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ANXIOGENIC EFFECTS OF AUDITORY STIMULI AS MEASURED WITH
ACOUSTIC STARTLE

A Thesis Presented

by

Jasmin Salam

to

The Faculty of the Graduate College

of

The University of Vermont

In Partial Fulfillment of the Requirements

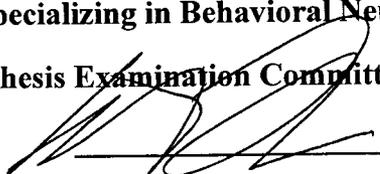
For the Degree of Master of Arts

Specializing in Psychology

February, 2008

Accepted by the Faculty of the Graduate College, The University of Vermont, in partial fulfillment of the requirements for the degree of Master of Psychology, specializing in Behavioral Neuroscience.

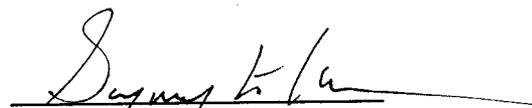
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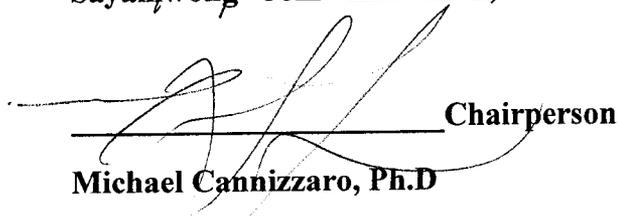
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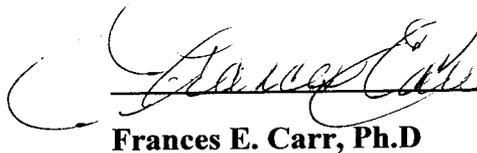
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Date: November 2, 2007

Abstract

Increased startle has been associated with increased levels of anxiety. Auditory stimuli facilitate startle suggesting that the auditory stimuli increase levels of anxiety. We have found that presentation of a moderate intensity (60 dB) tone facilitates startle in C57BL/6J mice and the facilitation persists after the offset of the tone. Because auditory stimuli activate neural regions related to anxiety, such as the bed nucleus of the stria terminalis (BNST), we investigated whether the persistent elevation in startle after the offset of a tone is due to a tone-induced anxiety state. In these three experiments, we examined if it was the tone that induced the elevation of inter-trial interval (ITI) startle amplitudes, whether this effect persisted over multiple tone presentations, and the effects of administering the anxiolytic drug buspirone on the ITI startle enhancement. The experimental session used for assessing the nature of the ITI startle elevations consisted of a series of consecutive startle stimuli (pre-tones, 20 msec noise burst) for measuring baseline startle followed by 27 tones (12 kHz, 60 dB, 30 sec) intermixed with 27 startle stimuli (same as pre-tones). Startle amplitude after the offset of tones was significantly higher than startle amplitude to the initial pre-tone stimuli. The elevation in startle after the offset of the tones was reduced by pre-test administration of the anxiolytic buspirone (4mg/kg). These data suggest that in mice, a moderate intensity tone produces a persistent elevation in startle that may be related to a tone-induced anxious state.

Dedication

I dedicate this to my younger sister. Thanks for always believing in me.

Acknowledgments

First and foremost, I would like to sincerely thank my advisor, Bill Falls, for all his help and support on this project and for being a constant source of strength and positivity the whole time I have worked with him. I also want to extend a warm thanks to my committee members, John Green, Jom Hammack, and Mike Cannizzaro for all of their insightful suggestions and encouragement throughout this entire process. In addition, I would specifically like to thank John Green for his thoughtful advice before my defense and for expressing such supportive enthusiasm afterwards.

I am thankful to those faculty, staff, and graduate students in the psychology department who openly shared their support and excitement about this accomplishment and to all those who attended my defense. I especially want to extend a gracious thank you to Kim Rhodes, Drina Vurbic, Andy Roering, and Analese Maddox for being such great friends, helping to keep me centered during the tougher times, and being so supportive during this endeavor. Most of all, I am grateful to my younger sister and best friend, Shahreen Salam, for her love and support with everything and for always having such strong faith in me. I don't know where I would be without her.

Table of Contents

	Page
Dedication.....	ii
Acknowledgments.....	iii
List of Figures.....	v
Introduction	
General Uses for Auditory Stimuli by Animals.....	1
Auditory Stimuli in Laboratory Settings.....	1
Auditory Effects on Startle Amplitudes.....	1-2
Learning and Memory.....	2
Stress and Anxiety Behaviors.....	2-4
Anxiety Neural Circuitry.....	4
Rationale for Study.....	4-6
Experimental Design and Purpose.....	6-7
Methods and Procedure	

Experiment 1.....	7-10
Experiment 2.....	11-12
Experiment 3.....	12-14
Results	
Experiment 1.....	15-16
Experiment 2.....	16-17
Experiment 3.....	17-19
Discussion.....	20-30
Bibliography.....	31-33

List of Figures

Figure 1a: Startle Amplitude to Pre-Tone Startle Stimuli.....	34
Figure 1b: Percent Changes in Startle Amplitude during Tones and ITI from Pre-Tones	34
Figure 1c: Startle Amplitude to Pre-Tone Startle Stimuli.....	35
Figure 1d: Percent Changes in Startle Amplitudes of Tones and ITI over Blocks	35
Figure 2a: Startle Amplitudes to Pre-Tone Startle Stimuli for Tones and No-Tone Procedures	36
Figure 2b: Tones- Percent Changes in Startle Amplitudes of Tones and ITI from Pre-Tones	36
Figure 2c: No Tones-Percent Changes in Startle Amplitudes of (would-be) Tones and ITI from Pre-Tones.....	37
Figure 3a: Startle Amplitudes to Pre-Tone Startle Stimuli for Vehicle and Buspirone Animals.....	38
Figure 3b: Percent Changes of Startle Amplitudes during Tones and ITI from Pre-Tones for Vehicle and Buspirone Animals.....	38
Figure 3c: Startle Amplitudes to Tones for Vehicle and Buspirone Animals	39

The behavior and physiology of animals are, by nature, adaptive and responsive to many environmental factors. Auditory stimuli are one class of environmental stimuli that impact animals. In natural situations, sounds are emitted by various species as a means of communication for the purposes of mating, defending territory, recognition of offspring/parents, foraging, warning signals, and discriminating size and age of conspecifics in their surroundings (George, Hara, & Hessler, 2006; Ghazanfar et al., 2007; Mudry & Capranica, 1987; Munhall & Byrne, 2007; Schmidt, Amrhein, Kunc, & Naguib, 2007; Schroeder & Ritters, 2006; Sebe, Nowak, Poindron, & Aubin, 2007; Smotherman, 2007). These observations indicate that auditory stimuli significantly affect the behaviors of animals and are essential for their survival and reproduction.

Auditory stimuli are an important feature in the surroundings of animals and have been studied extensively in laboratory settings. Auditory stimuli produce numerous effects on animals including altered arousal levels, changes in eating, activity, exploration, and sleeping time (Baker, Kentner, Konkle, Santa-Maria Barbagallo, & Bielajew, 2006; Billewicz-Stankiewicz & Golabek, 1977; Harris, Zhou, Youngblood, Smagin, & Ryan, 1997; Krebs, Macht, Weyers, Weijers, & Janke, 1996; Krebs, Weyers, Macht, Weijers, & Janke, 1997; J. Willott, 2007). They also produce changes in the number of regular estrous cycles, cardiovascular function, and can alter reflex activity (Baker et al., 2006; Manikandan et al., 2006). For example, background auditory stimuli have bi-directional effects on the startle reflex in rats. Low frequency (0-1 kHz) background stimuli facilitate acoustic startle while higher frequencies (10-20 kHz) inhibit acoustic startle (Campeau & Davis, 1992). The difference in low and high frequencies on

startle was argued to result from the effects of background noise on the central auditory system. It was argued that high frequency background stimuli mask the startle stimuli, which resulted in depressed startle responses. Low frequencies do not exhibit this masking effect and may facilitate startle by reducing auditory thresholds. If this suggestion is true, then the modulation of startle by auditory stimuli is strictly an auditory phenomenon due to a masking effect as perceived by the auditory system.

Auditory stimuli also impact learning and memory in animals. Exposure to loud (80-110 dB) or quiet (<35 dB) conditions resulted in decrements in learning and memory, while background noise levels (68-70 dB) facilitate these processes (Rabat, Bouyer, George, Moal, & Mayo, 2006). It was suggested that auditory stimuli affect learning and memory by modulating stress responses, including HPA axis activation and release of corticosterone. This rationale supports that the effects auditory stimuli have on stress levels induce changes in other behavioral and cognitive processes. In fact, one of the prominent effects of auditory stimuli is to induce stress.

Given that animals use auditory stimuli as warning signals during threatening situations, it is logical that auditory stimuli would elicit stress responses, preparing the animal to cope with danger. Auditory stimuli enhance stress induced sensitization (increased immobility) to a single 15 min session of repeated footshock administration in rodents (Stam, van Laar, Akkermans, & Wiegant, 2002). When exposed to auditory stimuli alone one week later, for three days, animals exhibited a persistence of the increased immobility as observed when previously administered footshocks. In non-exposed animals, immobility decreased and animals failed to show the persistence of this

effect, suggesting that auditory stimuli induce a persistence of stress-related responses. Perinatal exposure to auditory stimuli also elicited increased immobility in animals later during adolescence (Tokumo, Hirai, & Nishino, 2006), demonstrating that auditory stimuli induce stress-related responses that can be observed long after the initial exposure.

Consistent with these stress effects, auditory stimuli produce changes in stress hormones. Exposure to loud auditory stimuli (80-110 dB) has been found to increase corticosterone, adrenocorticotropin hormone (ACTH), corticotropin releasing hormone (CRH), noradrenaline, and adrenaline in animals, which has also been shown to last over a period of days (Armario, Montero, & Balasch, 1986; Atkinson, Wood, Kershaw, Bate, & Lightman, 2006; Baker et al., 2006; Burow, Day, & Campeau, 2005; Campeau, Akil, & Watson, 1997; Campeau & Watson, 1997; Gesi et al., 2002; Helfferich & Palkovits, 2003; Herry et al., 2007; Manikandan et al., 2006; Shors, 2001; Turner, Parrish, Hughes, Toth, & Caspary, 2005; van Raaij et al., 1997).

Increasing intensities as well as the presentation of unpredictable auditory stimuli were shown to induce anxiety-like behaviors using shuttlebox avoidance training, elevated plus maze, and open field tests (Herry et al., 2007; Hughes & Bardo, 1981). Moreover, Santis et al (1983) demonstrated, using the open field, that animals have a preferred place to groom, known as the G-site. In the presence of a 2 sec, 100 dB auditory stimulus, animals remained there if already in that location or if elsewhere, they escaped and ran to their G-site. Given the anxiolytic drug diazepam, mice showed the normal tendency to groom in the preferred place, but there were no escape attempts if

animals were outside their G-site, suggesting that the auditory-induced effect of escaping to the G-site might be mediated by anxiety.

Moreover, Atkinson et al (2006) has found that exposure to auditory stimuli results in increases of corticosterone levels, lasting 10-15 min after an auditory stimulus had been terminated. This resonating effect is consistent with the characterization of anxiety according to Walker et al, suggesting that anxiety is a persistent effect resulting from the presentation of a particular stimulus that continues long after the offset of the stimulus (Walker, Toufexis, & Davis, 2003). Since auditory stimuli appear to induce long lasting stress responses which are similar to the suggested description of anxiety, it is possible that auditory stimuli may directly affect levels of anxiety.

Auditory stimuli activate areas of the brain thought to be involved in anxiety. Studies suggest that the bed nucleus of the stria terminalis (BNST) is the neural substrate of anxiety (Walker & Davis, 1997; Walker et al., 2003). Animals exposed to auditory stimuli exhibited increases in c-fos mRNA induction, an indicator of neuronal activation, in the BNST (Burow et al., 2005; Campeau et al., 1997; Campeau & Watson, 1997; Walker & Davis, 1997; Walker et al., 2003). These data therefore suggest that auditory stimuli may be involved in increasing levels of anxiety.

We were interested in assessing the effects auditory stimuli have on anxiety in animals using the acoustic startle response. The acoustic startle reflex is easily measured and its neural circuitry is well known, consisting of a tri-synaptic pathway composed of cochlear root neurons (CRN), reticularis pontis caudalis (RPC), and spinal cord. It is also sensitive to drug effects, lesions, electrical brain stimulation, and environmental

manipulations (Davis, 1984). This makes it is a useful procedure for assessing behaviors, reflex activity, and emotionality in animals.

Consistent with the previously reported findings that auditory stimuli induce elevations in startle amplitude (Campeau & Davis, 1992; Schanbacher, Koch, Pilz, & Schnitzler, 1996), we have observed unconditioned elevations in startle amplitudes during the presence, versus the absence, of tones (30 sec, 60 dB). According to Campeau et al (1992), the ability of auditory stimuli to modulate levels of startle amplitudes results from effects within the auditory system (i.e. changes in auditory thresholds or hearing) induced by the presence of acoustic stimuli. We noticed that the observed elevation in startle amplitude in the presence of tones persisted into the ITI after their offset. If the increases in startle levels were due to an auditory system effect (i.e. changes in auditory thresholds), then it would seem that elevated startle levels would only occur during tone presentations as found in Campeau's study (1992), or perhaps for some short interval afterwards. Studies have also shown that increases in acoustic startle amplitudes are positively associated with levels of anxiety (Walker & Davis, 1997; Walker et al., 2003). Therefore, our observations of the persistent elevations in ITI startle levels after the offset of tones may be consistent with the characterization of anxiety responses (Atkinson et al., 2006; Walker & Davis, 1997). Thus, we became interested in investigating whether this effect may be resulting from anxiety induced by the tones.

The goal of our studies was to examine the nature of the ITI enhancement of startle amplitudes and determine whether it is mediated by anxiety. By conducting this series of experiments, we assessed whether the observed elevation in ITI startle

amplitudes after the offset of tones was attributable to the tones per se or a startle-stimuli induced sensitization, the duration of the ITI startle enhancement when given an extended number of tones, and whether this effect is mediated by an anxiety-like state brought on by the presentation of the tones. Addressing these questions further our understanding of the effects auditory stimuli have on animals and their influence on anxiety.

In Experiment 1, we found that startle amplitudes were elevated following the offset of the tone and examined the persistence of this effect over multiple tone presentations. If the increases in ITI startle responses were strictly due to an auditory system effect (e.g. changes in auditory thresholds) of the tone presentation, it was possible that over repeated presentations, elevated startle responses to the tone may have habituated or decayed over time and, in turn, would attenuate elevations in ITI startle. Therefore, we examined the effect of ITI startle responses when multiple tones were administered.

Davis has reported that repeated exposure to startle stimuli sensitizes startle responses (Davis, 1989). Experiment 2 addressed whether the increase in startle amplitude could be observed in the absence of tone presentations, indicating that this startle increase was due to a sensitization after repeated administration of startle stimuli. We directly compared startle sessions with and without tones, and analyzed whether there was sensitization during the startle-only procedure. If the tone presentations produced the increase in ITI startle amplitudes, this would support the hypothesis that this effect is mediated by an anxiety-like state.

Elevations in startle amplitudes have been suggested to be associated with increased anxiety levels (Walker & Davis, 1997; Walker et al., 2003). In the third experiment, we assessed whether the persistent elevation in ITI startle amplitudes resulted from an anxiety-like state induced by the onset of the tone by comparing the effects of the anxiolytic buspirone to vehicle on ITI startle responses. If elevations in ITI startle amplitudes were induced by an anxiety-like state, then an anxiolytic drug should attenuate this effect. Buspirone is a serotonin (5HT) agonist and is a clinically effective anxiolytic. 5HT receptors are prevalent in the BNST and if buspirone attenuated the ITI startle enhancement, it is possible it did so via the BNST, further suggesting that the attenuation in ITI startle responses was mediated by an anxiety-like state. Moreover, buspirone has been effective in attenuating increased startle amplitudes resulting from light-enhanced startle, a procedure where animals are exposed to prolonged illumination as an anxiogenic stimulus (Walker & Davis, 1997).

Method

Experiment 1

In previous experiments, startle amplitudes have been shown to increase during the presentation of tones. We also observed that the elevation in startle amplitudes persists during the inter-trial interval (ITI) after the offset of the tone. Therefore, Experiment 1 assessed whether the elevation in ITI startle amplitude was due to the presentation of the tone and if this effect would persist over an extended session with multiple tone presentations. In Experiment 1A, we presented a series of nine consecutive startle stimuli followed by 9 30 sec, 60 dB tones pseudorandomly intermixed with 9

startle stimuli alone. One or two ITI startle stimuli (one per min) were given between each tone administration yielding an ITI of 1-2 min. Startle was enhanced both in the presence of the tone and during the ITI between tones when compared to the initial startle stimuli. In Experiment 1B, we were interested in determining whether the elevation in ITI startle amplitudes persists over a longer session with multiple tone presentations. We administered nine consecutive startle stimuli as before, followed by a pseudorandom distribution of 27 30 sec, 60 dB tones intermixed with 27 startle stimuli alone, with a 1-2 min ITI as in Experiment 1A. If the elevation in ITI startle levels is mediated by an auditory system effect (e.g. changes in hearing thresholds), then there should be a decay in ITI startle responses over repeated tone presentations. However, if the elevation in ITI startle amplitudes persists over multiple tone presentations, habituation of ITI startle levels would seem unlikely, which would support the hypothesis that our effect may not be resulting from an auditory system effect (i.e. change in auditory thresholds).

Subjects

Eight week old, male C57BL6/J (n=32) mice were obtained from Jackson Laboratories in Bar Harbor, Maine. Mice were housed in groups of four and maintained in 12 hr light/dark cycles (lights on at 7:00 h). Food and water were available ad libitum. Prior to any experimental manipulations, a 14 day acclimation period was given.

Apparatus

Experimental procedures were carried out in eight sound attenuating cubicles measuring 58 (W) x 32 (D) x 55 (H) cm. Each cubicle was lined with black, sound absorbing foam with no internal source of light. Each cubicle contained a stabilimeter

device consisting of a load cell platform onto which the behavioral chamber was mounted. The chamber was constructed of clear acrylic, cylindrical in shape, 12.5 cm in length, with an inner diameter of 5 cm. The floor of the chamber consisted of a removable shock grid composed of six steel rods 3.2 mm in diameter, and spaced 6.4 cm apart (Med Associates, Georgia, VT). Startle responses were transduced by the load cell, amplified, and digitized over a range of 0-4096 units. Startle stimuli and tones were provided through a Radio Shack Supertweeter located 10 cm behind the behavioral chamber.

Data collection and the control and sequencing of all stimuli were controlled by Med-Associates startle reflex hardware and software (Georgia, VT). Startle amplitude was defined as the largest peak-to-peak value within 100 msec after the onset of the startle stimulus.

Procedure

Mice were placed in the startle apparatus and given a 5 min acclimation period preceding presentation of any stimuli. Immediately following, the acclimation period consisting of thirty startle stimuli of different intensities were presented in a pseudorandom order. The startle stimuli were 20 msec white noise bursts with a rise-decay time of 1 msec. Ten stimuli of each intensity level (95, 100, 105 dB) were presented with a mean ITI of 60 sec. A total of three initial startle sessions were used to acclimate the mice to handling, behavioral chambers, and startle stimuli.

Experiment 1A: Following startle stimulus habituation days, mice were returned to the startle chambers on day 4. Nine consecutive pre-tone startle stimuli (95, 100, 105

dB) were administered followed by 9 tone+startle stimulus trials (three at each 95, 100, and 105 dB) intermixed with 9 startle stimulus alone trials. Trials were presented in a pseudorandom order with the constraint that one of each intensity and trial type was presented in each block of six trials. The tone was a 30 sec, 12 kHz 60 dB pure tone. The startle stimulus occurred 29.75 sec after the onset of the tone, and the ITI between startle stimuli was 1 min.

Experiment 1B: To assess the effects of multiple tone presentations on ITI startle responses, three days later, mice were returned to the startle chamber to be tested with tones and startle stimuli. This session consisted of a 5 min acclimation period initially, then nine pre-tone startle stimuli at three different intensities (95, 100, 105 dB) were given, followed by 27 tone+startle stimuli intermixed with 27 startle stimulus alone trials with the constraint that each of the three intensities was given in each block of three startle noise bursts.

Data Reduction: Mean startle amplitudes were computed for each trial type: pre-tones, tones, and post-tones for each animal. Percent change was then calculated from pre-tones to tones and pre-tones to post-tones for each animal. For the extended procedure, data were reduced in the same manner except it was divided into three blocks: Block one consisted of startle amplitudes for the first three startle stimuli presentations for each intensity, block two was made up of the startle amplitudes for the second set of three startle stimuli for each intensity, and block three was composed of startle amplitudes for the last three startle stimuli given at each intensity. Statistical analyses were run using 3 x 2 ANOVA, with factors of Block and Trial Type, in SPSS 12.0.

Experiment 2

It has been shown that repeated presentation of startle eliciting stimuli sensitizes the startle reflex (Davis, 1989). It was possible that our observations of elevated ITI startle were independent of tone presentations and were the result of startle stimulus-induced sensitization of the startle response. To test this, we directly compared ITI startle in test sessions with and without tones. This enabled us to compare the effects of administering startle alone vs. tones and startle stimuli and verify whether our observed ITI effect was actually a result of the tone. If startle responses are only elevated during the ITI following tone presentations, this would support the hypothesis that the tones are responsible for the elevations in ITI startle responses. The alternative is that startle is enhanced in the absence of the tone, which would indicate the possibility that our effect of elevated ITI startle response is not necessarily induced by the tones but is due to a startle stimulus-induced sensitization.

Subjects

Eight week old, male C57BL6/J (n=24) mice were obtained from Jackson Laboratories in Bar Harbor, Maine. Mice were housed in groups of four and maintained in 12 hr light/dark cycles (lights on at 7:00 h). Food and water were available ad libitum. Prior to any experimental manipulations, a 14 day acclimation period was given.

Apparatus

Apparatus is identical to what is used in Experiment 1.

Procedure

This procedure was identical to Experiment 1B except that tones were omitted. Animals were placed in startle apparatus and administered a series of 63 startle alone noise bursts. Startle noise bursts consisted of 20 msec white noise bursts, having a rise-decay time of 1 msec. Startle stimuli were administered at three different intensities (95, 100, 105 dB) in a pseudorandom order.

Three days later, animals were placed in the startle apparatus and given a session with tones and startle stimuli (same as Experiment 1B). This procedure consisted of 9 startle alone stimuli, followed by 27 tones+startle intermixed with 27 startle alone stimuli with the constraint that each intensity (95,100,105 dB) was presented in every block of three startle stimuli.

Data from the no-tone procedure were analyzed identically to Experiment 1B, as though tones were actually given, in order to directly compare results from the no-tone and tone procedures. Percent changes of 'pre-tones' to 'tones' and 'pre-tones' to 'post-tones' over three blocks were calculated. For the experiment with tones, data were analyzed exactly as they were in Experiment 1B. Overall, data were compared between the two procedures of the experiment to determine if there was a significant potentiation of startle during tone presentations compared to when there were no tones presented at all.

Experiment 3

Increased startle amplitudes have been argued to reflect increases in anxiety (Walker & Davis, 1997; Walker et al., 2003). Therefore, to assess whether increases in startle amplitudes during the ITI were mediated by an anxiety-like state, we administered

the anxiolytic buspirone or vehicle to separate groups of animals. Animals were given nine initial startle noise burst stimuli, followed by 27 30 sec, 60 dB tones intermixed with 27 startle stimuli alone in a pseudorandom order (identical to Experiment 1B). This allowed us to observe the effects buspirone might have on the startle amplitudes during the ITI. If buspirone attenuated the elevation of ITI startle responses compared to those of vehicle animals, then this would support the hypothesis that the persistent elevation of ITI startle is mediated by an underlying anxiety-like state. If there would be no effect of buspirone, then this persistent elevation may be a result of changes in auditory thresholds rather than anxiety.

Subjects

Eight week old, male C57BL6/J (n=24) mice were obtained from Jackson Laboratories in Bar Harbor, Maine. Mice were housed in groups of four and maintained in 12 hr light/dark cycles (lights on at 7:00 h). Food and water were available ad libitum. Prior to any experimental manipulations, a 14 day acclimation period was given.

Apparatus

Apparatus is the same as used in previous experiments.

Procedure

This procedure was identical to Experiment 1B. Mice were placed in startle chambers to be tested with tones and startle stimuli. This session consisted of a 5 min acclimation period initially, then nine noise burst stimuli alone followed by 27 tone stimuli+startle stimuli intermixed with 27 startle stimulus alone trials. Three intensities were used during startle stimuli administration (95, 100, 105 dB) and given

pseudorandomly, with the constraint that each of the three intensities was given in each block of three startle stimuli. Animals were randomly placed in control or drug groups, and 4 mg/kg of buspirone was administered intraperitoneally (i.p.) to half of the animals while a saline vehicle solution was administered to the other half of the animals 5-10 min prior to testing.

Results

Experiment 1A:

Results from Experiment 1A (Figure 1a,b) show that startle responses during tone presentations are enhanced over pre-tone startle stimuli amplitudes given prior to tone administrations (% startle vs. $M=0$, $t(31)=4.40$, $p<.05$). Interestingly, startle responses remain significantly elevated during the ITI, after the offset of the tones, compared to pre-tones ($t(31)=2.56$, $p<.05$). Startle responses during tone presentations were also significantly enhanced when compared to ITI responses where startle stimuli alone were given ($t(31)=4.02$, $p<.05$). These findings indicate that the tone presentations, per se, enhanced startle compared to when no tones are presented. These outcomes illustrate that while tones themselves elevate startle, this startle enhancement persists into the ITI, after the offset of the tones.

Experiment 1B:

Experiment 1B demonstrated that the enhancement of ITI startle responses persists over an extended session with multiple tone presentations (Figure 1c,d). As in Experiment 1A, the tones significantly elevated startle responses (% startle vs. $M=0$; $t(31)=5.75$, $p<.05$), and this effect persisted into the ITI ($t(31)=4.55$, $p<.05$). The results further indicated that there was a significant difference between changes of pre-tone startle responses to tone responses compared to changes from pre-tones to ITI, indicating that the tone is enhancing startle levels more during their presentations than after their offset. ANOVA revealed that there was a significant effect of block ($F(2,62)=4.88$, $p<.05$) and trial type (tone or ITI startle stimuli), ($F(1,31)=33.68$, $p<.05$). The significant

block effect can be attributed to the change in pre-tones to ITI over blocks. All interaction effects were found to be non-significant ($p > .05$). As can be seen in Figure 1d, the percent change from pre-tones increases significantly over the three blocks ($F(2,62)=6.95, p < .05$), whereas this effect is not present when percent changes of tones from pre-tones was evaluated. In observing the persistent effect of ITI startle enhancement, we were prompted to investigate whether the ITI elevation in startle was being induced by a sensitization in startle responses over time or whether this effect is produced as a result of the tone presentations.

Experiment 2:

The results from Experiment 2 indicated that the tone was necessary for the elevation in startle during the ITI (Figure 2a,b). Mice given a startle test session in which the tones were omitted exhibited no change in startle amplitude from what would be the pre-tone startle stimuli to the tone startle stimuli ($p > .05$; Figure 2c), or from the pre-tone startle stimuli to the ITI ($p > .05$; Figure 2c). When these same animals were tested with the tones, we once again showed that the tones elevated startle (% startle vs. $M=0$; $t(23)=4.34, p < .05$) and this elevation persisted into the ITI ($t(23)=2.16, p < .05$). ANOVA showed that startle amplitude was greater in the presence of the tone than during the ITI ($F(1,23)=11.60, p < .05$) with a significant effect of block ($F(2,46)=5.44, p < .05$) as well (Figure 2b). All interaction effects were found to be non-significant, ($p > .05$). As in Experiment 1B, the block effect can be attributed to the significant increases in ITI startle responses over blocks ($F(2,46)=8.08, p < .05$), which was not observed with the tones. In the procedure without tones, there were no significant differences in startle responses to

what would be the pre-tones, tones, and ITI throughout the session ($p > .05$; Figure 2c), indicating that it is indeed the presentation of the tones which facilitates the persistent enhancement of startle levels. Figure 2b shows that startle enhancements to the tones and ITI were attenuated compared to previous experiments. Interestingly, these animals may have been less anxious to begin with as the tones continued to significantly facilitate startle responses, and ITI responses were significantly increased over blocks. In these data, the *pattern* of responding was consistent with what we have seen, although they are attenuated.

Experiment 3

Buspirone attenuated the elevation in ITI startle responses compared to animals given vehicle (Figure 3b). Results revealed a significant drug effect ($F(1,22)=5.39$, $p < .05$). They also showed that the tones significantly enhanced startle responses in buspirone and vehicle groups when compared to pre-tone responses (% startle vs. $M=0$; buspirone; $t(11)=2.85$, $p < .05$), vehicle ($t(11)=4.57$, $p < .05$)). T-tests further revealed that ITI startle responses for the vehicle animals were significantly different from pre-tones ($t(11)=4.36$, $p < .05$). On the other hand, t-tests for the buspirone group did not reveal any significant differences in the ITI startle responses from pre-tones ($p > .05$), which suggests that buspirone attenuated the ITI startle responses in these animals. ANOVA showed that the percent change in tones from pre-tones was significantly enhanced from the percent change in ITI startle responses from pre-tones ($F(1,22)=25.64$, $p < .05$) across both drug and vehicle groups as illustrated in Figure 3b. Since ITI responses of buspirone animals were found not to be significantly different from pre-tones as evidenced by the prior t-

tests, indicating they were not elevated, this indicates that the startle responses to the tones across groups were enhanced compared to the ITI startle responses.

We compared percent changes of tone responses from pre-tones between the two groups and found that there was a significant difference between the vehicle and buspirone animals ($F(1,22)=6.41, p<.05$). One interpretation of these data is that buspirone administration may have blunted all responses to tones and ITI stimuli, rather than selectively attenuating the ITI startle levels. However, in calculating raw values of startle amplitude in the presence of the tones in the two groups, we saw that the difference was not significant in the vehicle and buspirone animals (Figure 3c). ANOVA confirmed that there was *not* a significant difference in pre-tone responses between the groups ($F(1,22)=.530, p>.05$; Figure 3a), and the data suggest that the buspirone animals actually exhibited higher pre-tone startle responses than the vehicle animals, inconsistent with the hypothesis that buspirone may have attenuated responses to every stimulus type (Figure 3a). Therefore, the significant differences in percent changes of tone responses between buspirone and vehicle animals was likely due to buspirone attenuating an anxiety-like state produced by the tone presentations and evident in the ITI, and the significant enhancement of startle responses to the tones in the buspirone group was likely the result of a change in the response of the auditory system (e.g. changes in hearing thresholds) in the presence of the tones. This supports the hypothesis that the elevated ITI startle responses may be due to an underlying anxiety-like state, as the startle responses during the ITI were selectively attenuated by buspirone compared to vehicle

animals, and the pre-tone startle responses are actually higher in the buspirone animals, indicating that the attenuation resulting from buspirone was selective to the ITI.

Discussion

In this study, we found that tones enhanced startle amplitudes, and this enhancement not only occurred during tone presentations but persisted into the ITI after the offset of tones. Furthermore, our findings show that the elevation in ITI startle amplitudes were mediated by tone presentations and did not occur as a result of startle stimuli-induced sensitization over time. This elevation in ITI startle responses persisted over multiple tone presentations, which suggests that there was no habituation or decay in ITI startle responses over repeated tone presentations. We suggest that the tones induce a state of anxiety which persists into the ITI. When comparing the effects of buspirone vs. vehicle on the ITI startle responses, a distinct attenuation of ITI responses was observed in animals given buspirone, whereas vehicle animals did not exhibit this reduction. Thus, our findings are consistent with the elevation in ITI startle responses being due to an anxiety-like state introduced by the tones. The anxiolytic buspirone was chosen because this drug has been effective in attenuating increased startle amplitudes resulting from light enhanced startle, which uses prolonged illumination as an anxiogenic stimulus (Walker & Davis, 1997). Also, based on previous experiments conducted in our lab, buspirone does not exhibit the more intense sedative effects in rodents as observed with other anxiolytic pharmacological agents such as valium. Therefore, if any attenuation of elevated ITI startle amplitudes was observed as a result of administering buspirone, that effect was likely due to a decrease in anxiety rather than a sedative effect.

Interestingly, startle responses during tone presentations were consistently higher than responses during the ITI startle stimuli. This may result, in part, from a decrease in auditory thresholds from the tone presentations which carry over into the ITI. It has been shown that tones induce changes in auditory system sensitivity and thresholds specifically with respect to cochlear neurons (CN), inferior colliculus (IC), and the auditory cortex (Carlson & Willott, 1998; Ison & Allen, 2003; Willott & Turner, 2000; Willott et al., 1998). Depending on tone frequency and intensity, the tonotopic organization of these brain regions (the specific areas that are responsive to a particular range of frequencies and/or intensities) change over time, altering auditory thresholds and sensitivity which have been measured behaviorally using pre-pulse inhibition (PPI) and the startle reflex. Therefore, it is possible that during tone presentations in our experiments, the auditory system may exhibit tone-induced changes and become highly sensitive to all stimuli due to a reduction in auditory thresholds, which remain reduced after the offset of the tone, resulting in elevated responses during ITI startle stimulus alone presentations. However, there may be increased sensitivity of the auditory system during the 30 sec tones than the 20 ms noise burst startle stimuli given during the ITI. Therefore, while startle responses are elevated during the ITI compared to pre-tones, responses are enhanced more during tone presentations. This effect may also be influenced by a heightened anxiety-like state produced by the tone presentations. The presentation of the tones may activate neural systems mediating anxiety and as a result, cause a heightened anxious state in the animals, which is manifested behaviorally as enhanced startle responses during tone presentations. At the offset of the tones, this

anxiety-like state persists which elicits increased responsiveness during the ITI startle stimuli compared to pre-tones. However, the level of this anxiety-like state during the ITI startle stimuli is not as high as during the tones, resulting in a lesser ITI startle enhancement compared to that of tones. Furthermore, buspirone was found to attenuate startle responses to tones when percent changes from pre-tones were analyzed between groups, which may support the hypothesis that while tones may be affecting auditory systems (e.g. changes in thresholds), they may also be inducing an anxiety-like state which buspirone effectively reduces. Even considering the observed attenuation in startle responses to tones by buspirone, those startle responses were nonetheless significantly enhanced compared to pre-tones, which could reflect the tone-induced auditory system changes. We therefore believe that an anxiety-like state is, in part, mediating the enhancement of startle responses during tone presentations and that anxiogenic effect persists into the ITI producing the elevated ITI startle responses. Buspirone attenuates the anxiety-like state produced by the tones and abolishes the anxiety-mediated ITI startle enhancement.

The current experiments demonstrate that startle responses are enhanced during tone presentations and that this effect persists into the ITI. In Experiments 1B and 2 (w/Tones), we observed that percent changes in ITI startle responses from pre-tones *increased* significantly over blocks which was not observed with tones, indicating that the tone's ability to enhance ITI startle responses may increase over time. To explore the effect that a tone may have on increases in ITI startle responses over time, in a separate experiment, we administered six tones over a sixty minute session, with an extended nine

minute ITI imposed between each tone. This design allowed us to isolate the effect of each tone on the ITI and observe whether startle responses increased over the ITI. Results showed that startle responses did not increase during the ITI, and furthermore, that the ITI enhancement, was completely abolished. This suggests that single tone presentations are not sufficient to induce elevations in ITI startle responses. Instead, as demonstrated in the present experiments, multiple tones given within closer proximity to each other are necessary to induce the enhancement of ITI startle responses and the increase observed over blocks is an aggregate effect of tones. This indicates that multiple tones with shorter ITIs are required to induce anxiety-like states, and that tones have an additive effect on anxiety levels.

Our experiments have demonstrated that an elevation in ITI startle responses after the offset of the tones may be mediated by an anxiety-like state since previous studies have suggested that increases in acoustic startle amplitudes are positively associated with levels of anxiety (Walker & Davis, 1997; Walker et al., 2003). For example, when the stress hormone corticotropin releasing hormone (CRH) is injected into the lateral cerebral ventricle, a marked increase occurs in the amplitude of the acoustic startle response (CRH-enhanced startle). Consistent with the idea that these increases in startle amplitude reflect anxiety, the benzodiazepine anxiolytic, chlordiazepoxide, reduced the startle amplitude in CRH-treated animals but not in animals whose startle amplitudes were increased by non-anxiogenic agents such as strychnine and amphetamine. During CRH-enhanced startle, startle amplitudes have been reported to increase steadily beginning 30 min after CRH infusion and lasting for a 2 hr test period (Liang et al., 1992), further

supporting the hypothesis that increases in startle amplitude are positively correlated with levels of anxiety as produced by CRH infusions.

Furthermore, bright lights enhance startle amplitudes (Walker & Davis, 1997). Specifically, there are two test phases in this light-enhanced startle paradigm. In the first phase, the response to acoustic startle stimuli is measured in the dark. During the second phase, animals are either tested in the dark again or alternatively, in the presence of a bright light. Startle amplitudes increase from the dark to light test sessions but not during dark to dark test sessions. These startle enhancements resulting from light exposure have been argued to reflect states of anxiety because they are attenuated by the benzodiazepine, chlordiazepoxide and non-benzodiazepine anxiolytics, flesinoxan, buspirone, and propranolol (DeJongh, Groenink, Gugten, & Olivier, 2002). The tones used in our experiments may be producing a similar anxiogenic effect by increasing startle amplitudes which persist after their offset into the ITI, an effect which is attenuated by buspirone.

There has been debate over the distinction between fear and anxiety. Chemically induced lesions of the central nucleus of the amygdala (CeA) were found to completely eliminate fear-potentiated startle (conditioned fear), a procedure that involves initially conditioning animals to a neutral CS (e.g. tone) with an aversive US (e.g. footshock), and then testing them later with the CS alone, which should elicit a similar conditioned response (CR) as the US. Moreover, lesions of the CeA left intact the increase in startle produced by CRH-enhanced startle (Walker & Davis, 1997; Walker et al., 2003). On the other hand, chemically induced lesions of the bed nucleus of the stria terminalis (BNST)

abolished CRH-enhanced startle but left intact the increase in startle produced by fear. What is interesting is that both of these structures receive input from the basolateral amygdala (BLA), yet their involvement in fear-related behaviors can be clearly dissociated. Based on this evidence, there are two fear systems which can be anatomically dissociated, and the BNST is the integral component mediating the sluggish response system where once it is activated by a specific stimulus, it continues to influence behavior long after the initiating stimulus has been terminated, and this sustained response is what is referred to as anxiety. Fear, on the other hand, is mediated by the CeA, and can be characterized as a rapid response system that mediates short-term responses to specific threat cues.

In efforts to further investigate and clarify the hypothesis that the BNST is indeed involved in anxiety, Walker et al have (1997; 2003) shown that light enhanced startle was suppressed when the AMPA receptor antagonist, 2,3-dihydroxy-6-nitro-7-sulphamoylbenzo(F)-quinoxaline (NBQX) was injected into the BNST. In instances with CRH-enhanced startle which has been shown to be BNST-mediated and anxiogenic, it was found that when a visual CS was presented following CRH-enhanced startle, startle levels did not recede and remained elevated. This was distinct from results that were obtained when a specific fear eliciting stimulus (footshock) resulted in increases in startle which is mediated by the CeA, where during the subsequent presentation of the visual CS alone, startle levels were reduced to baseline levels (1997; 2003). These findings distinguish anxiety-related circuitry and behavioral responses from those associated with fear and further illustrate that the BNST is critical in mediating anxiety-like responses

whereas the CeA is the underlying neural mechanism for expression of fear. It is possible that anxiety is mediating the persistent elevations in ITI startle responses obtained in our experiments, and the BNST is the neural substrate responsible for that that prolonged enhancement.

Auditory stimuli have been shown to have many functions for numerous species and can be anxiety-provoking depending on their duration, intensity, and frequencies as previously mentioned. The utility of auditory stimuli inducing anxiety is that they can serve as warning signals which might provoke actions necessary for survival by organisms. The possibility of auditory stimuli being anxiogenic in the present experiments is suggested by their ability to augment startle responses (Campeau & Davis, 1992; Schanbacher et al., 1996), which has been established as being indicative of increases in anxiety levels. Moreover, animals that were exposed to auditory stimuli exhibited increases in c-fos mRNA induction in the BNST (Burow et al., 2005; Campeau et al., 1997; Campeau & Watson, 1997; Walker & Davis, 1997; Walker et al., 2003). Increases in c-fos mRNA in the BNST, a critical neural structure mediating anxiety, as a response to loud auditory stimuli, suggest that auditory stimuli may be involved in increasing levels of anxiety via activation of the BNST. It is possible that tone presentations during the present experiments produce increases in c-fos mRNA induction in the BNST, which may be associated with the enhanced startle responses during the tones. The activation of c-fos mRNA in the BNST may subside after the offset of the tones, but do not return to baseline levels observed during pre-tone startle stimuli presentations. Therefore during ITI startle stimuli presentations, startle responses are

enhanced relative to pre-tones although not to the same degree as during tone presentations, suggesting that the elevations in ITI startle responses are tone-induced and mediated by an anxiety-like state.

Interestingly, the current experiments, 60-dB tones, which constitute a background noise intensity, were able to induce persistent enhancements of ITI startle amplitudes, suggesting they are anxiogenic. In previous studies that have used background intensity noises, animals were not shown to exhibit anxious behaviors. One explanation for this is that the strain of mice used in the present experiments, C57BL/6J mice, are predisposed to being anxious (Clement et al., 2007; Jakovcevski, Schachner, & Morellini, 2007) and may have been hypersensitive to the intermittent tone presentations, resulting in increases in startle amplitudes. Prior experiments using background intensity noises typically used rats, which are less anxious by nature and therefore, may not be as responsive to auditory stimuli. Also, the main experimental paradigm used to assess the main elevated ITI startle amplitudes consisted of tones being administered randomly with variable 1-2 min ITI, making them unpredictable. In previous experiments in our lab, we noticed that giving systematic, predictable tones with a uniform ITI (3 min) throughout the session resulted in a less robust effect of ITI startle enhancement. These observations of predictability vs. unpredictability are consistent with studies that examined the effects of predictable vs. unpredictable auditory stimuli on anxiety levels using the place avoidance task (Herry et al., 2007). The design is such that on the first day, animals are allowed to freely explore two compartments via an alleyway. The compartment in which the animals spend more time in is designated as the preferred one. Results showed that

animals subsequently given unpredictable auditory stimuli in their preferred place displayed increased avoidance behavior compared to animals exposed to predictable stimuli, illustrating that unpredictability may induce higher anxiety-like responses. This suggests that the unpredictable distribution of the tones in our experiments may have induced increases in an anxiety-like state as well as indicated by the enhanced startle responses.

Increases in c-fos mRNA induction in the BNST parallel those found in the PVN in response to auditory stimuli (Burow et al., 2005). The PVN is a key structure in proximal effector circuits which appear to be responsible for activation of the HPA axis, a major component of the neuroendocrine system that regulates stress responses. Therefore, one question that may arise regarding the enhancement of ITI startle amplitudes observed in the present experiments is whether it is induced by stress, as defined by hypothalamic-pituitary-adrenal axis (HPA axis) involvement and/or anxiety and how we can distinguish them. Based on this evidence, it seems feasible that perhaps the elevation in ITI startle amplitudes we found may be at least partially due to stress effects and not entirely due to anxiety. While this might be the case, CRH-enhanced startle has been shown to be prevented with pretreatment with the CRH receptor antagonist, alpha-helical CRH. The effects of intracerebroventricular (i.c.v.) infusions were mimicked by intracisternal but not by intrathecal CRH infusions and were not disrupted by lesions of the paraventricular nucleus (PVN) of the hypothalamus (Liang et al., 1992). These findings suggest that the effects of CRH on startle were mediated directly by CRH receptors in the brain and did not involve activation of the CRH-

regulated HPA axis. This implies that if CRH-enhancement in startle amplitudes is not regulated by HPA axis activity, we might not expect that to be the case with our results either as our procedure does not interact with stress pathways as directly as CRH-enhanced startle might, and therefore suggests that the ITI startle enhancement observed in our experiments is mediated by an anxiety-like state instead.

Our experiments were conducted by measuring the acoustic startle reflex. Studies have found the acoustic startle pathway receives projections from neural substrates associated with fear and anxiety. Specifically, the basic acoustic startle circuit begins in the cochlear root neurons (CRN). The next synapse occurs in the area just medial to the ventral nucleus of the lateral lemniscus (VLL) which is known to receive projections from the CRN. From there, auditory information is sent to the ventromedial region of the nucleus reticularis pontis caudalis (RPC). Cell bodies in the RPC send their axons to all levels of the spinal cord by way of the reticulospinal tract. Fibers from the reticulospinal tract synapse in the spinal cord, forming the final synapse before the neuromuscular junction. The CeA and the BNST, mediating fear and anxiety responses, project directly into one of the brainstem nuclei, namely the RPC, essential for startle (Davis, 1989; Davis, Falls, Campeau, & Kim, 1993; Davis & Shi, 1999; Koch, 1999). This association between fear circuitry and the acoustic startle pathway enables us to use this procedure as a means of assessing levels of fear and anxiety. The tone presentations in our experiments may activate the amygdala and BNST that project into the RPC underlying the acoustic startle pathway, resulting in enhanced startle responses that are mediated by an anxiety-like state. Since buspirone attenuated this effect, it is feasible

that the anxiolytic drug may have reduced activations of the necessary anxiety-related structures, including the amygdala and BNST, which in turn attenuated startle responses during and after tone presentations, further supporting the hypothesis that our observed effect of enhanced startle responses is mediated by an anxiety-like state. In addition, because the acoustic startle response is straight forward and easily measured, this paradigm could provide a simple way for pharmaceutical companies to test the effects of drugs on anxiety levels in animals. The evidence here therefore supports the hypothesis that the startle procedure can provide a simple and reliable model for assessing anxiety.

Taken together, these results are consistent with our hypothesis that a persistent enhancement of startle amplitude after the offset of the tones is mediated by anxiety. It appears that anxiety is a longer lasting response that persists after the initiating stimulus is no longer present. Auditory stimuli may produce anxiety-like responses behaviorally and physiologically that are revealed by enhanced startle amplitudes caused by activation of the BNST. Although our results are consistent with effects of anxiety, further research needs to be conducted in order to validate the hypothesis that the elevation in ITI startle amplitudes is due to anxiety. Such studies would include lesioning the BNST and assessing the effects on ITI startle levels and testing the effects of different anxiolytic drugs such as valium or propranolol. Nevertheless, the elevation in ITI startle responses after the offset of the tones observed in the present experiments seem to reflect changes in affective states resembling anxiety introduced by the tones.

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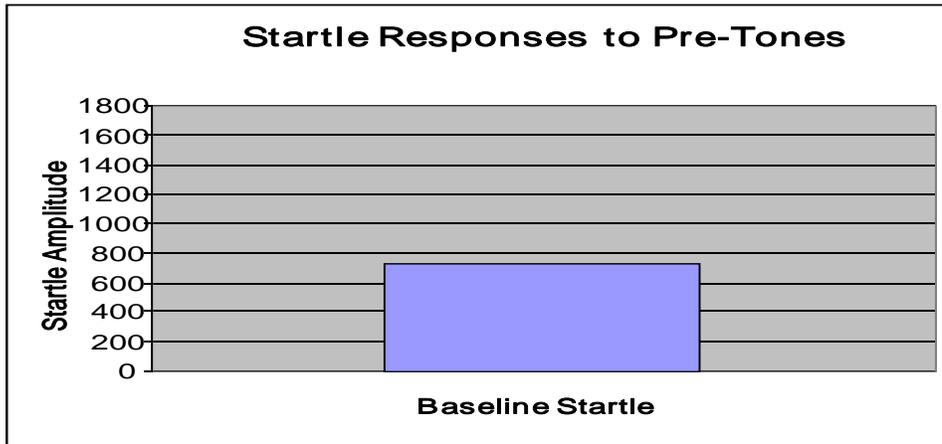
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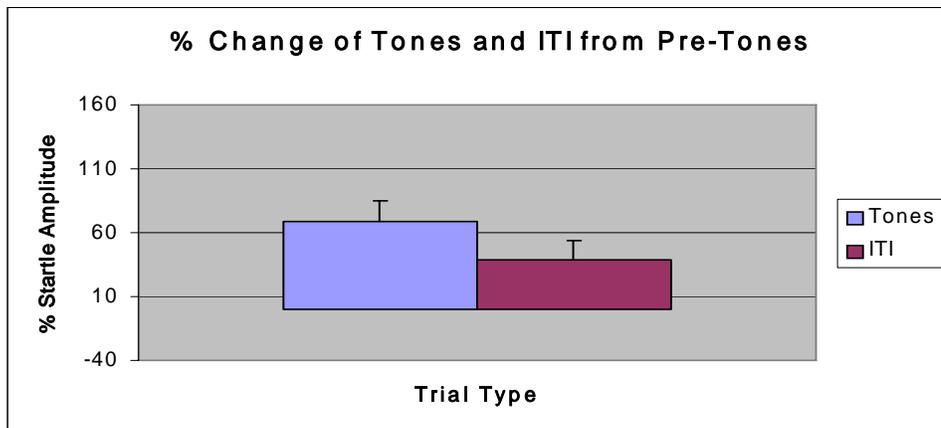
Figure 1:

Experiment 1A

a) Startle Amplitude to Pre-Tone Startle Stimuli



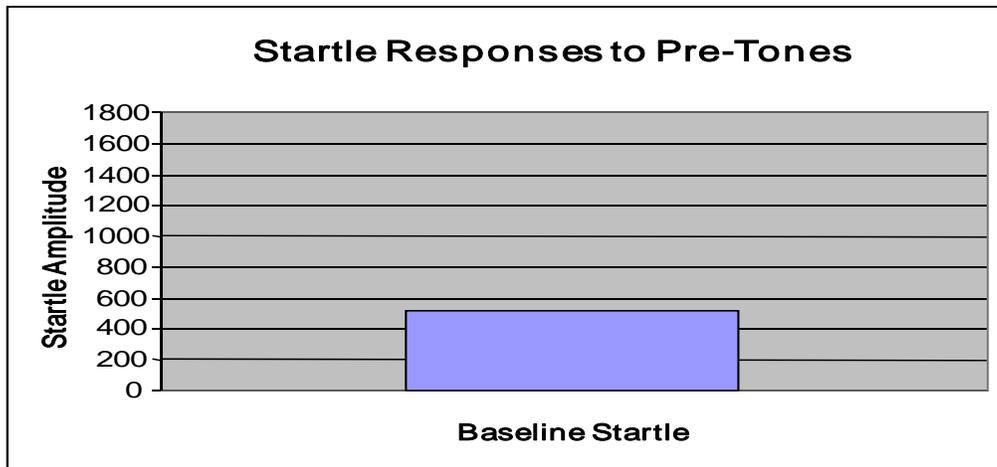
b) Percent Changes in Startle Amplitude during Tones and ITI from Pre-Tones



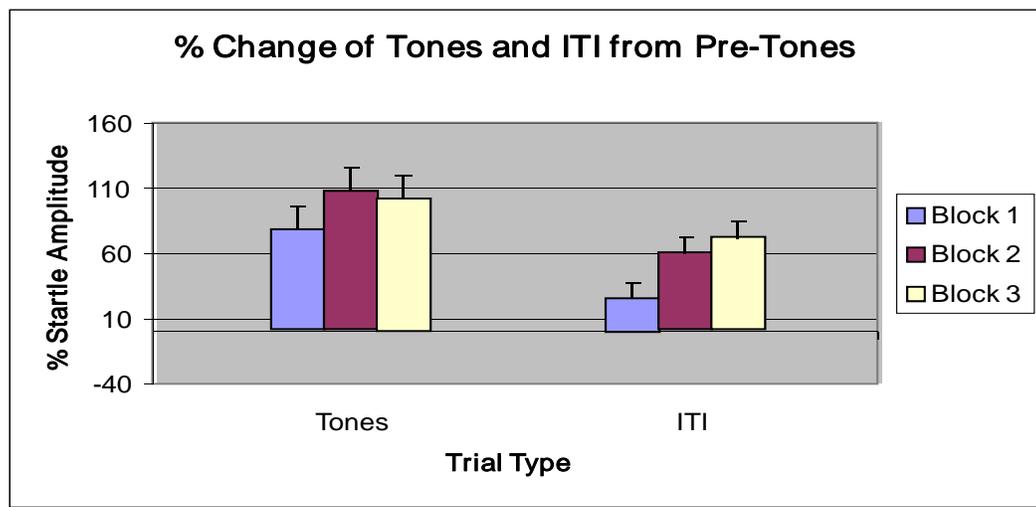
Figs. 1a and b: Percent changes from pre-tones show that startle responses during tones and ITI were significantly elevated. Tones also enhanced startle responses more than startle responses during ITI startle stimuli indicating that elevations in startle responses are tone-induced and elevated startle responses persist into the ITI.

Experiment 1B

c) Startle Amplitude to Pre-Tone Startle Stimuli



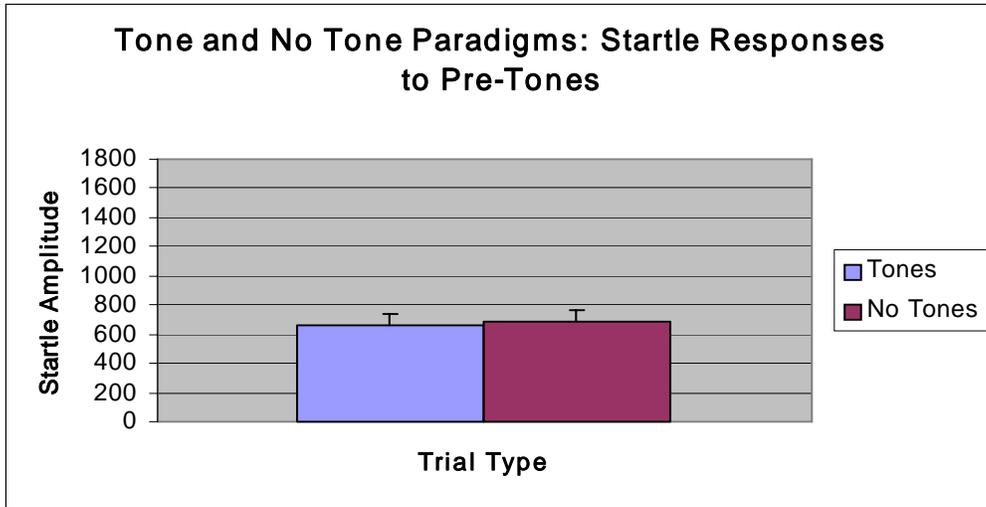
d) Percent Changes in Startle Amplitudes of Tones and ITI over Blocks (nine of each trial type per block)



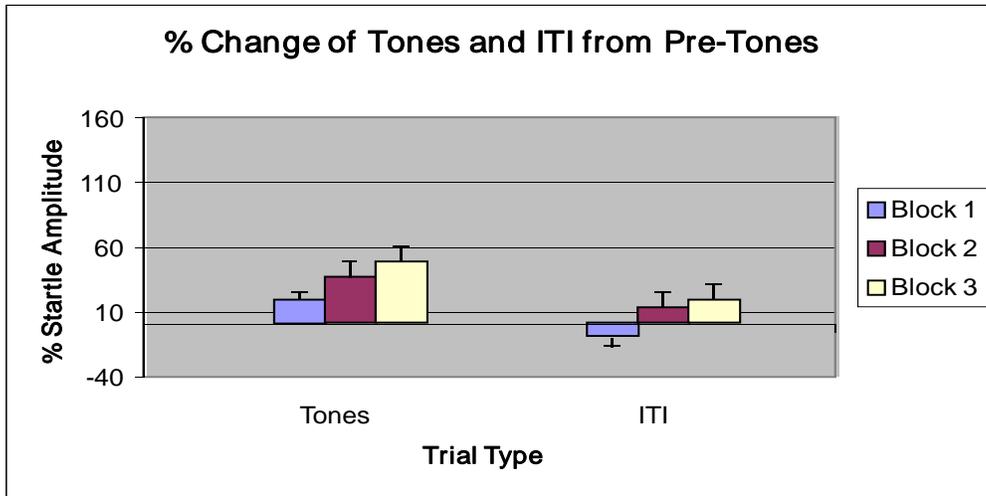
Figs 1c and d: Tone and ITI startle amplitudes were significantly elevated from pre-tones. In addition, ITI startle responses were shown to significantly increase over block.

Figure 2:

a) Startle Amplitudes to Pre-Tone Startle Stimuli for Tones and No-Tone Procedures



b) Tones- Percent Changes in Startle Amplitudes of Tones and ITI from Pre-Tones



c) No Tones-Percent Changes in Startle Amplitudes of (would-be) Tones and ITI from Pre-Tones

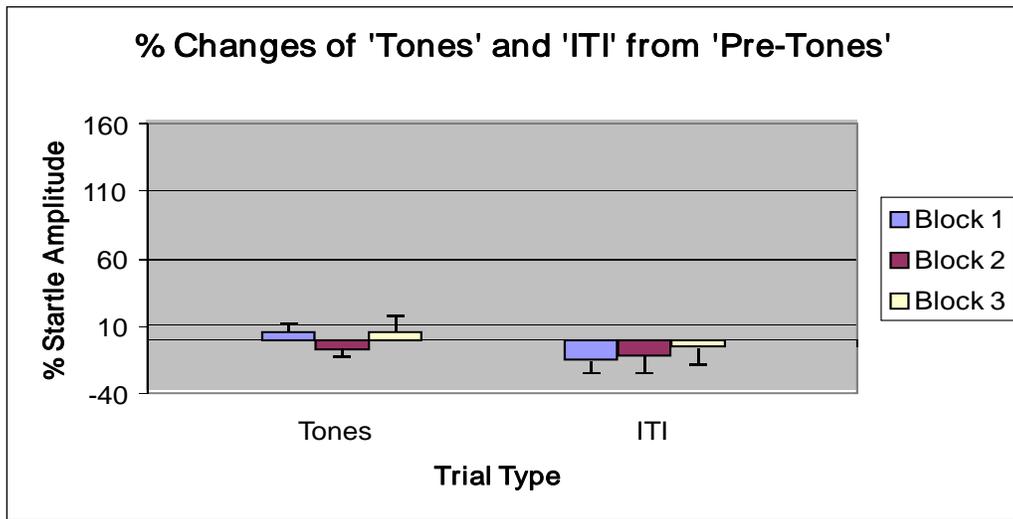
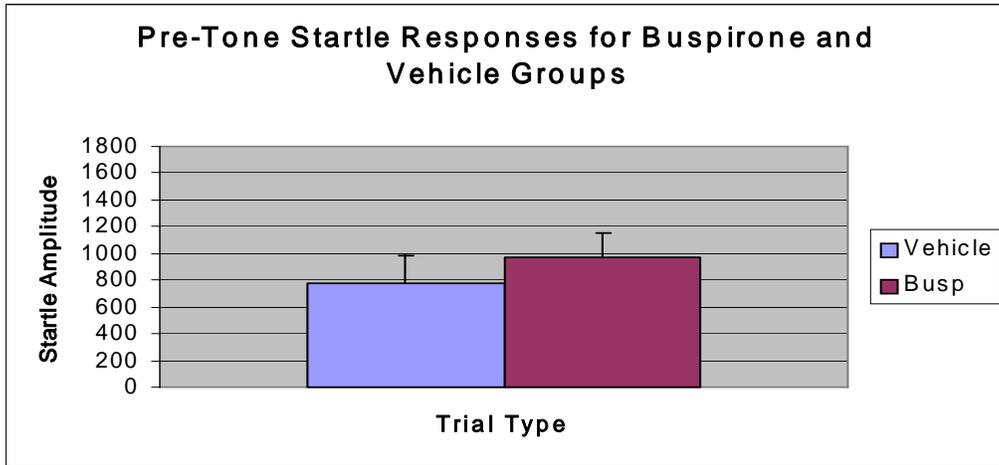


Fig 2: a) Pre-tones startle amplitudes were not significantly different from each other between Tone and No-Tone procedures. b) Tones and ITI were significantly elevated from pre-tones startle amplitudes. The ITI elevation also significantly increased over blocks. c) There was no significant difference in startle amplitudes across session.

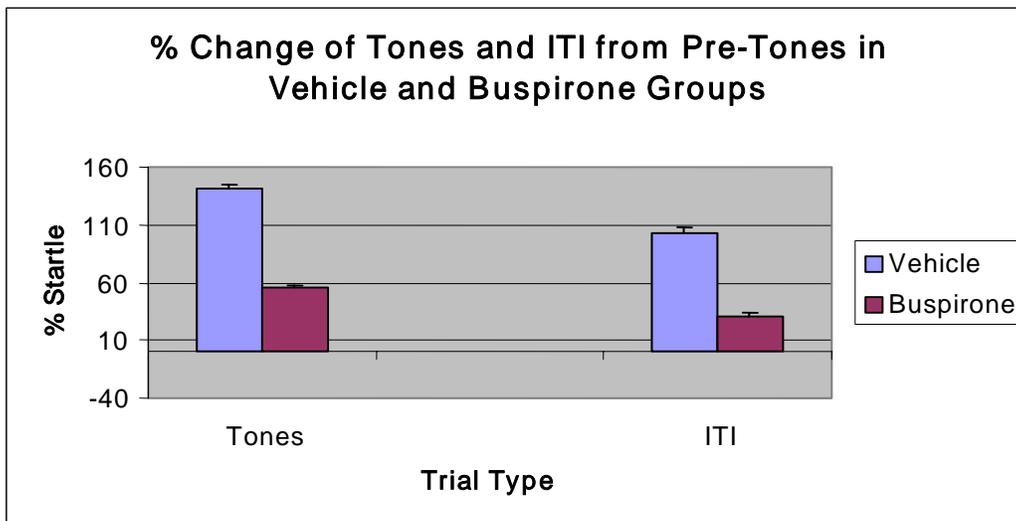
Figure 3:

a) Startle Amplitudes to Pre-Tone Startle Stimuli for Vehicle and Buspirone Animals



b) Percent Changes of Startle Amplitudes during Tones and ITI from Pre-Tones for

Vehicle and Buspirone Animals



c) Startle Amplitudes to Tones for Vehicle and Buspirone Animals

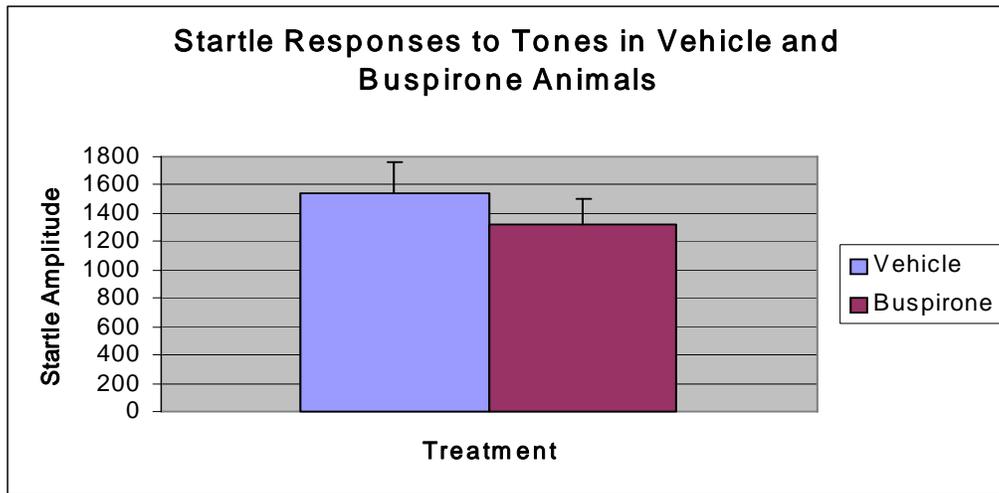


Fig 3: (*Vehicle: N=12, Buspirone: N=12*) a) Pre-tone startle amplitudes were not significantly different between buspirone and vehicle animals. b) Startle amplitudes of tones and ITI in both buspirone and vehicle animals were significantly enhanced from pre-tones. Percent changes in tone were significantly different from percent changes of ITI startle amplitudes between buspirone and vehicle animals. c) Startle amplitudes to tones were shown not to be significantly different between buspirone and vehicle animals.

