EFFECT OF STRESS ON ANIMAL MODELS OF ANXIETY
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ABSTRACT

In this study, the effect of stress exposure on phasic fear and immediate and prolonged anxiety was examined in rats. To test the equipment at hand and confirm basic neural processes, the acoustic startle response (ASR) and prepulse inhibition (PPR) tests were conducted. To study and analyze the effect of stress on anxiety, the rats underwent two tests, light enhanced startle (LES) and the zero maze test. The LES test is linked to sustained fear, or anxiety, which is a prolonged state of apprehension caused by a nonspecific, distant threat. The zero maze test served as a measure of anxiety for the rats as well. The rats underwent the tests before and after a stressor was presented to determine if the stressor increased anxiety-like responses. Fear potentiated startle was also explored in rats to model phasic fear. The purpose of the training session was to condition the subjects so that they paired a neutral stimulus with an aversive shock. Understanding the rats’ responses to these tests and linking the results back to the neural pathways under investigation could provide insight into human psychiatric and neurological diseases, specifically anxiety disorders.

INTRODUCTION

The presence of anxiety disorders has important implications on both mental and physical health. Anxiety is a natural emotion that plays an important part in the fight-or-flight response. However, a functionally damaging level of anxiety (characteristic of anxiety disorders) negatively affects daily living through hallucinations and re-experience of trauma. In addition, anxiety disorders have been associated with an increased presence of somatoform disorders, which are characterized by symptoms such as pain, nausea, weakness, and dizziness, as well as chronic physical disorders such as heart disease and respiratory disorders. Anxiety disorder is the most common mental illness in the United States and studies show that one in three people will suffer from anxiety. It is clear that anxiety is not only an individual issue but also an issue of public health (1). Therefore, it is important that researchers study anxiety and the anatomical mechanisms behind it. However, many tests for stress and anxiety require individuals to be distressed or experience psychological discomfort, thus making testing on humans unlawful. Animal models are a standardized alternative to this dilemma. Because rats and humans have relatively similar neural pathways, using rats in such studies can lead to a better understanding of human anxiety and fear.

Stress has been implicated as a strong environmental risk factor for the development of anxiety (2). Exposure to stress may cause changes along the neurological axis that may be phenotypically expressed as heightened anxiety. Stress triggers hyperactivation of the hypothalamic pituitary adrenal axis, resulting in an increased release of corticosteroids (3). The binding of corticosteroids to receptors may result in degeneration of dendrites, inhibition of
neurogenesis, and degradation of neuroplasticity, which is physically expressed as increased anxiety-like behavior in animals (4).

The objective of this study is to examine the effect of stress exposure on different animal models of anxiety. This study also examined individual differences in anxiety levels between rats. The restraint groups were exposed to one hour sessions of restraint stress. Phasic fear, tested with FPS, and sustained fear, tested with the zero-maze and LES, were measured after stress exposure. These results were compared to a control group that was tested similarly on the same days as the experimental group.

**Neuroanatomical Circuits for Phasic vs. Sustained Fear**

In both humans and rats, the amygdala of the brain plays a key role in fear responses and anxiety. These nuclei, located within the temporal lobes, function as the integrating center for cortical processing and pathways pertaining to learning, specifically fear conditioning. Neuroscientists categorize fear learning or anxiety into two classes: phasic fear and sustained fear.

**Acoustic Startle Response**

In this study, acoustic startle response was used to model phasic and sustained fear in rats. Startle response is a defensive response to sudden or threatening stimuli that has been observed in mammals using a variety of tests. The acoustic startle response (ASR) is often seen when mammals are presented with auditory stimuli over 80 decibels (6). In mammals, acoustic startle response is mediated by a neuronal circuit called the primary acoustic pathway. The primary acoustic pathway is located in the ponto-medullary brainstem and is near the primary auditory pathway. It consists of a small number of neurons connected by chemical synapses. The main components of the primary acoustic pathway are the auditory nerve, the ventral cochlear nucleus, the dorsal nucleus of the lateral lemniscus, the caudal pontine reticular nucleus (PnC), spinal interneurons and spinal motor neurons. During the primary acoustic pathway, the dorsal cochlear nucleus, ventral cochlear nucleus, or the cochlear root nucleus (which are all part of the central auditory pathway) first receive an acoustic startle stimulus from the auditory nerve. Reticulospinal neurons of the PnC then receive acoustic input from these nuclei or the lateral superior olive. The PnC then activates motor neurons which results in an acoustic startle response. The PnC contains large neurons that project to the spinal cord and to cranial and facial motor nuclei, which is why auditory stimuli result in whole body acoustic startle responses. Studies have shown that the fastest route of transmission of acoustic input into motor output occurs when the ASR is mediated by a serial trisynaptic pathway that consists of the cochlear root nucleus, the PnC, and motor neurons. However, other less direct pathways that convey excitatory auditory input to the PnC via reticular relay nuclei or interneurons in the spinal cord can also affect ASR. Although it is clear that the PnC plays a very important role in ASR, it is still not clear which subregion of the PnC mediates the ASR. Studies have shown that glutamate is most likely the excitatory transmitter of auditory input to PnC neurons. Scientists have also found that GABA inhibits ASR because blocking GABA receptors in the PnC enhances ASR (6). In this investigation, acoustic startle response of the rats was measured using a load cell platform that transfers the force of the rats’ startle to a waveform that can be analyzed. Detailed
explanation of this process will be presented in the Methods section. It is predicted that auditory stimuli of higher decibels will induce a greater ASR from the rats.

Prepulse Inhibition

Prepulse Inhibition is a measure of sensory gating, a process of sensory filtering that reduces the organisms’ response to a non-threatening stimulus. This neurological process reduces an organism’s, in this case a rat’s, reaction to a primary startle stimulus. By presenting a weaker acoustic noise that acts as a prestimulus, rats will tend to produce a less intense ASR than when presented solely with the primary startle stimulus of a loud burst of white noise. This weaker acoustic noise can be varied according to decibel intensity or loudness, frequency level, duration of the interstimulus intervals (ISIs), and length of the intra-trial stimuli (ITSs). By using a prestimulus, the amount of sensory information sent to the brain for the actual startle stimulus is limited, thereby allowing the rat’s brain to process the abridged input and produce a lessened, behavioral motor output. The brain’s ability to adjust its sensitivity to sensory input is a desired evolutionary trait that prevents organisms from overloading their finite cortical centers. Brains can only process so much information. By leaving sensory inputs ungated, brains would have to analyze an excessive amount of information, ultimately affecting and delaying the organisms’ motor responses. In humans, complications in sensory gating tend to be associated with symptoms of Schizophrenia, Autism, and Alzheimer’s Disease. Without sensory gating, people with these disorders have difficulty controlling their actions, focusing on everyday life, and remembering events (7).

Stressors

Stressors are crucial in affecting the startle response in rats and to see the effects of stress on anxiety. Exposing rats to a stressor for an extended period of time induces sustained fear (anxiety). Corticotropin-releasing hormone (CRH) is a neurotransmitter and peptide hormone involved in the stress response. There are published experiments testing whether amygdaloid CRHs mediate anxiety-like behaviors, but no conclusive evidence has been found (8). In this experiment, exposing the rats to a stressor differentiates the rats between those affected by phasic fear, and those affected by sustained fear. Notably, there are a variety of ways to expose rats to stress. One way to stress rats is to place them in a closed room with a predator or with predator pheromones. Another stressor is to confine the rats in a closed-tube for an extended period of time. It is important to isolate the rats being stressed because when stressed, rats release pheromones that may affect either the anxiety or hormone levels of others. If the rats were introduced to a stressor, then the same rats would show an increase in sustained fear by having a bigger startle response to the LES.

Light-Enhanced Startle

Light-enhanced startle is the process of exposing rats to bright light in order to increase their overall startle response. Rats often live in burrows and travel during the night. Thus, they are averse to bright light. To carry out light-enhanced startle, scientists first perform acoustic startle response tests on the rats in the dark. They then perform the same tests on the rats in the presence of bright light (1600 lumens). Finally, they perform the tests on the rats in the dark again. Scientists have found that exposing the rats to bright light for five to twenty minutes led to
significant increases in acoustic startle amplitude. Light-enhanced startle was reduced selectively by both benzodiazepine and nonbenzodiazepine anxiolytics. Unlike fear-potentiated startle, light-enhanced startle does not depend on learning and memory processes. It also leads to a state of uncertainty about potential danger for the rat because when the light comes on, the rat does not know when and whether something harmful may happen. Thus, the rat remains in a state of sustained fear which leads to an increase in startle amplitude (5). It is hypothesized that the rats who are stressed will display higher startle responses than the rats who are not stressed.

Zero Maze

The elevated zero maze is an alteration of the elevated plus maze model of anxiety in rats. The design encompasses an elevated circular platform with two opposite enclosed quadrants and two open. This removes any ambiguity in interpretation of time spent on the central square of the “plus” design, and allows clear and consistent exploration. The rats were tested on the zero maze to observe and analyze the amount of time they spent in the open areas versus the closed areas. The results from this test were used to analyze anxiety-like responses in the rats. The time spent in the open areas correlates with a lower anxiety-like response, while the time spent in the closed areas correlates with a higher anxiety-like response. It was hypothesized that after the rats are exposed to the stressor, they would spend more time in the closed areas of the zero maze, exhibiting a higher anxiety-like response.

Fear-potentiated Startle

Fear-potentiated startle is a model of phasic fear and involves Pavlovian conditioning. It has been used to measure conditioned fear. There are two sessions involved with fear-potentiated startle, a training session and a testing session. During the training session, the rats are presented with a neutral or a conditioned stimulus (CS), paired with an aversive or unconditioned stimulus (US). The CS is usually a pure tone and the US is usually a shock. After the training session, the rats begin to associate the US with the CS. During the testing session, the rats are presented with auditory pulses in the presence and absence of the CS. Since the rats now associate the CS with the US, presenting the auditory pulses in the presence of the CS elicits a higher startle response in the rats (5). It is hypothesized that the rats who are stressed will display higher startle responses than the rats who are not stressed.

Individual Differences

The role of individual factors is an important, yet often overlooked, area of behavioral neuroscience. After the data was analyzed to examine the effects of stress on the group of rats, the data for Session 1 was reanalyzed for individual differences. To better understand and analyze the individual differences in rats, the rats’ light enhanced startle responses were compared with their anxiety-like responses in the zero maze. In the zero maze, the percent time in the open arms of the maze was calculated. A higher percentage correlates with a low negative correlation between these two calculations would indicate that the rats that exhibited high anxiety in the zero maze also exhibited the most light enhanced startle. This would be a clear indication that individual factors, varying from rat to rat, contributed to the results for both tests. No correlation could indicate that the neural pathway involved with LES and the pathway involved in the zero maze are not connected. It could also indicate that individual differences
played a minimal role in the results and did not affect the data to a major degree. Understanding of the biological foundation for these individual factors could provide insights into human individual differences in fear.

**Anxiety/Startle Response Overview**

Studying the startle response in the twelve Sprague-Dawley rats through fear potentiated startle, light enhanced startle, prepulse inhibition and zero maze will explore the relationship between fear, anxiety, and stress. Developing an animal model to exhibit how stress enhances anxiety may identify the neural substrates involved in the acoustic startle response. In theory, the results of testing the startle response in twelve rats prior to adding a stressor variable, should be smaller than the startle response in post-stressed rats. The process of adding a stressor is to instill sustained fear in the rats. Similar to the sustained fear in rats, human anxiety disorders are characterized by nervous behavior and the constant expectation of future threat. By studying the pre-stress and post-stress startle response in the rats, different reactions to the light-enhanced startle can be identified. If an individual rat shows an abnormally high startle response in the Light-Enhanced startle, compared to its peers, tracking the startle response in the maze or Post-Stress may show whether some rats are intrinsically more prone to being startled. Studying the correlation of greater startle responses in the maze/LES tests with the respective post-stressor startle responses may also be useful for clinical purposes/applications. Stress is commonly associated with forms of mental illness; mapping out all the neural substrates involved in the startle responses may explain the mechanism behind why some people suffer from anxiety disorders and the relationship between stress and anxiety.

**MATERIALS & METHODS**

**Subjects**

This study was approved by the Institutional Animal Care and Use Committee. Twelve Sprague-Dawley rats (S1-S12) served as subjects (Ss) in this study. S1 was omitted from several of the later tests’ data and was used solely to test the functionality of the equipment. Five individuals--S2, S3, S4, S5 and S10--were in the control group. Six individuals--S6, S7, S8, S9, S11, and S12--were in the experimental group. Each rat was housed in its own clear plastic cage in a climate-controlled room set on a 12-hr light-dark schedule. All tests were conducted in the afternoon, during the light cycle. All Ss had free access to food and water throughout the study. Prior to the onset of testing, the rats were randomly assigned to either the Restraint Group or the Control Group.

**Apparatus**

*Acoustic Startle Response, Prepulse Inhibition, Fear Potentiated Startle and Light Enhanced Startle*

The conditioning chambers were Med Associates sound attenuating enclosures with sound attenuating foam. Within the chamber is the platform on which the rat stood that has a sliding piece to enclose the rat. Also within the chamber is a load cell to measure force,
speakers, a red light, and an amplifier turned to run and set at 2. The speakers produced 75 dB of white noise.

Zero Maze

The zero maze consisted of a large, raised, circular track. The track was designed so that two opposite quadrants were walled on the outer edge, and the other two quadrants were unwalled. The zero maze test was conducted in dim, even lighting.

Restraint Stress

Transparent plexiglass semi-circular cylinders, with radius of 4.445cm and length of 20.32cm, were used to restrain the restraint group Ss. While the cylinders allowed for limited movement, they did not restrict the subjects’ respiration.

Procedure

This study comprised of several tests: Acoustic Startle Response (ASR), Prepulse Inhibition (PPI), Light-Enhanced Startle (LES), Zero maze, and Fear-Potentiated Startle (FPS). The eleven Ss were first exposed to ASR. ASR was repeated again two days later. PPI was tested in all eleven Ss two days after the second session of ASR.

On the first day of the study, the eleven rats were taken individually from their cages and held in place for three-minute period to acclimate them to human contact.

In the second week of this study, the Ss were first pre-tested in LES and the zero maze. Subject groups were randomized to control for the effects of test order. Two days later, the six Ss in the Restraint Group received the experimental treatment: an hour of Restraint Stress. Immediately afterward, all eleven subjects were tested in LES and the zero maze. Three days later, LES and the zero maze were performed again.

Acoustic Startle Response

Ss in cages 1 through 4 were brought into the testing room on a cart. The four Ss were to be tested in four conditioning chambers with one rat in each chamber. Rat 1 was placed in chamber 1 and each of the next three Ss followed the same pattern. To put the Ss in the chambers, each cage was set in front of its respective cage. The Ss were picked up below their shoulders and placed onto a platform within the conditioning chamber. Each rat would only use one chamber for the duration of the experiment. The Ss were given a five-minute acclimation period on the platform before the forty trials began. The Ss listened to noise pulses at 80 dB, 90 dB, 100 dB, 110 dB, and 120 dB with a 15-20s interval between sounds. After the rat heard 120 dB noise, the rat heard the 80 dB noise, beginning the cycle of five tones again. The experiment was configured on the computer prior to the beginning of the 40 trials. At the conclusion of the experiment, the data needed to undergo post-processing. The data was saved and a post-analysis was run and an examination of the peaks was performed to check for errors. The experiment was repeated twice more with Ss 5-8 and then Ss 9-12. Rat 5 and rat 9 was tested in chamber 1 with the other three Ss filling the three chambers respectively. The procedure was repeated two days later, two more times.
Prepulse Inhibition

The prepulse inhibition (PPI) test used the conditioning chamber described in the ASR Apparatus section. Ss 2, 3, and 4 were placed in chambers 2, 3, and 4. After those three Ss completed their testing, they were exchanged for Ss 5-8 and then 9-12. The Ss were placed into their respective chambers and were given a one minute acclimation period with no trials administered. The experimenters used the SOF-826 prepulse inhibition startle pro software set. Each animal was given a number for each test chamber. The testing chambers were described in the ASR protocol. Each rat was placed in the conditioning chamber for 55 trials. Out of the first forty trials, 24 PPI trials were randomly interspersed between the 16 Noise Burst Only trials. The 24 Prepulse trials consisted of 8 trials of 20 kHz at 70 dB, 8 trials of 12 kHz at 70 dB, and 9 trials of 4kHz at 70 db. The Noise Burst trials were at 100 dB intensity level. The next fifteen trials consisted of five trials of three different intensity levels: 70, 80, and 90 dB. The speakers produced 75 dB level of white noise initially. Each trial is separated by a variable ITL with a range of 2-8 seconds between each trial. The speakers produced 75 dB level of white noise (9).

Light-Enhanced Startle

The same procedure that was used for the Acoustic Startle Response test was replicated in Light-Enhanced Startle test, except that each subject underwent three blocks, instead of one block, of 40 trials. Only Ss 2-12 were used in this test. The first block of trials was identical to the ASR test. During the second session, however, the subjects were tested in 1600 lumen bright light. For the third block, the Ss were tested in the absence of the bright light once again.

Zero Maze

After a two minute acclimation period in the testing room, the rat was placed on an open (unwalled) quadrant of the zero maze, facing away from the experimenter. The rat was allowed to explore the maze for a period of five minutes, during which the program AnyMaze recorded the amount of time the rat spent in each section of the maze and the amount of times the rat entered each section. In the event that the rat fell off of the maze, the recording was paused until the rat was returned to the track. Following the five minute period, the rat was returned to its cage and removed from the testing room.

Restraint Stress

After initial testing in LES and zero maze, the Ss of the restraint group were restrained in transparent plexiglass semi-circular cylinders. The Ss were restrained four at a time in separate devices, and each rat was placed into the cylinder for a one hour period.

Fear Potentiated Startle

Conditioning chambers were also utilized for the Fear Potentiated Startle (FPS) test. The FPS test consisted of two sessions: a training session and a testing session. The purpose of the training session was to condition the Subjects so that they associated a neutral stimulus with an
aversive shock. Subjects were given a 5 minute acclimation period before the training session, which consisted of 10 trials. Each trial consisted of a 30 second tone of 80 dB that coterminated with a half second shock of 0.5 mV. There was a four minute onset latency period between each shock.

After 48 hours, the subjects underwent a testing session. The testing session was conducted similarly to the training session. However, the subjects were only exposed to tones without shock.

RESULTS

The initial set of parametric experiments performed tested the acoustic startle response (ASR) of 12 subjects. The rats were exposed to auditory pulses (80-120 dB) in 15-20 second intervals, and their startle amplitudes were recorded. Data were plotted into a line graph and represented as means of repeated trials (n=8). Subjects S5-S8 were excluded due to malfunctions in the equipment. There is a clear positive trend (figure 3a), for as intensity increases, the average startle amplitude also increases. A one-way ANOVA statistical test was performed on the pulse intensity, and showed that the difference in average startle amplitude as a result of increasing intensity was statistically significant, with $F_{3,30} = 35.443, p < .001$. Results ultimately confirmed that not only that variability existed between individual rats, but furthermore that all rats contributed to an increase in startle amplitude with increasing pulse intensity with a peak at 110 dB and plateauing at intensity levels beyond it. Another aspect of the ASR observed was the effect of habituation on the rats over the three-session period. The startle amplitudes across all subjects were recorded and averaged for restraint sessions performed over three consecutive sessions in 24-hour intervals. Data were plotted on a line and represented as means of all rats in respective sessions ±SE (n=3). A direct correlation was observed between pulse intensity and startle amplitude for all three sessions, and a 2-way ANOVA was performed to determine whether or not there was a significant difference between intensity across three sessions. ($F_{4, 84}=56.195, p < 0.001$). Furthermore, startle amplitudes were noticeably higher across all pulse intensities during session 1 than in sessions 2 and 3, suggesting the presence of a long-term habituation effect due to the subjects’ repeated exposure to the stimuli.

The other parametric test was Pre-pulse inhibition. The data collected from this test proved that as prepulse did in fact lower the startle response. The rats were exposed to three different pseudorandomized prepulses with intensities of 70 dB and either 4, 12, or 20 KHz frequencies preceding a startle stimulus of 100 dB. The control of the 100 dB startle stimulus alone resulted in an average amplitude startle of 4.2372. The 70 dB prepulse at a frequency of 4 KHz resulted in an average amplitude startle of 3.0676, the 12 KHz prepulse resulting in an average amplitude startle of 3.5214, and the 20 KHz prepulse resulting in an average amplitude startle of 2.9883. All three prepulse startles were lower than the average amplitude of the startle. After the T-Test was calculated, the results for the 4 KHz, 12 KHz, and 20 KHz were 0.00456, 0.0184, 0.00147, respectively. This proves that the difference between startle response with the prepulses were statistically different to the control of the acoustic startle stimulus alone, since the calculations were below 5% ($p < 0.05$.)

The next set of experiments performed tested for LES and the effect of restraint stress on LES and startle amplitude. Figure 6 describes the average startle amplitude values for various decibel levels in the pre-test. At that point both the control and the restrained groups had not been treated differently, so a difference was not expected. A 2-way ANOVA confirmed that there was no significant difference between the two groups, although the clear positive
correlation between pulse intensity and startle amplitude are confirmed. Figure 7 details the average startle amplitude values for the different decibel levels in the delayed post-test. For each of the three blocks, the restrained group showed higher average responses than the control group. This supports the effect of stress of startle response, but a 2-way ANOVA test showed that the data was not statistically significant. Data was plotted on separate lines for each group. In each block, the restrained rats showed higher average startle response than the control rats, confirming the effect of a stressor on startle amplitude. However, a definite conclusion cannot be made because the data was calculated to not be statistically significant ($p > 0.05$). 11 rats (S2-S12), split into a control and restrained group as previously indicated, were exposed to 40 trials of pulse intensities for each of three blocks: dark, light, and dark again. Data were plotted on a bar graph and represented as percentages of startle amplitudes obtained in Block 1 ($n=3$). The graphs shown are only showing the relative percent increases for 90 dB, because at 80 dB the rats were not significantly startled to show an increase, and at 100 dB and 110 dB the ceiling effect began to impact the relative increases, bringing them down. Figure 8a displays the relative percent changes of the control rats and restrained rats of the pre-test. At block 2, the relative increase for the restrained rats was higher than the relative increase for the control rats, and the same was true for block 3. The fact that both blocks were higher than block 1 was evidence of LES. Between the two groups, no significant difference was calculated as at this point the two groups had been treated the same. However, a two-way ANOVA was performed to show that a statistically significant difference existed between Blocks 1 and 2, as well as between Blocks 1 and 3, only at 90 dB ($F_{3,34}=0.827, p < 0.01$). Next, figure 8b displays the relative percent changes of the control rats and the restrained rats of the immediate post-test after the restraint stress was applied. Block 2 shows an overall increase relative to block 1, but even after stress, the control group had a higher relative increase than the restrained rats. Block 3 showed essentially no increase upon block 1 for both the control and restrained group. Statistical analysis showed there to be no significant difference between the control and restrained rats group. Additionally, although block 2 was observed to be higher than block one, the data was shown to not be statistically significant, so a conclusion cannot be definitively made. Similarly, figure 8c describes the relative percent changes of the control rats and the restrained rats of the delayed post-test, three days after the restraint stress. Block 2 shows an overall increase relative to block 1, with the restrained rats showed a higher relative increase than the control group. This supports both LES and the effect of stress on LES. However, after performing a 2-way ANOVA, the data was shown to not be statistically significant, so a conclusion cannot be as definitively made. Block 3 shows no overall increase upon block 1 for both the control and restrained group.

Another experiment that was performed was the zero maze experiment. The zero maze experiment consisted of a pretest session, immediate post-test session, and delayed post-test session. 11 rats (S2-S12) were split into a control and restrained group as previously stated and subjected to the zero maze. Figure 9 shows the average percent of time that a rat spent in the open arm. In the pretest session, both the control and the restraint rats spent about the same amount of time in the open arm, meaning that there is no significant difference between the two groups. In the immediate post-test, the control and restraint spent an average of 10% less time in the open arm in the immediate post-test session than they did in the pretest session. This means that the stressor had no effect on the immediate post-test. The decrease seen in the immediate post-test session compared to the pretest session can be attributed to a decrease in exploratory activity in the rats as the rats have already been exposed to the maze in the pretest session. From figure 10, there seems to be a significant difference between the two groups in the delayed post-

[4-9]
test session. However after the data was analyzed using a T-Test \((p > 0.05\) for all sessions) and a Two-Way ANOVA \((p > 0.05\) for all sessions), there seemed to be no statistical difference within session, within group, and between group and session (session: \(F_{2,18} = 3.417, p > 0.05\); group: \(F_{1,9} = 0.026, p > 0.05\); session/group: \(F_{2,18} = 1.039, p > 0.05\)).

After the data from the LES and zero maze experiment were analyzed, they were then reanalyzed to determine if a correlation exists between the pretest LES block 2 intensity at 100 dB and the time spent in the open arm in the pretest session of the zero maze \((r = 0.243, p > 0.05, n = 11)\). Statistically, there is no correlation between the block 2 intensity at 100 dB and the pretest session of the zero maze. The data from the pretest LES block 3 intensity at 100 dB and the time spent in the open arm in the pretest session of the zero maze was also analyzed in order to determine if there was a correlation between the two experiments \((r = 0.397, p > 0.05, n = 11)\). Statistically, there is no correlation between the block 3 intensity at 100 dB and the pretest session of the zero maze (Fig. 10).

To test for the relationship of stress on phasic fear, a fear potentiated startle experiment was conducted. The FPS experiment consisted of two sessions, a training session where the rats were exposed to a conditioned stimulus (a thirty second tone) and an aversive unconditioned stimulus (a half second shock). 12 rats were split into a control and experimental group, with the experimental group being subjected to restraint stress. In the second (test) session, the conditioned stimulus was no longer paired with the shock but occasionally with a second noise blast to gauge the strength of Pavlovian conditioning of the tone with the shock. From Figure 9, contrary to prediction, the rats responded more strongly to the noise burst alone than to the combination of the noise burst and the tone. A further statistical analysis shows yielded some interesting results. Comparing the noise blast control group to the noise blast-tone control group, a p-value of 0.767 was obtained. Comparing the noise blast experimental group to the noise blast-tone experimental group, a p-value of 0.576 was obtained. Lastly, comparing the noise blast-tone control group to the noise blast-tone experimental group, a p-value of 0.136. All three p-values show that FPS was not statistically significant. However, because the rats did not show FPS, the effect of stress on phasic fear is inconclusive.
Figure 3. Prepulse Inhibition testing 70 dB Prepulses with varying frequencies. The Prepulse Inhibition experiment shows that the startle response was inhibited when a neutral tone was presented before the actual startle pulse. The asterisks signify the statistic differences between the T-Tests results.

Light-Enhanced Startle (LES)

Figure 4. Average startle response of the rats of control and experimental groups are not significantly different.

Figure 5. Delayed post-test: Average startle amplitude increases with decibel level and the presence of restraint stress.
Figure 6. Change in startle amplitude during Blocks 2 and 3 were expressed as percentages of those of Block 1 at 90 dB auditory pulses across all three sessions.

**Zero Maze**

Figure 7. Percent of time that a rat spends in an open arm of a zero maze. 11 rats (S2-S12) were subjected to a zero maze over the course of 3 sessions. Restraint rats were confined in a stress tube while the control rats were not exposed to any type of stressor.
Correlation Between the Zero Maze & LES

Figure 8. Correlation between pretest open arm of the zero maze vs. 100 dB block 2 of LES and pretest open arm of the zero maze vs. 100 dB block 3 of LES. The rats in the light box session are the rats that were subjected to the 100 dB block 2 of LES. The rats in the dark box session are the rats that were subjected to the 100 dB block 3 of LES.

Figure 9: Average pulse intensities for fear potentiated startle baseline, noise burst (NB), and tone with noise burst trials. The Baseline test refers to the training period of nine pulses with varying intensities without the tone that is associated with the shock. The Noise Burst Alone test refers to the pulses with an absence of the tone during the testing period. The Tone and Noise Burst test refers to the presence of the tone with the noise burst.

DISCUSSION

This experiment performed on animal models focused on studying the relationship between stress and anxiety. The team investigated the effect of restraint stress on both phasic and sustained fear. Additionally, parametric tests were performed to determine the effectiveness of the new equipment. Observations were collected in relation to Prepulse inhibition, intensity, and short and long term habituation.
Effect of Stress on Animal Models: Light Enhanced Startle and Zero Maze

In this study, researchers predicted that restraint stress would enhance anxiety measured by the LES and the zero maze. In the LES, researchers predicted that the results would affirm previous studies’ findings that startle is enhanced in the presence of light (5). Although our data does reflect these predictions, they cannot be affirmed as there was no control group that underwent a dark-dark-dark block design instead of the dark-light-dark design that was utilized. Researchers also wanted to examine whether restraint stress potentiated sustained fear during LES trials. Sustained fear was evaluated by an increased startle level during the third trial of each LES session that took place after a trial in a light environment. In both immediate post-stress testing and delayed post-stress testing, there was evidence of startle for both the restraint and control group at 90 dB. However, at levels higher than 90dB, there were no evidence of startle. This conclusion may be a result of the shortcomings of measuring systems used. The startle response of rats may have been larger than measurable for the system, creating a ceiling effect. As shown in Figure 7, our results suggested that sustained startle is not affected immediately by stress. However, when examining prolonged anxiety data, researchers concluded that there was a marked difference between the restraint group and control group from 80-120 dB, with the restraint group showing higher startle responses after exposure to light. This study showed that restraint stress does not affect immediate anxiety but may affect prolonged anxiety.

In the zero maze, results for both the restraint and control group were relatively similar across sessions. In all pretest sessions, the Ss preferred the closed arm to the open arm. Both groups spent less time in the open arms during the immediate anxiety test, reflecting that this change was not due to exposure to stress, but rather, a series of other possible environmental factors, such as decline in exploratory activity, fatigue from other tests, or decreasing appeal of novelty. During the delayed post-stress test, however, the restraint group spent less time in the open arms than the control group did, as illustrated in Figure 10. This data reflects, in agreement with LES results, that restraint stress affects only prolonged, not immediate, anxiety.

Individual Differences in LES and Zero Maze

In studying the correlation between the two different apparatuses used to test the effect of stress on anxiety, no evidence was found between a relationship of performance on the zero maze and the LES. (Figure 11) High anxiety is quantified by an increased percentage of time spent in the closed arms of the zero maze and higher startle responses in the LES; however, our results did not find any correlation between these two measures of anxiety. Anxiety is a multifaceted phenomenon, involving multiple different pathways, including the PnC, the amygdala, and the BNST. It may be possible that the zero maze and LES, though both testing for sustained fear, examine the effects of different pathways of anxiety.

Effect of Stress on Animal Models: Fear Potentiated Startle

The fear potentiated startle experiment, conducted to investigate the effect of stress on phasic fear, resulted in inconclusive data. While it was predicted that the rats would associate a 30 second tone with a 0.5 second shock, tested by pairing the tone with a second noise burst, the data revealed that instead of being startled more by the tone-noise burst pairing, the Ss were
startled more by the noise burst alone. (Figure 12) Although contrary to prediction, it is possible that the intensity of the noise burst could have been so strong that regardless of whether or not the tone was present before, the startle response may have been independent.

**Neuroanatomical Circuits for Phasic vs. Sustained Fear**

![Neural Circuitry involved in Acoustic Startle Response](image)

**Figure 10: Neural Circuitry involved in Acoustic Startle Response.** The red box refers to the sustained fear circuit, the blue box refers to the phasic fear circuit within the amygdala, and the green boxes refer to the primary startle pathway.

Phasic fear refers to fear that begins rapidly or suddenly, but dissipates quickly once the threat or stressor is removed. As seen in Figure 12, Phasic fear is primarily mediated by the central amygdaloid nucleus (CeA) which directly receives its input from the basolateral amygdaloid nucleus, and then propels the signal to the Nucleus Reticularis Pontis Caudalis (PnC) found in the tegmental part of the brainstem in order to execute physical motor responses like a startle response. Sustained fear is attributed to long-lasting anxiety caused by unpredictable threats (5). Sustained fear is also mediated by the amygdala; however, it is localized in the Bed Nucleus Stria Terminalis (BNST), a part of the extended amygdala connected to the central amygdaloid nucleus by a series of fiber tracts. The BNST also receives its input from the basolateral amygdaloid nucleus and ultimately discharges corticotropin releasing hormone (CRH) to the PnC. The Light Enhanced Startle in this experiment represented the sustained fear measure due to the long-term testing of the stressed rats with the dark-light-dark trials, using the CeA pathway. The Fear Potentiated Startle in this experiment represented the phasic fear measure due to the short-term testing of the stressed rats that associated a tone with a shock, using the BNST pathway.

In order to test the lab equipment, several parametric tests were run. These tests included a Prepulse Inhibition (PPI) experiment, short and long term habituation experiments and a startle-intensity experiment. The short and long term habituation experiments yielded results that
confirmed the effect of short and long term habituation. From the data obtained, the amplitudes of the startle response gradually decline from repeated stimulation of the startle.

The acoustic startle experiment was conducted to correlate the amplitude of the startle response with the intensity of startle. The experiment’s results showed a positive association between the two variables. From 80 dB to 110 dB, the amplitude of the startle response was directly proportional to the intensity of the startle. However, the transition between a 110 db to a 120 dB showed no positive correlation. In fact, it showed a generally negative correlation.

The PPI experiment revealed two significant results in testing for phasic fear. First, the experiment tested for a correlation between the intensity of the startle (70 dB, 80 dB, 90 dB) and the amplitude of the startle response. According to the data collected, there is a positive association between startle intensity and startle response amplitude. Second, the experiment tested for the effect of a Prepulse on the startle response. The data gathered shows that the prepulse, a sound of varying frequency, had the smallest inhibition on the startle response at 12 kHz. The two other frequencies used, 4kHz and 20 kHz, resulted in startle responses that were more inhibited than without the prepulse. These results show that of the 11 subject rats used, they responded to more extreme frequencies.

**Experimental Error**

The study was designed to reflect an associative model of stress and anxiety, in which incidence of stress affects anxiety measures. However, there were certain limitations in our study that may have attenuated this result. The conventional zero maze test model includes walling of both the outside and inside ledges of the track. However, this model was not utilized because the AnyMaze tracking system could not pick up the rats’ movements when the rats were in fully walled quadrants. The half-walled track may not have provided as much environmental contrast to the open track than a traditional full-walled track may have. This model was not a very definitive measurement of anxiety, as the two placement options for the rats—the half-walled quadrants and the open quadrants—did not contrast definitively.

In addition, our stress procedure was also modified from previously published restraint procedures to accommodate the research timeframe. In general, published restraint procedures have been more intense than our modified procedure, which consisted of a single one-hour restraint session. For example, Zhang et al. 2014 utilized a repeated restraint stress model, where rats were exposed to stresses five consecutive days. Intensified restraint procedures may yield more intensified anxiety responses, as increased levels of corticotropin-releasing factor (CRF), a primary mediator of stress responses, dissipates after one hour with transient restraint stress models, but may persist when more intense procedures, such as the repeated restraint model, are utilized (10) (11).

Another environmental factor that may have affected the anxiety levels of the rats was construction. Throughout the duration of the experiment, facilities in the Hall of Sciences at Drew University were under construction. Certain deviations from normal testing conditions due to construction included loud noise and tremors. These deviations may have contributed to the rats’ anxiety levels, the sensitivity of the chambers that collected the data, and were factors we did not account for during data analysis. Finally, due to limited number of rats, we could only
use a very small sample size of twelve individuals: one as a trial rat, five as the control group, and six as the restraint group. As a result, it is difficult to say whether the trends found in this study are applicable to a general population.

**Future Experimentation**

The study was a preliminary investigation into the different ways stress affect anxiety and fear in animal models. This study was a behavioral study, examining the effect of stress on anxiety based on individual performance on tests. A step to further understand this phenomenon would be to examine the biological mechanisms behind our results. Future researchers should analyze the correlation between corticotropin releasing factor (CRF) that is normally released in response to stress, and performance of anxiety tests in rats after stress. This may give insight as to how manipulation of this peptide could be used in anti-anxiety drugs for treatment of diseases such as post traumatic stress disorder, generalized anxiety disorder, and seasonal anxiety disorder (5). The amygdala should also be investigated in the future at a neurobiological level. The amygdala analyzes the presence of danger or constraint and results in multiple stress responses such as rapid breathing, and adrenal action. Identifying changes in the structure of the amygdala after stress exposure may also lead to the understanding of the amygdala as a substrate of both stress and anxiety.

To evaluate phasic fear through a biological perspective, the caudal pontine reticular nucleus (PnC) is another pathway that could be further explored. An important component in the processing for phasic fear, the PnC is involved in the ASR and helps mediate the response pathway. This study was conducted to measure the intensity or amplitude of the startle shown in rats (behavioral expression). However, future scientists may want to look at the biological and neural aspect as well. In addition, previous studies have shown the similarity between human PnCs and rat PnCs, especially the existence of giant neurons that connect to the spinal cord and cranial and facial nuclei (12). Future experimentation may want to explore the specific biological substances that are found both in humans and rats, so that experimentation on rats can be converted into a comprehensive understanding of neurological issues in humans.
REFERENCES


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