fear (Muller et al., 2015). Unlike wildtype mice that begin to extinguish a fear memory after a set amount of trials, the GAD-65 knockout mice do not show extinction behavior. Their levels of freezing remain stable across trials whereas freezing in wildtype mice drops dramatically (Muller et al., 2015; Sangha et al., 2009). The GAD-65 knockout mice do seem to mirror the behavior seen in PTSD patients, where their fear does not diminish over multiple trials. In light of these findings, one would be able to assume the importance of GAD-65 in the amygdala during extinction.

The amygdala, specifically the basolateral amygdala (BLA) is important in the acquisition and expression of fear memory during fear conditioning (Fanselow, 1994; Poulos et al., 2009). Compared with acquisition during fear conditioning, extinction of fear memories leads to higher amounts of c-Fos positive cells in the amygdala (Jiao et al., 2015). c-Fos is an immediate early gene used to assess the neural activation in a given brain structure stained. The BLA also contains many GABAergic neurons that may be activated under certain instances (Jiao et al., 2015). Together, these studies demonstrate the importance of studying the BLA in all aspects of fear conditioning.

While many studies have examined the differences in responses to a recent and remote test following fear conditioning, few have examined the effect of testing the same animals at a recent and remote interval. Here, we used two main groups of animals: a within subjects group (WIS) that was tested at recent and remote intervals that were either previously shocked or not. To further understand the neural underpinnings associated with any effects caused by the multiple tests, GAD-65 immunoreactivity and c-Fos positive cells in the BLA were quantified using florescent immunohistochemistry. We hypothesized that the intensity of fear memories over multiple context exposures is associated with the amount of GAD-65 and c-Fos positive cells in the BLA.

Materials and Methods

Subjects

16 male, 60 day C57/BL6 mice were obtained from Taconic Farms (Albany, NY). Mice were housed four to a cage on a 12:12 hour light: dark cycle with food and water available *ad libitum.* All experimental procedures complied with the National Institute of Health Guide of the

Care and Use of Laboratory Animals and were approved by the University at Albany Institutional Animal Care and Use committee.

Handling

After arriving at the facility, mice were housed 4 to a cage with wood-shavings as bedding. Two days later, the mice were habituated to transport to the testing site and being handled by experimenters. Each animal was taken to the room adjacent to where they were going to be tested and placed in a beaker for 5 minutes on three consecutive days.

Behavioral Procedures

Apparatus

The fear conditioning boxes (Med Associates Inc.) measured 30 x 24 x 21 cm and consisted of two stainless steel side walls, a plastic rear wall and roof, and a hinged Plexiglas door. The floor was made up of 30 stainless steel rods (2mm diameter) spaced 1 cm apart. Boxes were within a sound attenuating cabinet that included a white light, an infrared light, and a camera mounted on the cabinet doors. Before and after testing, boxes were wiped down with a 50% ethanol solution and a drop pan sprayed with a solution of 50% Simple Green® cleaning fluid was placed inside. Footshocks were delivered through the grid floors.

Acquisition

On day 1 of training, mice were placed in the same room handling took place in 15 minutes prior to conditioning. Then the mice were individually placed within 4 identical conditioning boxes located within a sound-attenuating cabinet. Mice in the shocked group were each delivered 3-footshocks (1 sec, 1 mA, 1 min ISI, 2 min pre-shock interval [PSI]), and were

in the conditioning chamber for a total of 7 minutes. The no shock animals were placed in the conditioning box for 7 minutes.

Testing

Testing consisted of placing the animals back into the conditioning chamber for 4 minutes while measuring how much of that time was spent freezing by the animals. The first testing session took place 3 days after acquisition included the WIS shock and WIS no shock groups. An outline of the behavioral procedures is displayed in figure 1.

31 days after acquisition both of the WIS groups underwent the second testing trial which was identical to the first. During acquisition and testing freezing was measured using freezing analysis software (Med Associates Inc.).

Fluorescent Immunohistochemistry

Ninety minutes after completing testing, mice were administered an overdose of sodium pentobarbital (i.p.). They were then transcardially perfused using 0.1M potassium phosphate buffered saline (KPBS) followed by 4% paraformaldehyde (PFA) to fix the brain. Once the brains were removed, they were placed in 4% PFA for 24 hours and then placed in a 30% sucrose-70% KPBS solution for 76 hours at 4º C.

After 76 hours, brains were set in agarose and sectioned using a moving vibratome in KPBS. Slices were stored in cryoprotectant (50% KPBS, 30% ethylene glycol, 20% glycerol) within well-plates at -20º C until ready to be stained. The tissue was rinsed in KPBS and then incubated in a 0.3% H_2O_2 solution with KPBS. After rinsing again the tissue was put into a solution of 5% normal goat serum and 0.3% Triton-X detergent to block non-specific antibody binding sites. The tissue was incubated for 24 hours at 4º C in a 1:1000 antibody ratio for both

GAD-65 and c-Fos. After the incubation, the tissue was rinsed in a solution of 2% normal goat serum, 0.1% Triton-X, in KPBS. The tissue is then incubated in the secondary anti-bodies of c-Fos and GAD-65 for 3 hours at a ratio of 1:200. Once the 3 hours has passed, the tissue is rinsed with KPBS and incubated for 50 minutes in a Nissl body stain at a ratio of 1:200. The tissue was then rinsed with KPBS. Once the tissue was mounted onto slides and cover-slipped with a glycerol solution, they were scanned with a fluorescent microscope (Olympus) at a magnification of 10X.

Quantification

c-Fos

Upon analysis of scanned images, c-Fos positive cells were hand-counted in the BLA using a brain atlas (Dong, 2008). The tissue ranged from plate level 63-78, and the area of focus was the BLA. To confirm c-Fos positive cells were not an artifact on the slide or microscope, the Nissl stain was used to double label cell bodies. Once each piece of tissue was counted, the total amount of c-Fos positive cells was determined for each animal. These totals were then averaged together to obtain the average number of c-Fos cells per group.

GAD-65

GAD-65 is not contained within the cell body like c-Fos or Nissl, it is in the cytosolic vesicles of the presynaptic terminals. In order to analyze the GAD-65 for the purpose of the study, investigation into other tools of analysis was needed. Using the cropping tool on the ImageJ imaging software (Rasband, 2015) I was able to select the BLA. Once cropped out of the original image, the color threshold tool was used to completely saturate all pixels present inside the BLA. The measure tool was used to count the amount of pixels selected by the threshold

tool, which provided the total amount of pixels that make up the BLA for that piece of tissue. The hue, saturation, and brightness were modified to best match the color of the GAD-65 in the tissue and only those pixels were selected and counted. This new amount of pixels were taken as a percent of the total amount of pixels in the BLA. The mean percent brightness of GAD-65 in the BLA was found for each animal, then similar to c-Fos, the animals were averaged together to obtain the group average for GAD-65.

Statistical Analysis

To analyze freezing data, a one-way between subjects ANOVA was performed using SPSS (IBM) to compare the effect of the shock. The WIS group had a repeated measures ANOVA performed to identify any difference between the recent and remote tests. Also, the WIS group was divided into an extinction (EXT) or incubation (INC) based on the percent freezing of the remote test.

The average number of c-Fos positive cells for each group were compared using a one-way between subjects ANOVA for shock and no shock groups. An ANOVA was also performed between the INC and EXT groups for average c-Fos. GAD-65 averages for each group were compared using a one-way between subjects ANOVA for shock and no shock. The EXT and INC groups were also compared.

Results

Behavioral Data

A one-way between subjects ANOVA was performed using SPSS to determine the effect of shock on the freezing of the 3 shock WIS group compared to the no shock WIS group at both the recent and remote intervals. The shocked group froze significantly more than the no shock

group at both the recent and remote time points ($P<0.05$). A repeated measures ANOVA with the WIS shocked group was done for the recent and remote tests. There was no significant difference between the recent and remote test for the shocked group, it appeared their freezing stayed stable across both time points (Figure 2).

Upon further analyzing the average freezing at the remote interval for the shocked mice, there was high variability within the group. After performing a median split for whether the freezing increased or decreased for each animal at the remote interval, they were placed into an incubation (INC) (n=4) or extinction (EXT) (n=4) group. The INC group all had higher levels of freezing at the remote interval versus their recent test. The EXT group all had lower levels of freezing at the remote time point. A one-way between subjects ANOVA was used to analyze the difference between the INC and EXT groups' freezing at both the recent and remote tests (Figure 3). There was no significant difference between the two groups at the recent test. At the remote test however, the INC group froze significantly more than the EXT group ($P=0.015$). This unexpected phenomenon in the shocked group needs to be explored further with more animals.

GAD-65 and c-FOS

A one-way between-subjects ANOVA was performed for GAD-65 to examine any effect shock has on GAD-65 levels. There was no significant difference between the shock and no shock groups in amounts of GAD-65 in the BLA (Figure 5). Similarly when comparing the INC and EXT groups for levels of GAD-65, there is no significant difference. An ANOVA was also performed to examine the effect shock has on the amount of c-Fos positive cells in the BLA. There was no significant difference between the shock and no shock groups (Figure 4), but data showed the difference in c-Fos positive cells was nearing significance ($P=0.052$).

Discussion

This study measured the effects of multiple tests at a recent and remote time point on context fear conditioned mice. Previous studies examining conditioning over time have shown a variety of results. Some studies show an increase in the conditional response (McAllister & McAllister, 1967; Poulos et al., submitted), while others show a decrease or no change in the behavior (Gale et al., 2004). After showing similar levels in fear after the first test 3 days following acquisition, the mice displayed two distinct patterns of freezing; those that increased freezing and those that decreased freezing. In the current study animals that showed decreased freezing at the remote time point could have lower freezing at the recent time point, and vice versa for the higher freezing animals. After interpreting the freezing data from the recent test and comparing the individual animals in the shocked group, there was no significant difference. The difference only occurred after the 31 days had passed suggesting that animals either showed incubation or extinction during this time.

Animals undergoing extinction are usually put back into the context of acquisition for extended amounts of time over multiple trials (Chang et al., 2009). Alternatively, placing the animal back into the context 1 day after acquisition for a brief amount of time leads to an increase in response (Inda et al., 2011; Nader et al., 2000). This study used a 4 minute test 3 days after acquisition that may fall somewhere in the middle of being able to increase or decrease the fear response. To further investigate this question, animals can be put through a longer test at 3 and 31 days to determine if a longer test would lead to more extinction-like behavior.

There was no significant difference between groups for the amount of c-Fos positive cells in the BLA. In past research more c-Fos activation has been found in the BLA of a fear conditioned mouse when compared to a homecage mouse (Hellsten et al., 2015). One possibility

is that the florescent immunohistochemistry was not able to stain all cells correctly. Shortly after this experiment, the incubation times for the secondary antibodies were lengthened from 3 hours to 24 hours. Considering how close to significant the c-Fos levels were for the shocked versus no shock groups, it would be valuable to scan more tissue using the new methods and run more animals under the same conditions to fully explore the possibility of changes in c-Fos activation in the BLA.

There was also no significant difference in the levels of GAD-65 in the BLA between any of the groups. Although it is generally thought GAD-65 is used to make GABA in the moment, it has also been found to synthesize GABA more metabolically similar to GAD-67 (Muller et al., 2015). In the present study, all animals were sacrificed 90 minutes after testing, which is ideal for gathering c-Fos data; 90 minutes might not be when GAD-65 expression is highest. A study looking at GAD-65 at various time points after testing would be beneficial in understanding if there is a change in GAD-65. One more possibility is that the method used to measure the brightness of GAD-65 might be at fault. More tests need to be performed to test its reliability in determining the amount of GAD-65 present in the BLA.

Along with staining for GAD-65, staining for an antibody to parvalbumin (PV) would be a beneficial choice. PV is a protein that is expressed to regulate GABAergic neurons (Wang et al., 2014). In a study comparing GAD-65 and GAD-67, parvalbumin was co-localized 100% of the time with GAD-65 and 97% of the time with GAD-67 (Wang et al., 2014). Also, parvalbumin staining is static unlike GAD-65. In considering parvalbumin's presence inside the cell, it would be reasonable to quantify c-Fos expression within these neurons to study its role in inhibition.

Another area that would be interesting to study would be the dorsal hippocampus. The dorsal hippocampus has been shown to be involved in spatial information and is essential for contextual fear conditioning (Moustafa et al., 2013; Phillips and LeDoux, 1992). It also appears that the hippocampus is involved in recent memory retrieval and its role in retrieval may change after 50 days (Anagnostaras et al., 1999). The dorsal hippocampus has been shown to have strong connections to the amygdala during fear conditioning (McDonald and White, 1993; Izquierdo and Medina, 1997). Looking at c-Fos activation in the hippocampus would be constructive in adding to the structural knowledge involved in the behavioral paradigm presented here.

The infralimbic area of the prefrontal cortex (IL-PFC) has also been shown to be involved in expression fear memories (Giustino & Maren, 2015). It is an area that sends projection to the amygdala and plays an important role in the inhibition of fear memories (Quirk & Mueller, 2008). Because of its role in extinction, there is the possibility of changes in GAD-65 in the IL-PFC in animals that show incubation or extinction behavior.

This study's first purpose was to observe the effects of multiple tests at recent and remote time points for a shocked group of animals. To our surprise the mice within this group did not perform the same, with patterns of increasing or decreasing in freezing. This study will be replicated with a new group of mice, while also looking at other areas of the brain involved in the retrieval of a fear memory.

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Figure Legends

Figure 1. On day 1 animals were either shocked or placed into the context for 7 minutes. 3 days later, animals were put back into the context for 4 minutes. 28 days after the first test (31 days after acquisition) animals were tested again for 4 minutes.

Figure 2. Time spent freezing at recent and remote intervals for the shock and no shock group. At both the recent remote interval the shock group froze significantly more (*) than the no shock group. There is no significant difference between the shock animals at the recent and remote tests.

Figure 3. Shock animals were put into the extinguish (EXT) or incubate (INC) group and freezing at the recent and remote tests were analyzed. At the recent test, there was no difference between the EXT and INC groups. At the remote test INC animals froze significantly (*) more than EXT animals, suggesting a change in perception of fear over time.

Figure 4. After performing an immunohistochemistry on the selected tissue, c-Fos positive cells were counted in the BLA. The amount of c-Fos in the shocked group approached significance to be higher than the no shock group.

Figure 5. After performing an immunohistochemistry on the selected tissue, GAD-65 was quantified using the methods mentioned above. There was no significant difference between the two groups for GAD-65 present in the BLA.

Figure 1

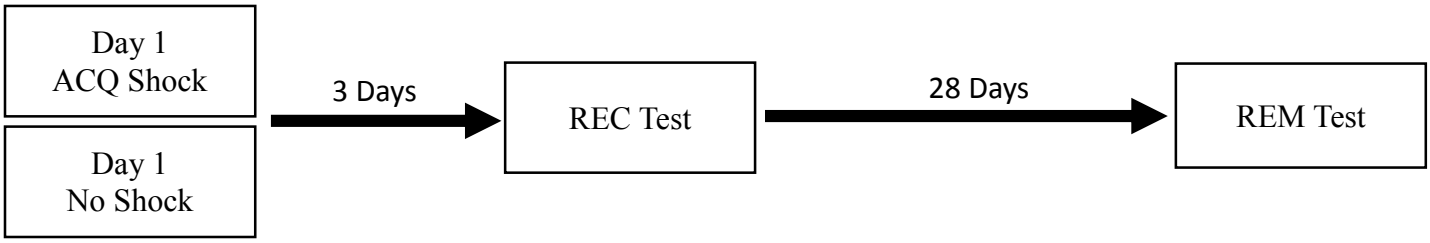
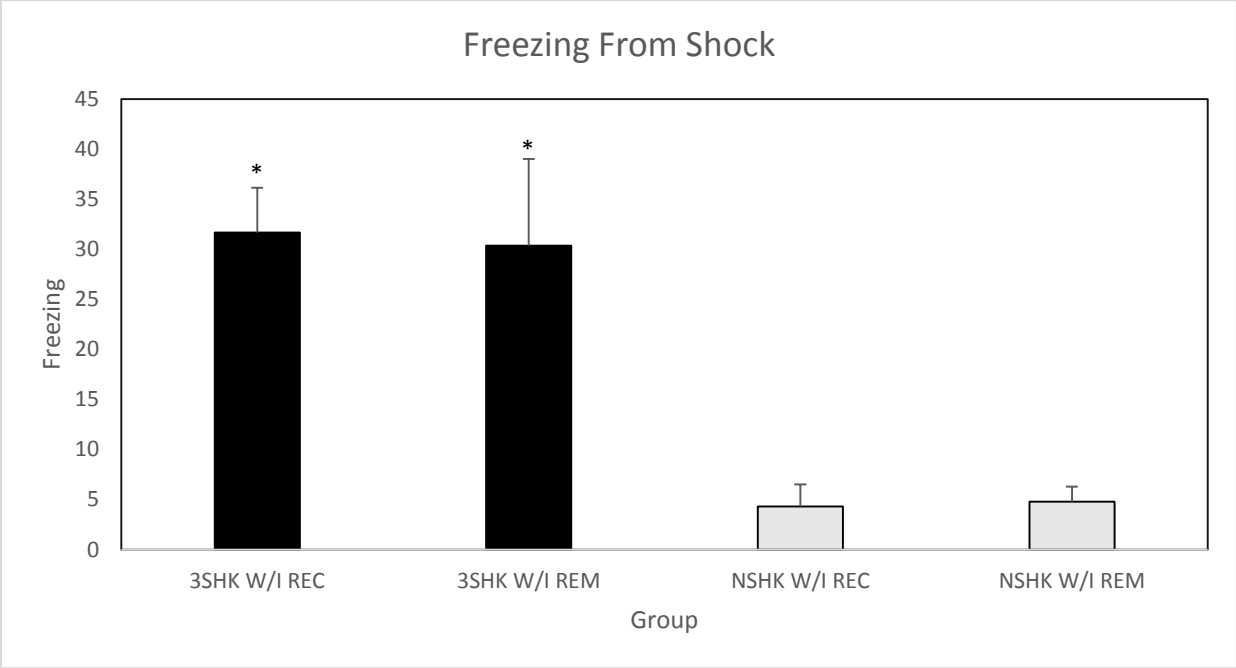


Figure 2


Freezing From Shock
45
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3SHK W/I REC
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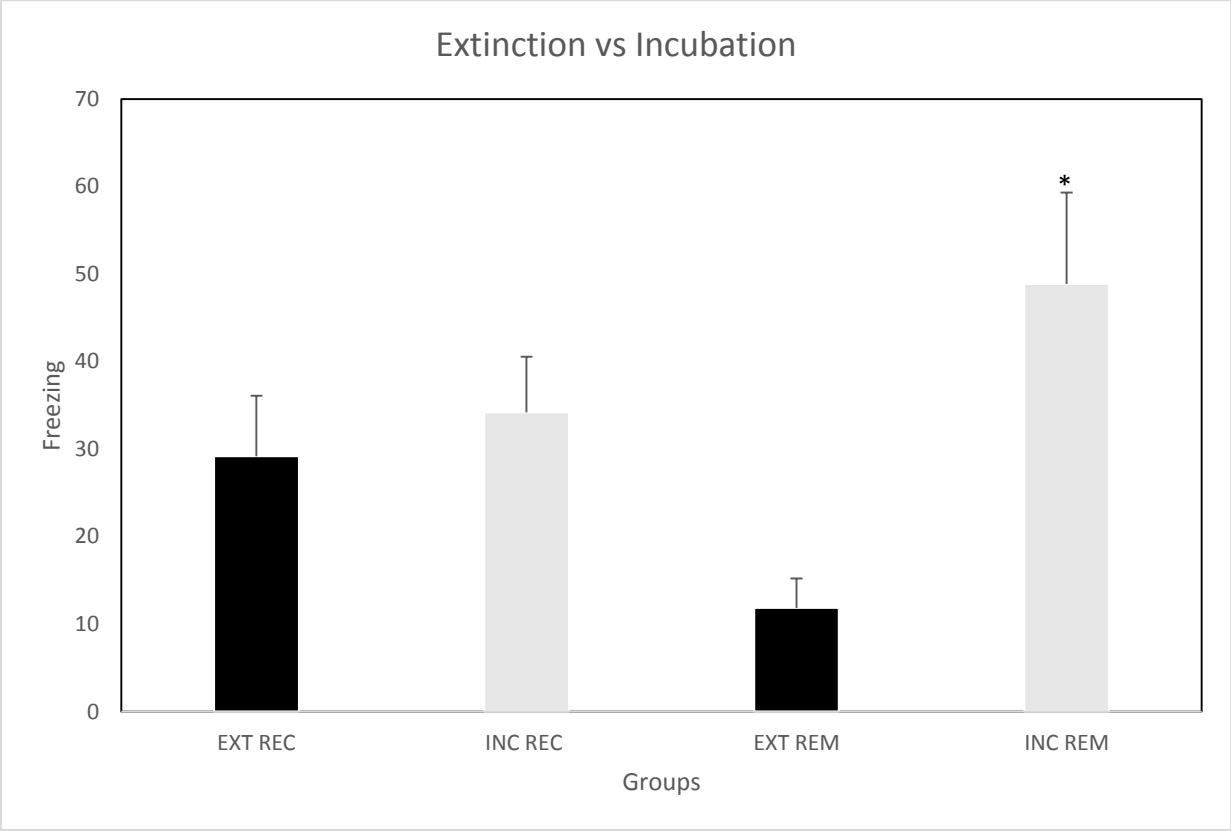
NSHK W/I REC
NSHK W/I REM
Group

Figure 3


Extinction vs Incubation
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Freezing
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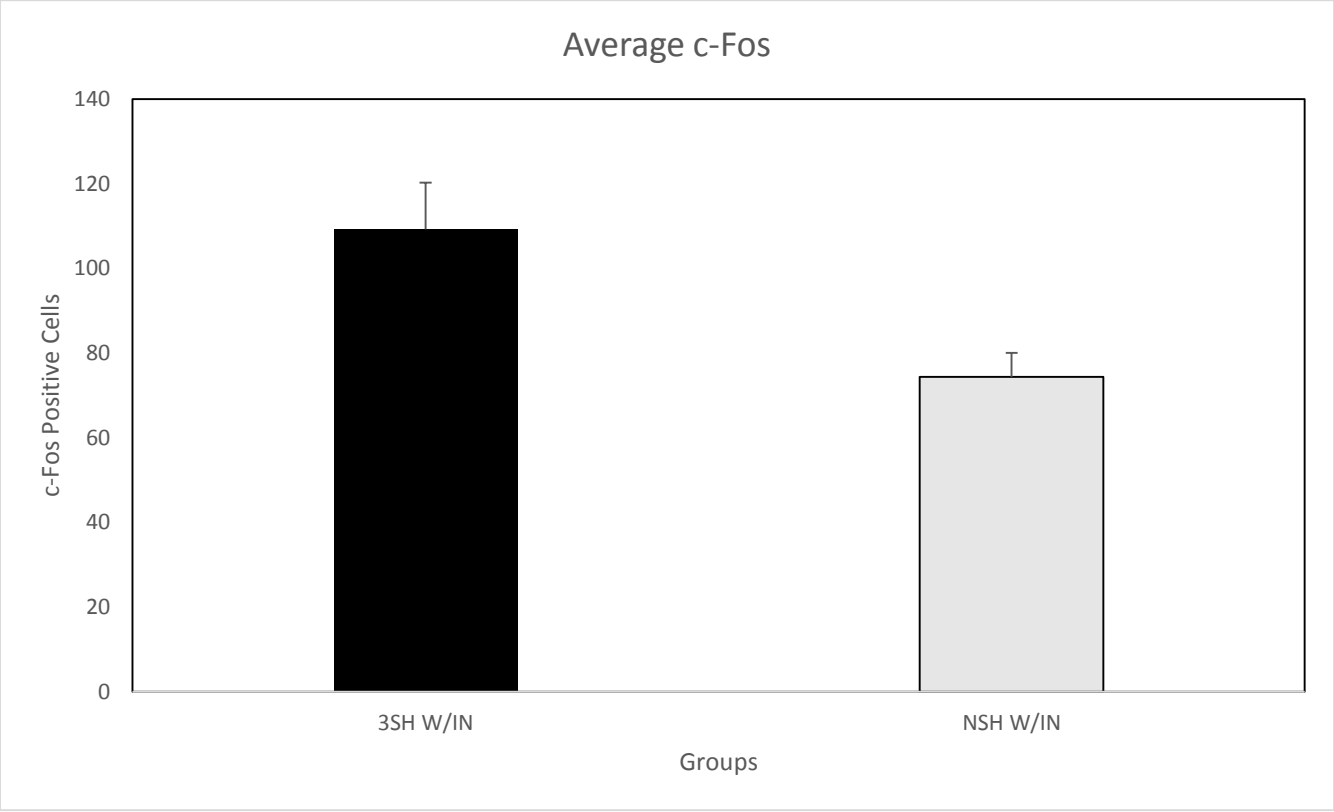
EXT REC
INC REC
EXT REM
INC REM
Groups

Figure 4


Average c-Fos
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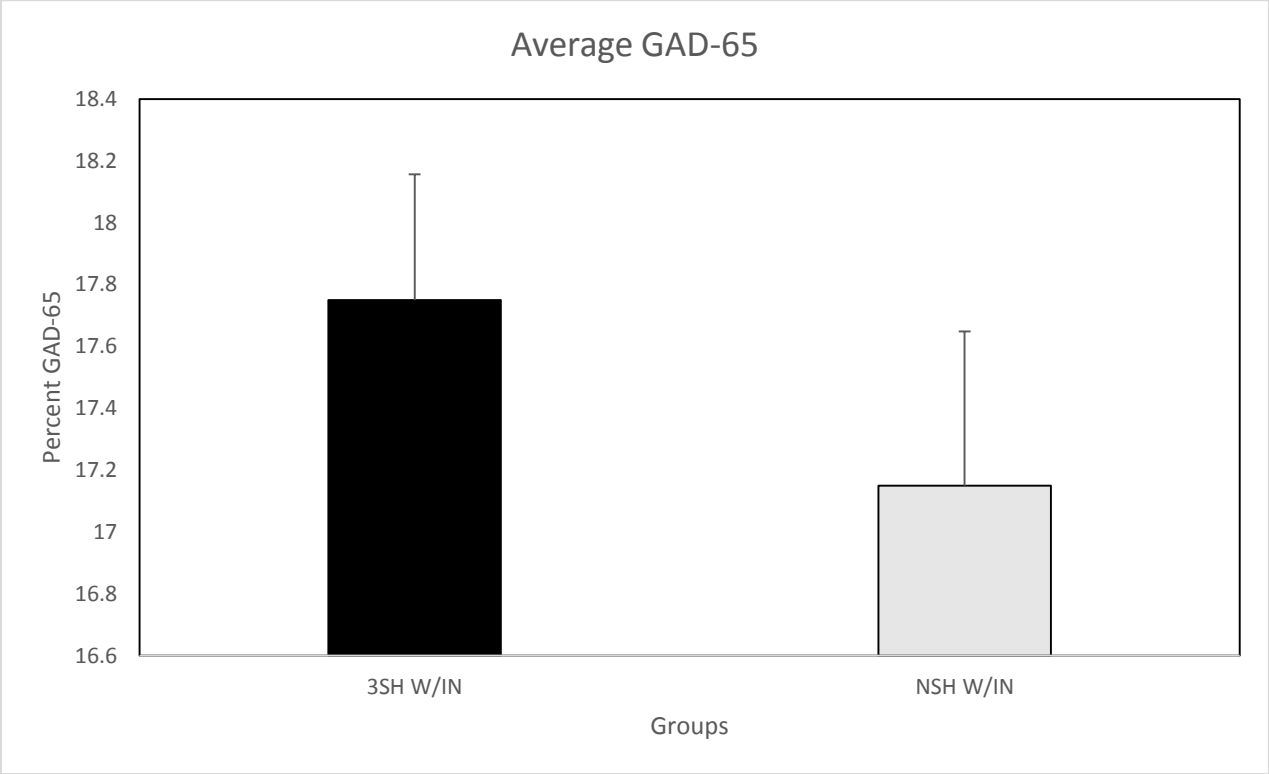
100
80
60
40
20
0
c-Fos Positive Cells
3SH W/IN
NSH W/IN
Groups

Figure 5


Average GAD-65
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Percent GAD-65
3SH W/IN
NSH W/IN
Groups