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ASSESSMENT OF NEUROTENSIN RECEPTOR AGONIST EFFECTS ON FEAR-POTENTIATED STARTLE

By

Mark Aaron Vanden Avond

THESIS

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Title of Thesis: ASESSMENT OF NEUROTENSIN RECEPTOR AGONIST EFFECTS ON FEAR-POTENTIATED STARTLE

This thesis by Mark Vanden Avond is recommended for approval by the student's Thesis Committee and Department Head in the Department of Psychology and by the Assistant Provost of Graduate Education and Research.

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ABSTRACT

ASSESSMENT OF NEUROTENSIN RECEPTOR AGONIST EFFECTS ON FEAR-POTENTIATED STARTLE

By

Mark Aaron Vanden Avond

Systemic administration of the NTS_1 receptor agonist PD149163 has exhibited anxiolytic effects in male rats. The present study sought to further evaluate the potential anxiolytic effects of PD149163 by assessing this compound in both male and female C57BL/J6 mice using the fear-potentiated startle (FPS) paradigm. Startle chambers were equipped with a shock-grid floor, fluorescent light, and an acoustic startle speaker. Conditioning took place between the light and floor shock, and test sessions measured startle to a 90 dB noise burst while the light was on (FPS) or off. Startle magnitude did not differ between the male and female mice. PD149163 produced a significant difference between male and female mice startle response and a significant reduction in FPS in females. The NTS_2 receptor agonist β-Lactotensin produced a sex difference at an intermediate dose. The anxiolytic and partial 5-HT_{1A} agonist buspirone did not produce a significant difference in FPS. The reduction in FPS by PD149163 coincides with previous studies conducted in male rats. The reduction in FPS found in female mice suggests that more research is needed to examine the neurotensin system and sex differences. Overall, these findings support targeting the neurotensin system for the development of novel strategies for treating anxiety disorders.

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INTRODUCTION

Anxiety Disorders

The Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5) defines anxiety as the exaggerated anticipation of a future threat and is associated with muscle tension and preparation for future danger. While anxiety and fear are related, the DSM-5 clarifies that fear is an emotional response to a real or perceived imminent threat with autonomic arousal necessary for fight or flight, whereas anxiety is an exaggerated response to a future threat (American Psychiatric Association, 2013). A substantial amount of research shows that anxiety disorders can persist throughout one's lifetime if left untreated (Beesdo, Knappe, and Pine, 2009; Beesdo, Pine, Leib, and Wittchen, 2010; Burstein, Beesdo-Baum, He, and Merikangas, 2014; Kessler, Andrade, Bijl, Demler, and Stein, 2002; Kessler et al., 2005; Lieb, Becker, and Altamura, 2005; Mohr & Schneider 2013; Wittchen, 2002).

Separation anxiety disorder, selective mutism, specific phobia, social anxiety disorder, panic disorder, agoraphobia and generalized anxiety are defined as anxiety disorders in the DSM-5. While each anxiety disorder has a specific definition, there are common symptoms among each disorder. These common symptoms are: a reaction that is more intense, to a stimulus, event, or perceived stimulus, actively avoiding the stimulus, event, or perceived stimulus, and occurs for six months or longer (American Psychiatric Association, 2013).

Anxiety disorders not only cause a burden on the individual, but also on society. About forty-two percent of adults have or have had anxiety disorders and anxiety disorders are the second highest prevalence for mental disorders (Kessler, Petukhove, Sampson, Zaslavsky, and Wittchen, 2012; Lieb et al., 2005). Lieb et al. (2005) estimates that generalized anxiety disorder costs around $250 per month for an individual, and Eaton, Martins, Nestadt, Binevenu, Clarke, and Alexandre (2008) estimates around $11 billion per year for specific phobia. Anxiety can cause a decrease in work production and quality of life, an increase in seeking medical practices, and impairment and disability (Leib et al., 2005; Wittchen, 2002).

Anxiety disorders are more prevalent in women in their lifetime. In their lifetimes, about thirty-three percent of women will be diagnosed with an anxiety disorder compared to twenty-two percent of men (McLean, Asnaani, Litz, and Hofmann, 2011). Girls have been shown to have rates of anxiety disorders twice that of boys at as early as the age of six (Lewinsohn, Gotlib, Lewisohn, Seeley, and Allen, 1998). Women with anxiety disorders are more likely than men to seek medical help and will miss more days of work (McLean et al., 2011).

Neurocircuitry of Anxiety

The neurocircuity mediating anxiety involves complex interactions between a number of structures, including the amygdala, septum, ventral tegmental area, periaqueductal gray (PAG), hypothalamic-pituitary-adrenal axis, and the orbitofrontal cortex.

The amygdala, in particular, has long been considered a "fear center" in the brain. In humans, higher amygdala volume is correlated with more anxiety (Qin, Young, Daun, Chen, Supekar, and Menon, 2014). The amygdala mediates fear and anxiety in animal behavioral models. The basolateral amygdala responds to cues that predict danger (Amano, Duvarci, Popa, and Pare, 2011). The basolateral amygdala projects to the central nucleus of the amygdala (Tye et al., 2011). Hitchcock & Davis (1986) found that in male rats bilateral lesions of the central nucleus of the amygdala blocked the potentiation of the startle reflex using a fear-potentiated startle paradigm in their experiment. An external cue, which was previously combined with an aversive stimulus, was used to produce an exaggerated startle response in the fear-potentiated startle paradigm. In male rats, an electrolytic lesion of the pathway between the central nucleus and the caudal lateral hypothalamus also blocked the fear-potentiated startle response, providing further evidence that the amygdala is necessary. Moreover, the lateral hypothalamus may play a role as well (Hitchcock & Davis, 1991). The central nucleus of the amygdala also projects to the nucleus reticularis pontis caudalis (Rosen, Hitchcock, Sananes, Miserenino and Davis, 1991). The nucleus reticularis pontis caudalis is important for the production of the fear-potentiated startle response. Davis, Gendelman, Tischler and Gendelman (1982) lesioned this area in male rats which abolished the acoustic startle, and lesions more rostral, caudal or dorsal did not abolish the startle, providing evidence that this area is important for the fear-potentiated startle response.

The septum is another important region in the production of anxiety-related behaviors. The lateral septum has been shown to connects the amygdala to the hypothalamus in a neural circuit implicated in anxiety-related behaviors (Calhoon & Tye,

2015). The elevated-plus maze is another behavioral test used to measure levels of anxiety. Anxiety is assessed in an elevated-plus maze by recording the number of entries and total time spent in open arms (i.e., those without walls) compared to closed arms of a maze positioned at a certain height (e.g., 50 cm) above the floor. An increase in entries and time spent in the open arm of the elevated-plus maze shows a decrease in anxiety-related behavior. A group of male rats with the lateral septum lesioned showed an increase in the percentage of open arm entries and percentage of time spent in the open arm. Similar results were found in male rats with medial septal lesions (Menard & Treit, 1995).

The ventral tegmental area (VTA) in the midbrain is also important for stress and anxiety-related behaviors. Quinpirole, a dopamine $D_{2/3}$ agonist, administered into the VTA has blocked fear-potentiated startle. Another study showed that lesions of the medial ventral tegmentum also blocked fear-potentiated startle. These findings provide evidence that dopamine neurons in the VTA are important for anxiety (Munro & Kokkinidis, 1997; Borowski & Kokkinidis, 1996). Mukherjee et al. (2010) showed that when circadian locomotor output cycles kaput (Clock) genes are deleted in mice, $Clock^{-/-}$ mice had an increased firing rate of dopaminergic neurons in the VTA. The $Clock^{-/-}$ mice showed lower anxiety behavior indicatd by increased time spent in the open arm of the elevated-plus maze and time spent in the middle of an open field compared to wild-type mice. These results suggests that there is less anxiety in the $Clock^{-/-}$ mice. Reduced anxiety was no longer evident after Clock protein levels in the VTA of $Clock^{-/-}$ mice returned to levels comparable to wild-type mice via viral-mediated gene transfer (Roybal et al., 2007).

Lesions of the PAG before or after fear-conditioning training (light + shock conditioning sessions) provided evidence that the PAG is implicated in the expression of fear-potentiated startle response. Lesioning the PAG of male rats before or after training inhibited potentiated startle caused by a light cue (Fendt, Koch, and Schnitzler, 1996). Pharmacologically, intra-PAG infusion of the serotonin $(5\text{-HT})_{2B/2C}$ receptor agonist, meta-chlorophenylpiperazine (mCPP), decreased anxiety-like behavior in male mice using the elevated-plus maze. Pretreatment of the $5\text{-HT}_{2A/2C}$ receptor antagonist, ketanserin, blocked the anxiolytic effects of mCPP which provides evidence that the 5-HT_{2C} receptor is important for anxiety in the PAG (Nunes-de-Souza, Nunes-de-Souza, Rodgers, and Canto-de-Souza, 2008).

The hypothalamic-pituitary-adrenal axis mediates sympathetic nervous system activity. The corticotrophin-releasing hormone neurons in the hypothalamus activate the anterior pituitary gland. The pituitary gland, in turn, releases adrenocorticotropic hormone, which causes the adrenal gland to release cortisol. The corticotrophin-releasing hormone neurons in the hypothalamus are activated in preparation for an urgent situation. Flandreau, Ressler, Owens, and Nemeroff (2011) have shown that a hyperactive hypothalamic-pituitary-adrenal axis can cause behavior associated with anxiety. This study examined Wistar rats using a battery of anxiety tests, including the open field test, elevated plus maze, defensive withdrawal, and forced swim test and showed an increase in adrenocorticotropic hormone concentrations.

The orbitofrontal cortex is important in processing reward and punishment, which assigns value to stimuli. The medial orbitofrontal cortex examines the reward value of stimuli, and the lateral orbitofrontal cortex examines the aversive properties of a stimulus

(for review, see Kringlebach and Rolls, (2004). Thus, the amygdala and orbitofrontal cortex act together to assign fear memories to conditioned stimuli.

Common Pharmacological Treatments for Anxiety Disorders

Historically, barbiturates were some of the first drugs used to treat anxiety (Lopez-Munoz, Ucha-Udabe and Alamo, 2005). Barbiturates bind to an allosteric site on the $GABA_A$ receptor, causing a conformational change that increases chloride conductance when the receptor is activated by an agonist (Sankar, 2012). Dixon, Rosahl and Stephens (2008) used $GABR_{A2}$ knockout mice, which are missing the genes that encode the $GABA_A$ α_2-subunit, and showed that pentobarbital hydrochloride did not have any anxiolytic effects. This provides evidence that the $GABA_A$ α_2-subunit is important for the allosteric site that barbiturates bind to, and therefore is important for the anxiolytic effects (i.e., anti-anxiety effects) of barbiturates. However, barbiturates have negative effects. Barbiturates have a high abuse potential (Lopez-Munoz et al., 2005; McClane & Martin, 1976) and a marginal therapeutic range during chronic use. Thus, chronic barbiturates use can easily lead to overdose (Lopez-Munoz et al., 2005). Moreover, barbiturates have been linked to many suicides (Gunnell & Eddleston, 2003).

Benzodiazepines were discovered in the 1960's. Benzodiazepines, like barbiturates, affect the GABA receptor (Sigel & Buhr, 1997). Like barbiturates, benzodiazepines will bind an allosteric site on the $GABA_A$ receptor and increase the rate of which Cl^- channels open to increase chloride conductance (Sankar, 2012). The benzodiazepine chlordiazepoxide increased the amount of entries to the open arm of the elevated-plus maze in mice when compared to saline, providing evidence that chlordiazepoxide decreases anxiety, which is also consistent with clinical evidence

(Belzung, Le Guisquet, and Griebel, 2000). The benzodiazepine diazepam decreased fear-potentiated startle in male mice (Risbrough, Brodkin, and Geyer, 2003) and increased time spent in the open arms of an elevated plus maze (Cole & Rodgers, 1995). Benzodiazepines replaced barbiturates for the treatment of anxiety disorders because the risk of abuse potential is relatively lower compared to barbiturates (Smith & Rudolph, 2012). Benzodiazepines can cause sedation and cognitive deficits and long-term use can lead to dependency and withdrawal symptoms (Durham, 2007; Glombok, Moodley, and Lader, 1988). Also, benzodiazepines can cause psychomotor retardation, which can produce slower reaction times that can impair driving skills and can cause anterograde amnesia (Longo and Johnson, 2000).

Antidepressants were used in the 1960's for the treatment of anxiety disorders. The first antidepressants used for anxiety were monoamine oxidase inhibitors (MAOI), but tricyclic (TCA) and serotonin reuptake inhibitor (SSRI) antidepressant drugs are more widely used for long-term treatment of anxiety disorders (Sargant and Dally, 1962; Durham, 2007). MAOIs increase monoamines in the synapse by inhibiting the enzyme that breaks down the monoamines. TCAs increase synaptic serotonin and norepinephrine by blocking the reuptake mechanism, along with binding to other receptors, such as the histamine H_1 receptor (Owens, Morgan, Plott, and Nemeroff, 1997). SSRIs are effective by inhibiting the serotonin reuptake transport, which increases serotonin in the synapse. MAOIs and TCAs are usually prescribed when SSRIs are not treating anxiety disorders effectively, and are second- or third-line treatments due to their potential side effects. (Sayed, Horn, and Murrough, 2014). For example, MAOIs interact with foods containing tyramine, such as cheese and wines, and can lead to hypertension (Gardner, Shulman,

Walker, and Tailor, 1996). Teixeira, Zangrossi, and Graeff (2000) showed that acute administration of the antidepressant imipramine increased escape latencies, while chronic imipramine reduced escape latencies in male rats. Similar acute effects were found using sertraline, an SSRI. Sertraline increased startle in a fear-potentiated startle procedure, which could be an indication of increased anxiety. Fluoxetine treatment did not show a significant difference (Steiner, Lecourt, and Jenck, 2012). While efficacy for both tricyclic antidepressants and SSRIs are similar, SSRIs are prescribed more frequently due to their safety and tolerability (Zohar, 2000). Antidepressant drugs take a few weeks for any therapeutic effects to occur. Along with delayed activation, antidepressant drugs are only effective for about sixty percent of patients (Prus, 2014). This could be because male rats given acute administration of fluoxetine, sertraline, and the 5-HT agonist mCPP displayed decreased social interactions and increased self-grooming (Bagdy, Graf, Anheuer, Modos, and Kantor, 2001). Decreasing social interactions between rats and increasing self-grooming is an indication of high levels of anxiety.

Neurotensin

Neurotensin (NT) is a 13-amino-acid neuropeptide found in the central nervous system and peripheral nervous system. As many other neuropeptides, NT acts as a neuromodulator in the nervous system and is closely associated with dopamine systems (St-Gelais, Jomphe, and Trudeau, 2006). In the VTA and substantia nigra NTS_1 receptors are expressed on about eighty to ninety-five percent of dopamine neurons (Binder, Kinkead, Owens and Nemeroff, 2001; Dana et al., 1989). Dopamine neurons either increase or decrease firing depending on the abundance of NT; high concentrations of NT will increase dopamine firing while low concentrations of NT will decrease dopamine

firing (Jiang, Pessia, and North, 1994; Farkas, Chien, Shigehiro and Nakajima, 1997; Wu & Wang, 1995). NT utilizes three receptor iosforms, NTS_1, NTS_2 and NTS_3/sortilin receptors, and has the highest affinity for NTS_1 receptors followed by NTS_2 receptors. The neurotensin receptors are g-protein coupled receptors (Luca et al, 2003), which interact with dopamine receptors to decrease D_2 receptor agonist binding affinity (Binder, Kinkead, Owens, and Nemeroff, 2001).

The NTS_1 receptor can be found throughout many brain areas, which corresponds to evidnece that NT plays a role in anxiety, schizophrenia, drug abuse, neurodegenerative diseases, pain, and many other disorders (St-Gelais et al., 2006; Prus, Hillhouse, and LaCrosse, 2014). Boudin, Pelaprat, Rostene and Beaudet (1996) were the first to image the NTS_1 receptor in the whole mammalian brain using immunohistochemistry to identify the receptor (see table 1). Of particular relevance to anxiety, NTS_1 receptors were found in the posterior cortical nucleus of the amygdala on perikarya, dendrites, and axon terminals. The hippocampus also contains NTS_1 receptors on cell bodies, dendrites, and axon terminals. In the diencephalon, the thalamus and anterior dorsal nucleus found perikarya labeled for NTS_1 receptors. The hypothalamus contained NTS_1 receptors on axon terminals throughout the medial and lateral subdivisions and in the median eminence.

Table 1: Neurotensin receptor locations

Brain area	Dendrites	Perikarya/ Cell Body	Axon	Terminals
Frontal Cortex: Layer II-III		+	+	
Frontal Cortex: Layer IV	+			
Frontal Cortex: Layer V		+		
Parietal Cortex: Layer II-III		+		

Parietal Cortex: Layer IV	+			
Parietal Cortex: Layer V		+		
Anterior Cingulate Cortex: Layer IV				+
Endopiriform Cortex: Layer IV				+
Insular Cortex: Layer IV				+
Perirhinal Cortex: LayerI-III and VI				+
Entorhinal Cortex		+		
Retrosplenial Cortex: Layers I				+
Retrosplenial Cortex: Layers II-III		+		+
Caudate Putamen		+		+
Nucleus Accumbens: Core and Shell			+	
Anterior Commissure			+	
Islands of Calleja	+	+		
Septum		+	+	
Broca		+		
Preoptic Nucleus	+			
Bed Nucleus of the Stria Terminalis		+	+	
Amygdala	+	+		+
Thalamus		+		
Optic Tract	+	+		
Hypothalamus				+
Suprachiasmatic Nucleus			+	
Lateral Mammillary Nucleus			+	
Subthalamus	+	+		
Epithalamus		+		
Habenula		+		
Substantia Nigra	+	+		
Pars Compacta	+	+		
Ventral Tegmental Area	+	+		
Interfascicular Nucleus	+	+		
Nucleus Raphe Linearis Caudalis	+	+		
Periaquductal Gray	+	+		
Dorsal Raphe	+	+		
Latrodorsal Tegmental Nuclei	+	+		
Tegmentum			+	
Locus Coeruleus			+	
Tegmental Nucleus		+		
Medulla		+		
Pontine Nuclei		+		
Reticular Formation		+		
Inferior Olivary Nucleus	+	+		
Paragigantocellular Nucleus	+	+		
Vagus	+	+		
Solitary Tract				+

While NT is found throughout the brain and has different behavioral implications, little has been studied with NT and differences between males and females. NT expression is similar in male and female rats until puberty when sex hormones begin to change NT levels (Bello et al., 2004; Ciofi, 2000). Ovariectomized female rats given estradiol treatments expressed more NT when compared to ovariectomized female rats that were not given estradiol treatments (Ciofi, 2000). However, Dufourny & Warembourg (1999) did not find ovariectomized female guinea pigs to have a significant change in NT immunoreactivity when subjects were given estradiol treatments. The differences in NT expression post estradiol treatment could be due to species differences. Mice could have a more similar NT system to primates than rats. In areas of the brain, such as the subthalamic nucleus, mice and primates express NT mRNA while rats do not. While rats did not have a NT containing neurons in some areas, rats also had neurotensin containing neurons in areas where mice and humans did not. A neurotensin-dopamine pathway projects to the prefrontal cortex, the nucleus accumbens and amygdala in rats, but is not found in mice or humans (Smits, Terwisscha, van Scheltinga, van der Linden, Burbach, and Smidt, 2004). NT concentrations were found to be different between males and females in a number of brain regions, including: the prefrontal cortex, nucleus accumbens, hippocampus, and substantia nigra. Due to the estrous cycle of female rats, NT concentrations also vary in the VTA, nucleus accumbens, and anterior caudate/putamen depending on where the female is during the cycle (Kinkead et al., 2000).

Neurotensin Pharmacological Agents in Anxiety Models

Few studies have examined the potential effects of NT on anxiety. Fitzpatrick et al. (2012) have found that NTS_1 receptor knockout male mice traveled less and spent less time in the center using an open field test compared to wild-type controls. These effects have been associated with higher levels of anxiety. However, a significant difference was not found between the knockout mice and controls using an elevated plus maze. These findings show that the 'anxious' phenotype of the knockout mice might be dependent on the environment and context. Further research needs to examine the effects that environment and NT has on anxiety. Ollmann et al. (2015) demonstrated an increase in time spent in the open arms of an elevated plus maze after bilateral microinjections of NT into the ventral pallidum in male rats showing an anxiolytic effect. PD149163, a NT_1 receptor agonist, has been shown to decrease conditioned footshock-induced ultrasonic vocalizations, which is an indication of anxiolytic effects (Prus et al., 2014). Shilling & Feifel (2008) found that PD149163 reduced fear-potentiated startle in male rats, but also decreased the startle magnitude. This suggests that PD149163 may produce unintended effects, such as decreased locomotor activity, which could explain the decreased fear-potentiated startle effect. An even smaller amount of research has been conducted on the pharmacology of the NTS_2 receptor and the effects on anxiety. Male wild-type mice were given β-lactotensin, a NTS_2 receptor agonist, and time spent in the open arms of the elevated plus maze increased (Hou et al., 2011). Further research needs to examine the effects of NTS_2 receptor agonists using other paradigms for anxiety.

Fear-Potentiated Startle Paradigm

The fear-potentiated startle paradigm was first introduced in 1951 partly on the anecdotal observation that patients with an anxiety disorder had an exaggerated startle response to a sudden loud noise (Brown, Kalish, and Farber, 1951). Initially, rats were conditioned using a light-buzzer conditioned stimulus (CS) presented for five seconds with a unconditioned stimulus (UCS) shock initiating for the last two seconds of the CS. It was believed that the CS-UCS pairing would lead to an anticipatory fear reaction. To test this, a startle stimulus was presented in place of the shock and the magnitudes of the jumps were recorded using a stabilimeter-like apparatus. The magnitudes of the jumps were compared to a group that did not have the CS-UCS presented simultaneously, but were presented the same amount of light-buzzer and shocks as the experimental group. The experimental group produced a higher startle magnitude to a sudden sound when compared to the control group (Brown et al., 1951).

Further studies have used pharmacological agents to study the effects on fear-potentiated startle. Extensive research has evaluated treatments that alter neurotransmitters and their effects on potentiated startle (see table 2) (Cassella & Davis, 1985; Chi, 1965; Davis, 1979; Davis, 1986; Davis, Cassella, and Kehne, 1988; Davis, Falls, Campeau, and Kim, 1993; Davis, Redmond, and Baraban, 1979; Hijzen & Slangen, 1989).

Table 2: Effect of drugs on FPS

Drug	Receptor site	Agonist or antagonist	Effects on potentiated startle
Clonidine	α_1 adrenergic	Agonist	Blocked Startle
Imipramine (acute	SERT & NET; Histamine H1; cholinergic muscarinic	Reptake inhibitor/antagonist	No Effect
Piperoxane	α_2 adrenergic/histamine H_1	Antagonist	Increase Startle
Propranolol	β_1 adrenergic	Antagonist	Decrease Startle
WB4101 (2-(2,6-Dimethoxyphenoxyethyl)aminomethyl-1,4-benzodioxane hydrochloride)	α_1 adrenoceptor	Antagonist	No Effect
Yohimbine	α_2 adrenoceptor	Antagonist	Increase Startle
Amobarbital	$GABA_A$	Positive modulator	Blocked Potentiation
Diazepam	$GABA_A$	BZ site agonist (positive modulator for $GABA_A$)	Blocked Potentiation
DMCM (methyl-6,7-dimethoxy-4-ethyl-beta-carboline-3-carboxylate)	GABA	Antagonist	Increased Startle
Flumazenil	$GABA_A$	Antagonist	No Effect
Flurazepam	$GABA_A$	BZ site agonist (positive modulator for $GABA_A$)	Blocked Potentiation
Midazolam	$GABA_A$	BZ site agonist (positive modulator for $GABA_A$)	Blocked Potentiation
Nicotine	Cholinergic Nicotinic	Agonist	Decreased Startle

Scopolamine	Cholinergic Muscarinic	Antagonist	No effect
Raclopride	Dopamine $D_{2/3}$	Antagonist	Decreased Startle
SCH23390	Dopamine D_1	Antagonist	Decreased Startle
SCH23390 + 8-OH-DPAT	Dopamine D_1 + Serotonin 5-HT_{1A}	Antagonist + Agonist	Blocked Potentiation
SCH23390 + Ipsapirone	Dopamine D_1 + Serotonin	Antagonist + Partial Agonist	Blocked Potentiation
Cocaine	DAT/SERT/NET	Reuptake inhibitor	Increase Startle
d-amphetamine	Dopamine releaser	VMAT & DAT blockers	Increase Startle
Morphine	Opioid Mu receptor	Agonist	Blocked Potentiation
Naloxone	Opioid Mu	Antagonist	No Effect
Buspirone	Serotonin 5-HT_{1A}	Partial Agonist	Blocked Potentiation
Cinanserin	Serotonin 5-$HT_{2A/2C}$	Antagonist	No Effect
Cyproheptadine	Histamine H_1	Antagonist	No Effect
Gepirone	Serotonin 5-HT_{1A}	Partial Agonist	Blocked Potentiation
Tropisetron	Serotonin 5-HT_3	Antagonist	Decreased Startle
Ipsapirone	Serotonin 5-HT_{1A}	Partial Agonist	Decreased Startle
Ketanserin	Serotonin 5-$HT_{2A/2B/2C}$	Antagonist	No Effect
m-CPP	Serotonin 5-HT_{2C}	Agonist	No Effect
MDL73005EF	α_1 adrenoceptor	Antagonist	Blocked Potentiation
Methysergide	Serotonin 5-$HT_{2B/2C}$ & 5-HT_{1A}	Antagonist & Agonist	Decreased Startle
Ondansetron	Serotonin 5-HT_3	Antagonist	Decreased Startle
p-Chloroamphetamine	Serotonin releaser		No Effect
Fenclonine (para-	Serotonin depleter		Decreased

chlorophenylalanine)			Startle

Drugs that affect the adrenoceptors were shown to have different effects on potentiated startle. Agonists, such as clonidine or propranolol, blocked potentiated startle, while antagonists, such as piperoxan and yohimbine, increased potentiated startle. Imipramine (acute and chronic) and WB4101, an agonist and antagonist respectively, had no effects on potentiated startle (Davis et al., 1979; Davis et al., 1993; Cassella & Davis, 1985).

Drugs that facilitate GABA neurotransmission were found to inhibit potentiated startle. Positive modulators of the GABA receptor, such as amobarbital, diazepam, flurazepam and midazolam, blocked potentiated startle, while DMCM and flumazenil, $GABA_A$ receptor antagonists, increased and had no effect on potentiated startle, respectively (Chi, 1965; Davis, 1979; Davis et al., 1979; Davis et al., 1993; Hijzen & Slangen, 1989).

Drugs that affect dopamine receptors have shown a differential effect. Dopamine releasers, such as cocaine and *d*-amphetamine, increased potentiated startle, while dopamine receptor antagonists, such as raclopride and SCH23390, decreased potentiated startle. Dopamine receptor antagonists in combination with serotonin receptor agonists, SCH23390 + 8-OH-DPAT, SCH23390 + ipsapirone, have blocked potentiated startle (Davis et al., 1993; Borowski & Kikkindis, 1998).

Many drugs have been used to study the effects of the 5-HT receptor and their effects on FPS. Partial agonists at $5\text{-}HT_{1A}$ receptors, such as buspirone, gepirone, and

ipsapirone, either block or decrease potentiated startle. Cinanserin, cyproheptadine and ketanserin, all 5-HT receptor antagonists, had no effect potentiated startle, while other 5-HT receptor antagonists, tropisetron, methysergide, ondansetron, and fenclonine, decreased potentiated startle (Mansbach & Geyer, 1988; Davis et al., 1988; Davis et al., 1993). The differential effects of the 5-HT receptor antagonists may be due to the different receptor subtypes affected.

Lesion studies have identified structures important for FPS. Tischler & Davis (1983) have found that lesions of the dorsal nucleus of the lateral geniculate nucleus, deep layers of the superior colliculus, visual cortex, and posteroventral region of the nucleus of the lateral lemniscus attenuated or eliminated potentiated startle, while lesions to the pretectal nuclei, superficial layers of the superior colliculus, thalamic reticular nucleus, nucleus reticularis pontis caudalis or dorsal nucleus of the lateral lemniscus did not attenuate potentiated startle. Lesions of the amygdala blocked a potentiated startle while lesions to the cerebellum or red nucleus did not (Hitchcock & Davis, 1986). Lesions to the caudal ventral amygdalofugal pathway and substantia nigra blocked potentiated startle, while lesions to the rostral ventral amygdalofugal pathway and 6-OHDA lesions of substantia nigra did not block potentiated startle (Davis, 1986). With the main "fear center" in the brain being the amygdala, Campeau & Davis (1995) showed that lesions to the central nucleus and basolateral complex of the amygdala blocked potentiated startle. When the hippocampus was lesioned freezing was attenuated, but fear-potentiated startle was not affected (McNish, Gewirtz, and Davis, 1997). Thus, lesion studies strongly implicate the amygdala as necessary for FPS.

Most FPS research has involved rodents as test subjects; however, primates and humans have also been studied and can exhibit a FPS response (Grillon & Davis, 1997). Diazepam and morphine decrease potentiated startle in a dose-dependent manner in rhesus monkeys, an effect previously found in rodents (Winslow, Nobel, and Davis, 2007). Norrholm et al. (2006) were the first to show within-session fear extinction and reinstatement using startles in humans. This is important, because humans and non-human animals show similar physiological effects; there is a greater translational value in studying when studying non-human animals. The next logical step would be to examine how anti-anxiety drugs affect FPS in humans, and Patrick, Berthot, and Moore (1996) showed that diazepam, a clinically used benzodiazepine, blocked potentiated startle, an effect previously found in rodents and non-human primates (Davis et al., 1993). The FPS paradigm was even used to test new types of drugs for clinical use. Grillon, Cordova Levine Charles, and Morgan (2003) examined the effects of LY35470, a glutamate receptor agonist, on FPS in humans, and found a reduction in potentiated startle along with subjective data suggesting a decrease of overall anxiety levels. Hormones have also been tested. Female participants were given injections of testosterone which reduced potentiated startle. Hermans, Putman, Baas, Koppeschar, and van Honk (2006) were able to study sex differences, and further supported the notions that testosterone mediates sex differences in fears.

Given that clinically used anti-anxiety drugs, such as diazepiam and buspirone, and lesion studies have shown to block or decrease potentiated startle, this gives the FPS paradigm evidence for support to study anxiety. Further support in using the FPS paradigm is the translational value between non-human animal test subjects and humans.

Rationale

As mentioned previously, anxiety disorders are prevalent in society and are the second most diagnosed mental disorders. Current treatments, benzodiazepines and antidepressants, have considerable side effects. Benzodiazepines can be addictive and can cause sedation and cognitive deficits. Antidepressants are effective in only about sixty percent of people with an anxiety disorder and have many different side effects. NTS_1 receptor agonists have been shown to have a potential anxiolytic effect. Research also suggests a role for the NTS_2 receptor having anxiolytic effects (Hou et al., 2011). The majority of behavioral studies using neurotensin drugs have been studied in male rodents, which limits the translational value of research to humans. Using NTS_1 and NTS_2 receptor agonists in male and female mice is the next logical step for advancing exploration for treating anxiety.

Therefore, the present study was conducted to examine the NTS_1 receptor agonist PD149163, and the NTS_2 agonist β-Lactotensin, on fear-potentiated startle in male and female mice. We hypothesize that the NTS_1 receptor agonist, PD149163, and the NTS_2 receptor agoninst, β-Lactotensin, will significantly decrease FPS, buspirone, previously shown to decrease FPS, will act as our positive control, and male and female mice will have a different FPS and be affected differently with the drugs.

METHODS

Materials

Subjects

Forty-five male and 45 female wild-type C57/BL6 mice (Mus musculus) (Charles River, Portage, MI) were used as subjects. Subjects were about two months old upon arrival and weighted between 18 and 25 grams before drug tests. Animals were housed three to a cage with food and water provided *ad libitum*. Animals were maintained in a climate-controlled room with a 14/10-h light/dark cycle (lights on at 7.30). Behavioral training and testing occurred two to three weeks after arrival and between 8.00 and 16.00. Animal care and experiments were conducted in accordance with The Guide to Care and Use of Laboratory Animals and was approved by the Institutional Animal Care and Use Committee (protocol 254) at Northern Michigan University, Marquette, MI.

Test Compounds

PD149163 and β-lactotensin were generously provided by RTI International (Piedmont, NC) and administered at doses of 0.1, 0.3 and 1.0 mg/kg (Carey, 2014; Hou et al., 2011). Buspirone was purchased from Sigma-Aldrich (St. Louis, MO) and administered at doses of 1.25, 2.5, and 5 mg/kg (Risbrough et al., 2003). The salt forms of the drugs were used. All test compounds were dissolved in saline, and was administered subcutaneously at a volume of 10 ml/kg 30-min prior to testing.

Equipment

Two startle chambers were commercially built (Med Associates Inc., St. Albans, VT) and consisted of a Plexiglas cage with steel rod floor bars. A scrambled current was delivered to the steel rod floors to serve as footshocks. The cages rested on a platform that transduced animal movements into digital recordings via the Startle Reflex software (Med Associates Inc.). Florescent lights were placed next to the cages to serve as a conditioned stimulus (see below). Speakers were placed alongside the cages and produced a startle stimulus (0.20 sec, 90 dB, white noise burst) and a red light on the top. The cages and other instruments were placed in sound-attenuated cabinets equipped with fans for ventilation and masking noise. A computer controlled and recorded all data from the startle chambers using Startle Reflex (Med Associates Inc.) in the experimental room.

The open-field consisted of two rectangular, open-top boxes (built from laminated melamine). Each box measured 30 x 30 x 27cm. A camera was mounted 71cm from the center of each box and recorded and analyzed locomotor activity using Noldus EthoVision video software (Leesburg, VA). A lightbulb was placed 80cm from the center of each box, providing light.

Procedure

Training

Training procedures were similar to those described by Risbrough et al. (2003). The purpose of these conditioning trials was to pair the light (conditioned stimulus) with the elicitation of shock (unconditioned stimulus). The expected result was that the stimulus light (CS) will cause the mice to have a greater startle response (conditioned

response) when the light is on. A conditioning session consisted of ten trials. A session began with a 5-min acclimation period, consisting of a red chamber light and ventilation fan turning on, but no experimental events. Following the acclimation period, ten trials (separated by 120-180 sec) began and each trail consisted of a stimulus light activating for 10 s and co-terminating with a 0.30 mA shock (0.25 sec duration) delivered to the floor of the nonrestrictive cage (Figure 1a).

Testing

A series of drugs were tested in the mice, with N = 15 per group. One drug was tested in each group, but mice within each group were tested with three doses of the test drug, in addition to a saline test given before testing drug doses and a final saline test given after testing drug doses. The three doses of each drug were tested in a counter-balanced, ascending order. For example, the test order for mouse FPS5 was saline, PD149163 0.3 mg/kg, PD149163 1.0 mg/kg, PD149163 0.1 mg/kg, and saline. Test sessions were separated by six to seven days. After a dose has been tested, one training session was conducted the day prior to the next test session in order to maintain conditioning with the light-shock pairing.

A testing session consisted of 24 trials. A session began the same as a training session. Following the acclimation period, ten startle stimuli (0.20 sec, 90 dB, white noise burst) separated by 20 s occurred in the dark to habituate the subject to the startle burst before the light cue turned on. Then, 24 trials (separated by 120-180 sec) consisted of either a stimulus light activated for 10 sec preceding the activation of a startle stimulus or no stimulus light being activated for a ten sec period prior to a white noise burst. Half

of the trials consisted of the light-startle stimulus pairing and half consisted of the startle stimulus only (i.e., with no preceding stimulus light (Figure 1b).

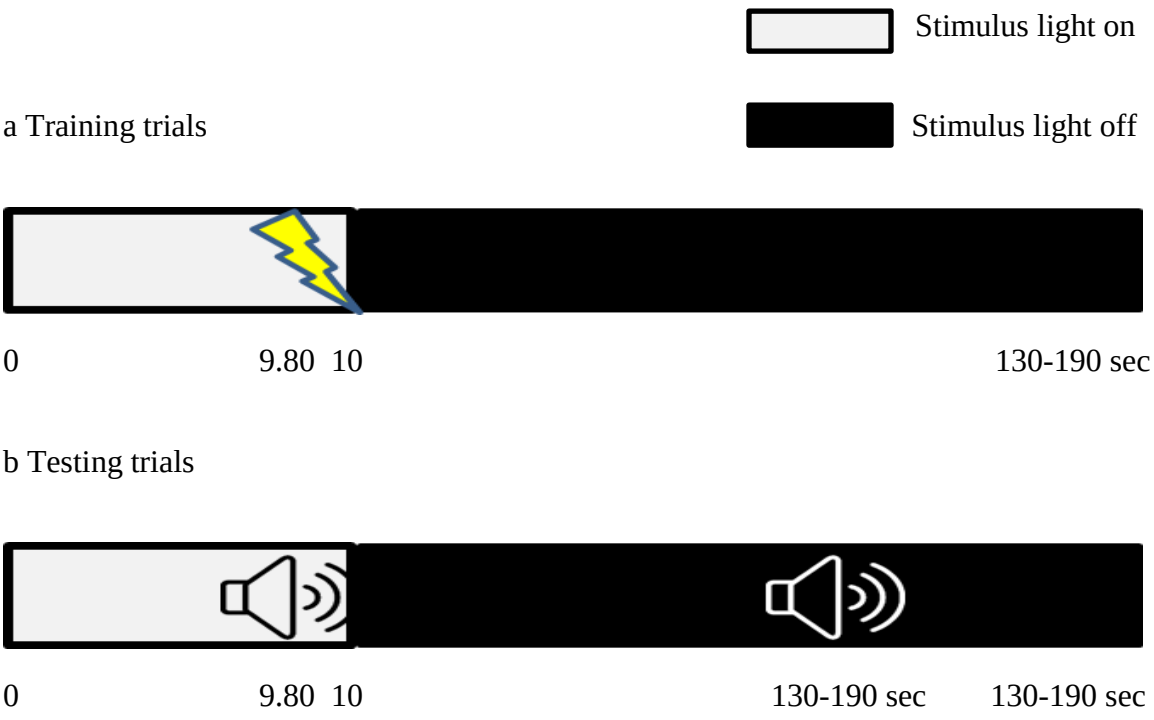

Figure 1: Schematic description of stimulus presentation during training and test trials. (a) Training trials. Ten training trials consisted of a ten sec light cue co-terminating with a 0.30 mA scrambled footshock during the last 0.25 sec. A dark period followed the light period which varied 120 to 180 sec. (b) Testing trials. Twenty-four trials consisted of a stimulus light turning on for ten seconds and co-terminating with a 0.20 sec, 90 dB, white noise burst startle stimulus, followed by a dark period of 120-190 sec. Twelve of the trials consisted of the stimulus light on, while twelve trials consisted of the stimulus light off.

Open-Field Test

Immediately following fear-potentiated startle tests, mice were placed in the center of the open-field for five minutes. During this open-field session, total path-length of movements, total time spent in the center of the box, and total number of times mice

entered and left the center of the open field was measured. Following each trail, the open-field was cleaned using isopropyl alcohol.

Data Analysis

The first ten startle stimuli of the test session were used to habituate the animals to the startle stimulus and were not used in the data analysis. The dependent variables measured for the FPS test sessions were FPS (+/- standard error of the mean [SEM]) and mean startle magnitude (+/- SEM). The FPS was calculated as follows:

$$\left(\frac{startle\ magnitude\ for\ light\ noise\ trials - startle\ magnitude\ for\ noise\ only\ trials}{startle\ magnitude\ for\ noise\ only\ trials}\right) * 100$$

(Shilling & Feifel, 2008; Walker & Davis, 2002; Winslow, Nobel and Davis, 2007). This calculation provides the percentage of startle occurring from the difference between the white noise burst when the stimulus light was on and off above the intensity of startle occurring from the white noise burst when the stimulus light was off. The dependent variable for the open field tests were total path length (cm) total time spent in the center of the box (sec), and total number of times mice entered and left the center of the open field. All dependent variables for the open field tests were reported as means (+/- SEM).

As noted earlier, the subjects in all groups were treated with saline before and after drug treatment. This allowed for a determination whether there was an increase or decrease in FPS or startle magnitude after weeks of drug testing. The FPS for saline before versus after drug testing were compared using a paired-samples t-test. A paired-samples t-test was used to compare the startle magnitude during light-noise test trials and

noise-only trials to see if potentiation did occur as a result of activating the light stimulus. A two-factor mixed measures ANOVA was used, with sex as the between-subjects factor and drug dose as the within-subjects factor for each group, to determine if there was a sex difference and/or an interaction between sex and drug dose for FPS. Because it was also of interest to determine the effects of each drug dose within each sex alone on FPS and startle magnitude, a one-way repeated measure ANOVA was used to analyze the effects of each dose on FPS and startle magnitude within each group of male or female subjects. Any statistically significant differences were further analyzed using Bonferroni post hoc tests.

Total distance traveled in the open field was analyzed using a one-way repeated measure ANOVA for each group to assess if locomotor activity was also affected. Total time spent in the center was assessed using a one-way repeated measure ANOVA for each group. Total entries and exits from the center were analyzed using a one-way repeated measure ANOVA. All statistical analyses were conducted using GraphPad Prism version 6.0 (GraphPad Software, La Jolla, CA).

RESULTS

PD149163

There was no statistical difference in FPS between saline before (M=12.30, SEM=3.79) and after (M=17.65, SEM=3.73) testing PD149163 in male mice, t(14)=0.99, p=0.34 (data not shown). There was no statistical difference in percent FPS between saline before (M=16.81, SEM=4.07) and after (M=12.67, SEM=4.35) testing PD149163 in female mice, t(14)=0.66, p=0.52 (data not shown).

The light-noise startle magnitude was compared to the noise only startle magnitude for saline (i.e., the mean of saline values before and after testing PD149163) in the male PD149163 group. There was a statistically significant increase in the startle magnitude in the light-noise (M=965.7, SEM=61.72) condition compared to the noise-only (M=803.8, SEM=49.32) condition for males, t(29)=5.83, p<0.0001 (Figure 2 top). The light-noise and noise-only startle magnitude was also compared for saline in the female PD149163 group. There was also a statistically significant increase in startle magnitude in the light-noise (M=736.7, SEM=36.94) condition compared to the noise-only (M=613.1, SEM=27.12) condition for female; t(29)=5.115, p<0.0001 (Figure 2 bottom).

A two-way mixed factor ANOVA for FPS between sex and dose of PD149163 revealed a statistically significant effect of dose, F(3,84)=6.84, p=0.0004, and sex, F(1,28)=13.79, p=0.0009, but no interaction, F(3,84)=2.18, p=0.10 (Figure 3). Bonferroni post hoc test confirmed that doses of 0.1 and 1.0 mg/kg for females

significantly decreased when compared to males. Figure 4 (top) shows the FPS for saline or PD149163 administration to male mice. PD149163 administration significantly altered the FPS, $F(2.35, 32.86)=3.56$, $p=0.034$, in male mice. This was due to a significant increase in FPS at the 0.1 (mg/kg) dose when compared to saline. Figure 4 (bottom) shows the FPS for saline or PD149163 administration to female mice. PD149163 administration significantly altered the FPS, $F(2,27.90)=5.22$, $p=0.01$, in female mice. This was due to a significant decrease in FPS at the 1.0 (mg/kg) dose when compared to saline.

A two-way mixed factor ANOVA for startle magnitude between sex and light-noise trials revealed a significant effect for dose, $F(3,84)= 112.0$, $p<0.0001$, sex, $F(1,28)= 8.56$, $p=0.0067$, and interaction, $F(3,84)= 4.16$, $p=0.0085$ (data not shown). Further analysis showed saline an 0.1 mg/kg to be significantly decreased in females compared to males. A two-way mixed factor ANOVA for startle magnitude between sex and noise-only trials revealed a significant effect for dose, $F(3,84)= 95.28$, $p<0.0001$, sex, $F(1,28)= 4.237$, $p=0.049$, and interaction, $F(3,84)= 4.14$, $p=0.0087$ (data not shown). Further analysis showed saline to be significantly decreased in females compared to males. A one-way repeated measures ANOVA for startle magnitude during the light-noise trials for male mice was significantly different across doses of PD149163; $F(2.28, 31.92)=49.15$, $p<0.0001$ (Figure 5 top). This was due to a significant decrease in startle magnitude at the 0.3 and 1.0 (mg/kg) doses compared to saline. Startle magnitude during the noise-only trials for male mice was also significantly different across doses of PD149163; $F(2.07, 29.00)=46.12$, $p<0.0001$ (Figure 5 bottom). This was due to a significant decrease in startle magnitude at the 0.1, 0.3, and 1.0 mg/kg doses compared to

saline. Startle magnitude during the light-noise trials for female mice was significantly different across doses of PD149163; $F(2.54, 35.54)=93.50$, $p=0.0015$ (Figure 6 top). This was due to a significant decrease in mean startle magnitude at the 0.1, 0.3, and 1.0 mg/kg doses compared to saline. Startle magnitude during the noise-only trials for female mice was significantly different across doses of PD149163; $F(2.57, 35.97)=60.64$, $p=0.0027$ (Figure 6 bottom). This was due to a significant decrease in startle magnitude at the 0.1, 0.3, and 1.0 mg/kg doses compared to saline.

A two-way mixed factor ANOVA for total distance traveled (cm) between sex and dose of PD149163 revealed a statistically significant effect of dose, $F(5,140)=181.8$, $p<0.0001$, but neither sex [$F(1,28)=0.26$, $p=0.62$] nor the interaction, $F(5,140)=1.40$, $p=0.23$. Figure 7 (top) shows the total distance traveled (cm) after saline or PD149163 administration to male mice. PD149163 administration significantly altered the total distance traveled in male mice, $F(2.33, 32.60)=150.0$, $p<0.0001$. There was a significant decrease in total distance traveled at doses of 0.1, 0.3, and 1.0 mg/kg when compared to saline in male mice. Figure 7 (bottom) shows the total distance traveled (cm) after saline or PD149163 administration to female mice. PD149163 administration significantly altered the total distance traveled in female mice, $F(3.17, 44.35)=61.99$, $p=0.0007$. There was a significant decrease in total distance traveled at doses of 0.1, 0.3, and 1.0 mg/kg when compared to saline in female mice.

A two-way mixed factor ANOVA for total time (s) spent in center between sex and dose of PD149163 revealed a statistically significant effect of dose, $F(5,140)=16.92$, $p<0.0001$, and interaction, $F(5,140)=4.26$, $p=0.0012$, but not sex, $F(1, 28)=3.80$, $p=0.06$. Further analysis revealed a significant decrease in total time spent in center for female

mice when compared to male mice at the doses of 0.3 and 1.0 mg/kg. Figure 8 (top) shows the total time spent in center (sec) after saline or PD149163 administration to male mice. PD149163 administration did significantly alter the total time spent in center in male mice, $F(1.88, 26.41)=15.89$, $p=0.0018$. This is due to an increase in total time spent in the center at a dose of 1.0 mg/kg compared to saline in male mice. The total number of entries and exits of the center area was significantly altered in male mice, $F(2.49, 34.79)=21.74$, $p<0.0001$ (Figure 9 top). This was due to a significant decrease at the 0.3 and 1.0 mg/kg doses. Figure 8 (bottom) shows the total time spent in center (sec) after saline or PD149163 administration to female mice. PD149163 administration significantly altered the time spent in center in female mice, $F(1.93, 26.97)=5.95$, $p=0.0078$. This is due to an decrease in total time spent in the center at a dose of 0.3 mg/kg in female mice. The total number of entries and exits of the center area was significantly altered in female mice, $F(2.58, 36.15)=15.74$, $p<0.0001$ (Figure 9 bottom). This was due to a significant decrease at the 0.3 and 1.0 mg/kg doses.

PD149163 Startle Magnitude: Saline

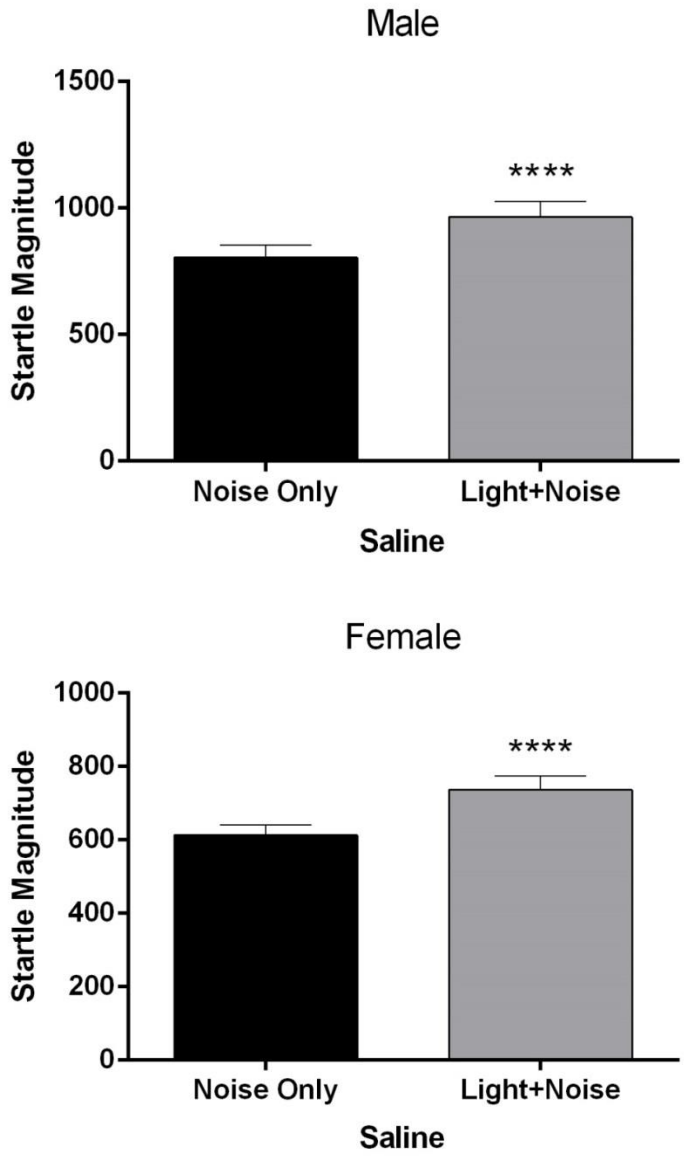

Figure 2: The light-noise and noise-only startle magnitude during saline administration for the PD149163 group in male (top) and female (bottom) mice. ****p<0.0001 light+noise versus noise-only. Data are expressed as mean +/- SEM, N=15.

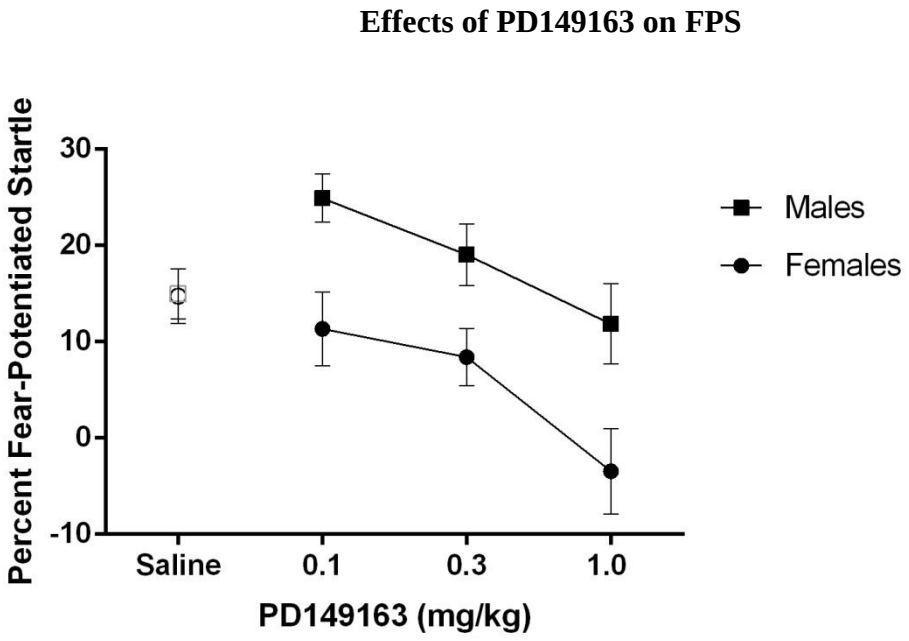

Figure 3: The effect of PD149163 administration on FPS in male (square) and female (circle) mice. Data are expressed as mean +/- SEM, N=15.

Effects of PD149163 on FPS

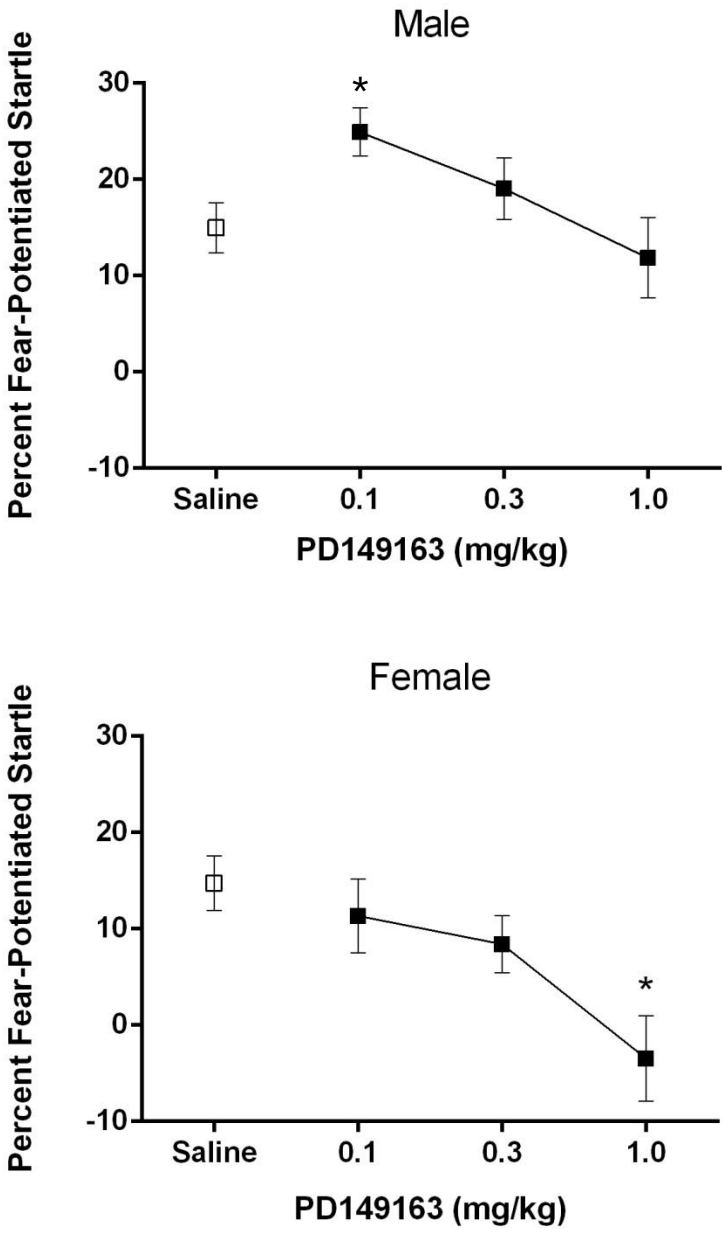

Figure 4: The effect of PD149163 administration on FPS in male (top) and female (bottom) mice. *p<0.05 versus saline. Data are expressed as mean +/- SEM, N=15.

PD149163 Startle Magnitude: Male Light-Noise and Noise-only

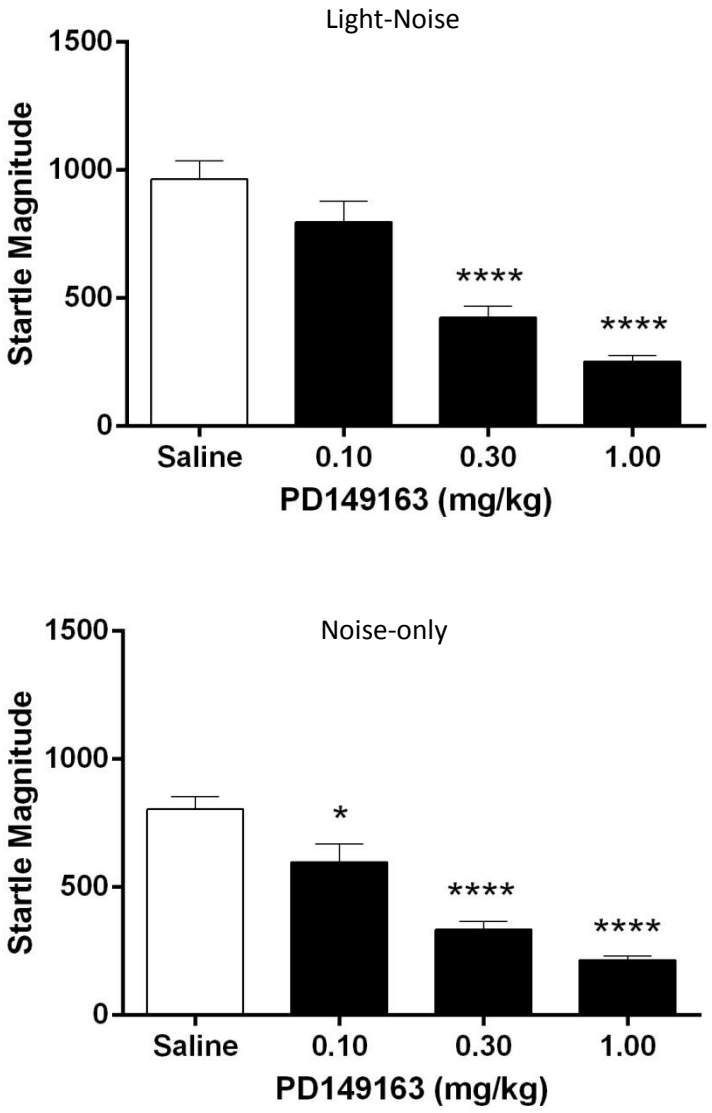

Figure 5: The light-noise (top) and noise only (bottom) startle magnitude during PD149163 administration compared to saline in male mice. *p<0.05 & ****p<0.0001 versus saline. Data are expressed as mean +/- SEM, N=15.

PD149163 Startle Magnitude: Female Light-Noise and Noise-only

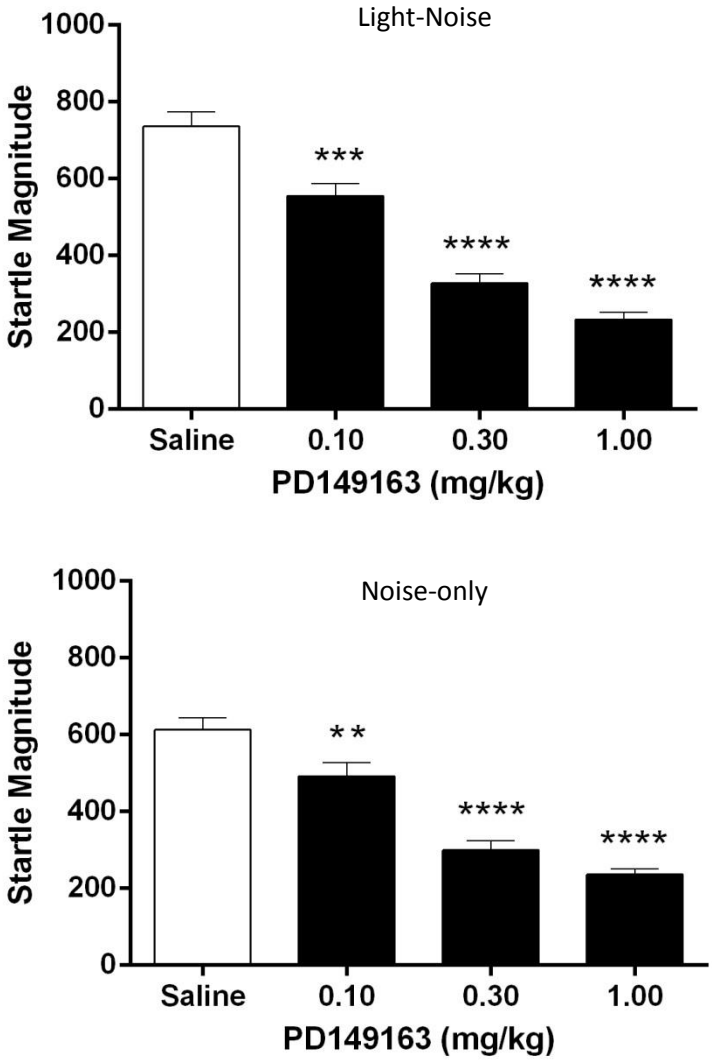

Figure 6: The light-noise (top) and noise only (bottom) startle magnitude during PD149163 administration compared to saline in female mice. *p<0.05 & ****p<0.0001 versus saline. Data are expressed as mean +/- SEM, N=15.

PD149163: Distance Traveled

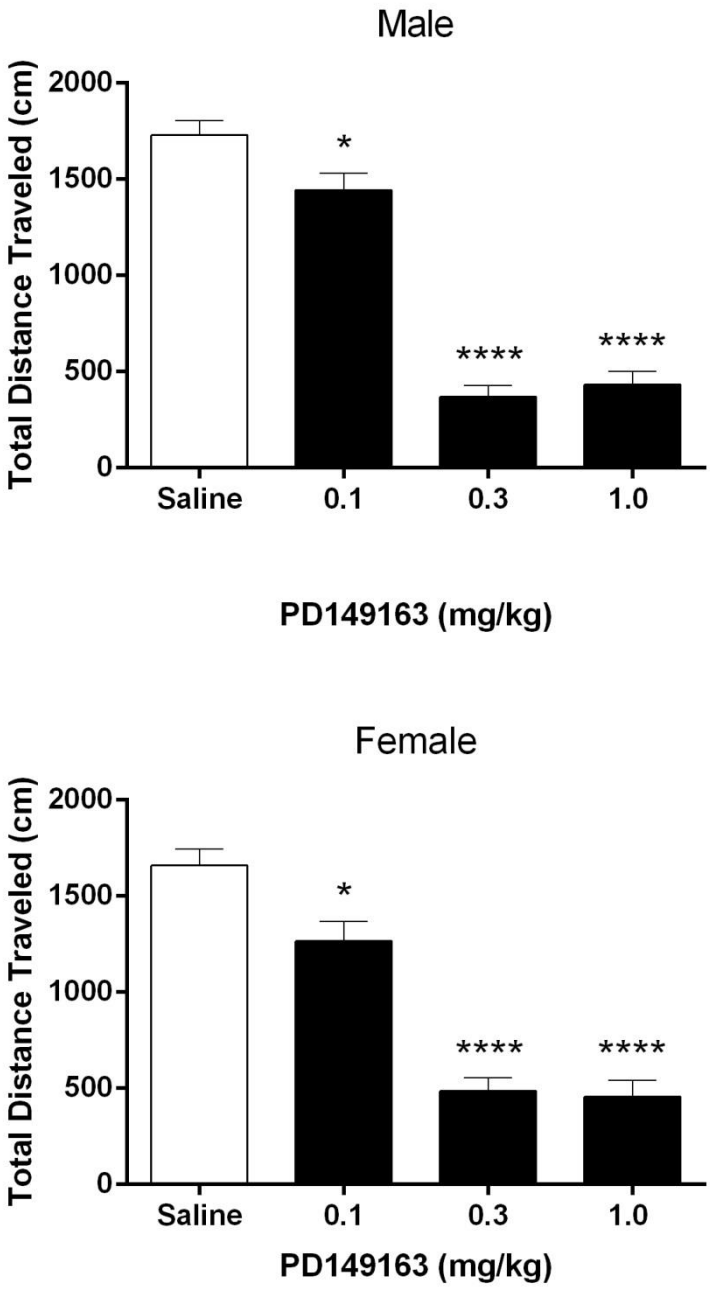

Figure 7: The effects of PD149163 on total distance traveled (cm) in the open field apparatus in male (top) and female (bottom) mice. *p<0.05 & ****p<0.0001 versus saline. Data are expressed as mean +/- SEM, N=15.

PD149163: Time Spent in the Center

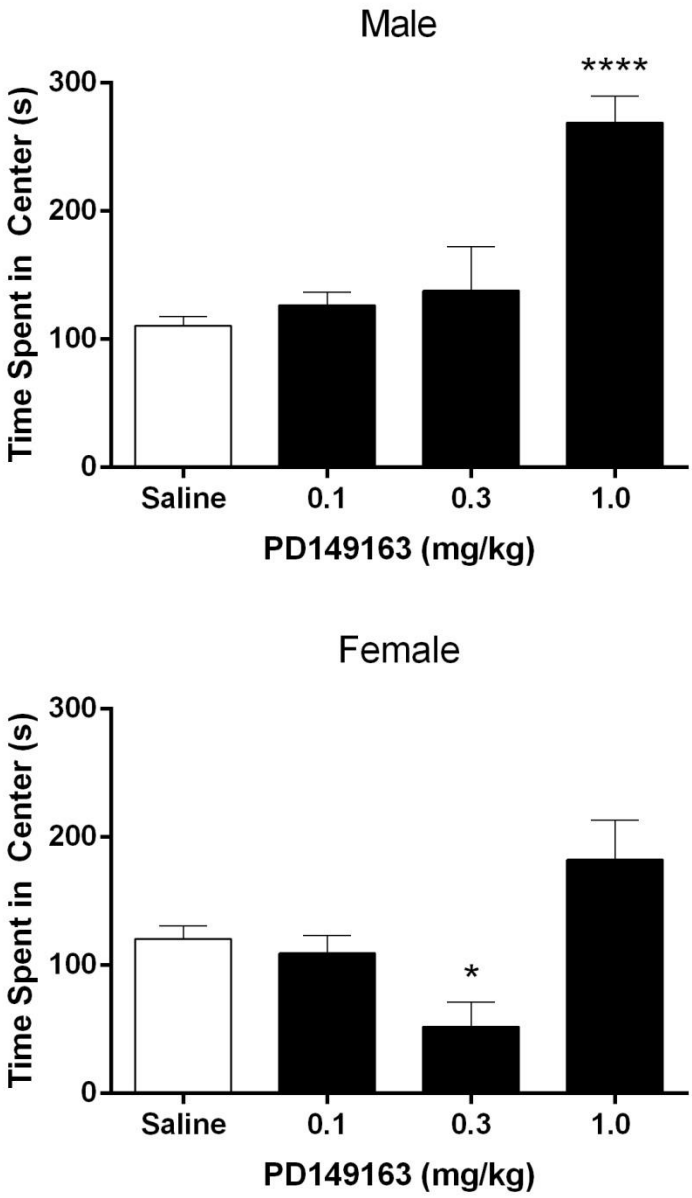

Figure 8: The effects of PD149163 on the total time (s) spent in the center of the open field apparatus compared to saline in male (top) and female (bottom) mice. *p<0.05 & ****p<0.0001 versus saline. Data are expressed as mean +/- SEM, N=15.

PD149163: Total Entries and Exits of the Center

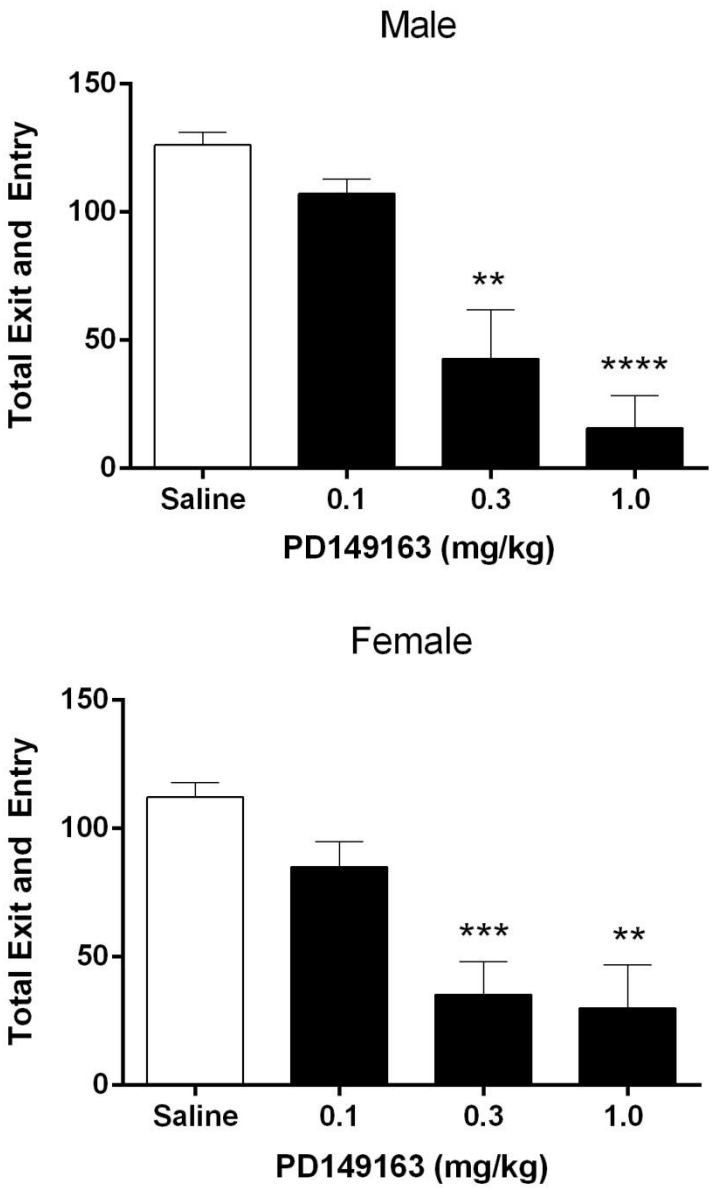

Figure 9: The effects of PD149163 on the total exits and entries of the center of the open field apparatus compared to saline in male (top) and female (bottom) mice. **p<0.01, ***p<0.001 & ****p<0.0001 versus saline. Data are expressed as mean +/- SEM, N=15.

β-Lactotensin

There was not a statistical difference in FPS between saline before (M=13.28, SEM=2.67) and after (M=19.42, SEM=3.68) testing β-Lactotensin in male mice, t(14)=1.61, p=0.13 (data not shown). There was not a statistical difference in FPS between saline before (M=6.78, SEM=5.11) and after (M=16.62, SEM=3.14) testing β-Lactotensin in female mice, t(14)=1.65, p=0.12 (data not shown).

The light-noise startle magnitude was compared to the noise-only startle magnitude for saline (i.e., the mean of saline values before and after testing β-Lactotensin) in the male β-Lactotensin group. There was a statistically significant increase in the startle magnitude in the light-noise (M=1064, SEM=41.39) condition compared to the noise-only (M=884.7, SEM=37.10) condition for males, t(29)=6.31, p<0.0001 (Figure 10 top). The light-noise and noise-only startle magnitude for saline was compared in the female β-Lactotensin group. There was also a statistically significant increase in the startle magnitude in the light-noise (M=762, SEM=43.54) condition compared to the noise-only (M=661.6, SEM=37.13) condition for female, t(29)=4.07, p=0.0003 (Figure 10 bottom).

A two-way mixed factor ANOVA for FPS between sex and dose of β-Lactotensin revealed a statistically significant effect of sex, F(1,28)=8.09, p=0.008, interaction, F(3,84)=2.74, p=0.049, but not dose, F(3,84)=2.033, p=0.11 (Figure 11). A Bonferroni post hoc test confirmed that the dose of 0.3 mg/kg for females significantly decreased compared to males. Figure 12 (top) shows the FPS for saline or β-Lactotensin administration to male mice. β-Lactotensin administration did not statistically significantly alter the FPS in male mice, F(2.54, 35.49)=0.11, p=0.93. Figure 12

(bottom) shows the FPS for saline or β-Lactotensin administration to female mice. β-Lactotensin administration significantly altered the FPS in female mice, $F(2.27,31.75)=3.75$, $p=0.03$. The post hoc analysis did not identify doses that differed statistically from saline.

A two-way mixed factor ANOVA for startle magnitude between sex and light-noise trials revealed a significant effect for dose, $F(3,84)= 3.401$, $p=0.0214$, sex, $F(1,28)= 20.39$, $p=0.0001$, but no interaction, $F(3,84)= 1.675$, $p=0.1786$ (data not shown). Further analysis showed saline, 0.1, 0.3, and 1.0 mg/kg to be significantly decreased in females compared to males. A two-way mixed factor ANOVA for startle magnitude between sex and noise-only trials revealed a significant effect for sex, $F(1,28)= 14.83$, $p=0.0006$, but not for dose, $F(3,84)= 2.239$, $p=0.0897$, nor interaction, $F(3,84)= 0.7623$, $p=0.5184$ (data not shown). Further analysis showed saline, 0.1 and 0.3 mg/kg to be significantly decreased in females compared to males. A one-way repeated measure ANOVA for startle magnitude during the light-noise trials for β-Lactotensin was not significantly different in male mice across doses; $F(2.23, 31.14)=1.05$, $p=0.37$ (Figure 13 top). Startle magnitude during the noise-only trials for β-Lactotensin was not significantly different in male mice across doses; $F(2.64, 36.96)=1.53$, $p=0.23$ (Figure 13 bottom). Startle magnitude during the light-noise trials for β-Lactotensin was significantly different in female mice across doses; $F(2.92, 40.90)=5.64$, $p=0.0027$ (Figure 14 top). This was due to a significant decrease in startle magnitude at the 0.3 mg/kg dose compared to saline. Startle magnitude during the noise-only trials for β-Lactotensin was significantly different in female mice across doses; $F(2.65, 37.13)=7.69$, $p=0.0009$ (Figure 14 bottom).

This was due to a significant decrease in startle magnitude at the 0.1 mg/kg dose compared to saline.

A two-way mixed factor ANOVA for total distance traveled (cm) between sex and dose of β-Lactotensin revealed a statistically significant effect of dose, $F(5,140)=11.62$, $p<0.0001$, but neither sex, $F(1, 28)=0.004$, $p=0.95$, nor the interaction, $F(5,140)=0.28$, $p=0.92$. Figure 15 (top) shows the total distance traveled (cm) after saline or β-Lactotensin administration to male mice. β-Lactotensin administration significantly altered the total distance traveled in male mice, $F(3.22, 45.02)=7.92$, $p=0.0002$. A significant decrease of total distance traveled was shown at doses of 0.1, 0.3 and 1.0 mg/kg compared to saline in male mice. Figure 15 (bottom) shows the total distance traveled (cm) after saline or β-Lactotensin administration to female mice. β-Lactotensin administration significantly altered the total distance traveled in female mice, $F(2.78, 38.93)=4.75$, $p=0.0076$. The post hoc analysis did not identify doses that differed statistically from saline.

A two-way mixed factor ANOVA for total time (sec) spent in center between sex and dose of β-Lactotensin revealed no statistically significant effect of dose, $F(5,140)=2.02$, $p=0.08$, sex, $F(1,28)=1.10$, $p=0.30$, and interaction, $F(5,140)=1.27$, $p=0.28$. Figure 16 (top) shows the total time spent in center (sec) after saline or β-Lactotensin administration to male mice. β-Lactotensin administration did not significantly altered the total time spent in center in male mice, $F(3.20, 44.75)=2.17$, $p=0.10$. The total number of entries and exits of the center area was not significantly altered in male mice, $F(2.70, 37.75)=0.61$, $p=0.60$ (Figure 17 top). Figure 16 (bottom) shows the total time spent in center (sec) after saline or β-Lactotensin administration to

female mice. β-Lactotensin administration did not significantly altered the time spent in center in female mice, $F(2.97, 41.64)=1.26$, $p=0.29$. The total number of entries and exits of the center area was not significantly altered in female mice, $F(2.15, 30.12)=1.64$, $p=0.21$ (Figure 17 bottom).

β-Lactotensin Startle Magnitude: Saline

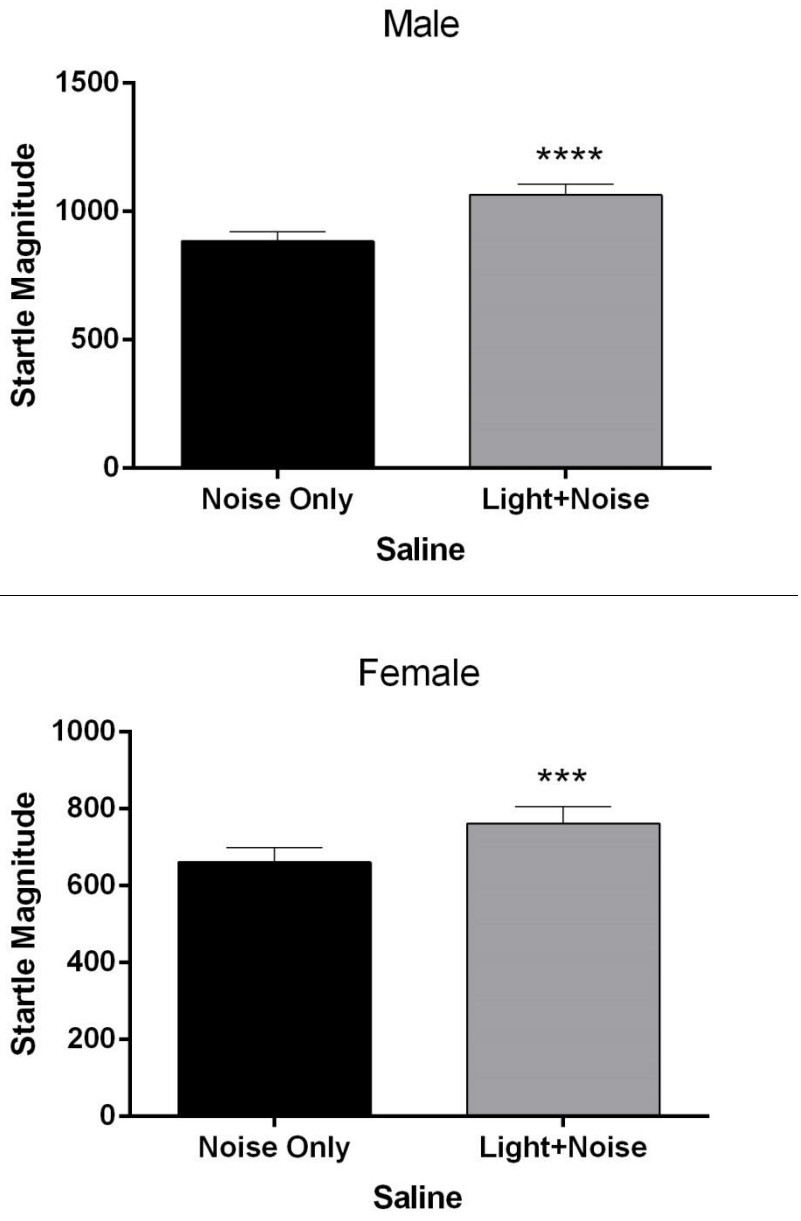

Figure 10: The light-noise and noise-only startle magnitude during saline administration for the β-Lactotensin group in male (top) and female (bottom) mice. ***p<0.001 & ****p<0.0001 light+noise versus noise-only. Data are expressed as mean +/- SEM, N=15.

The Effects of β-Lactotensin on FPS

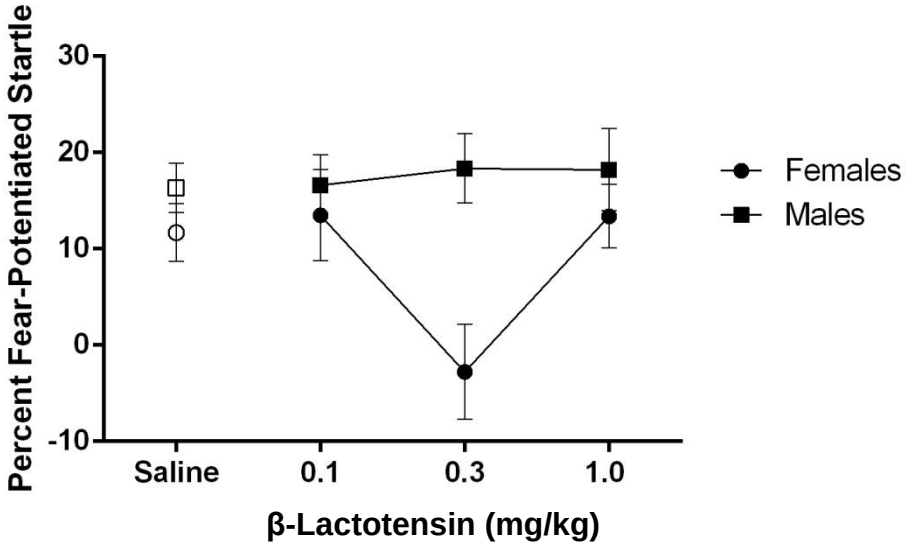

Figure 11: The effect of β-Lactotensin administration on FPS in male (square) and female (circle) mice. Data are expressed as mean +/- SEM, N=15.

The Effects of β-Lactotensin on FPS

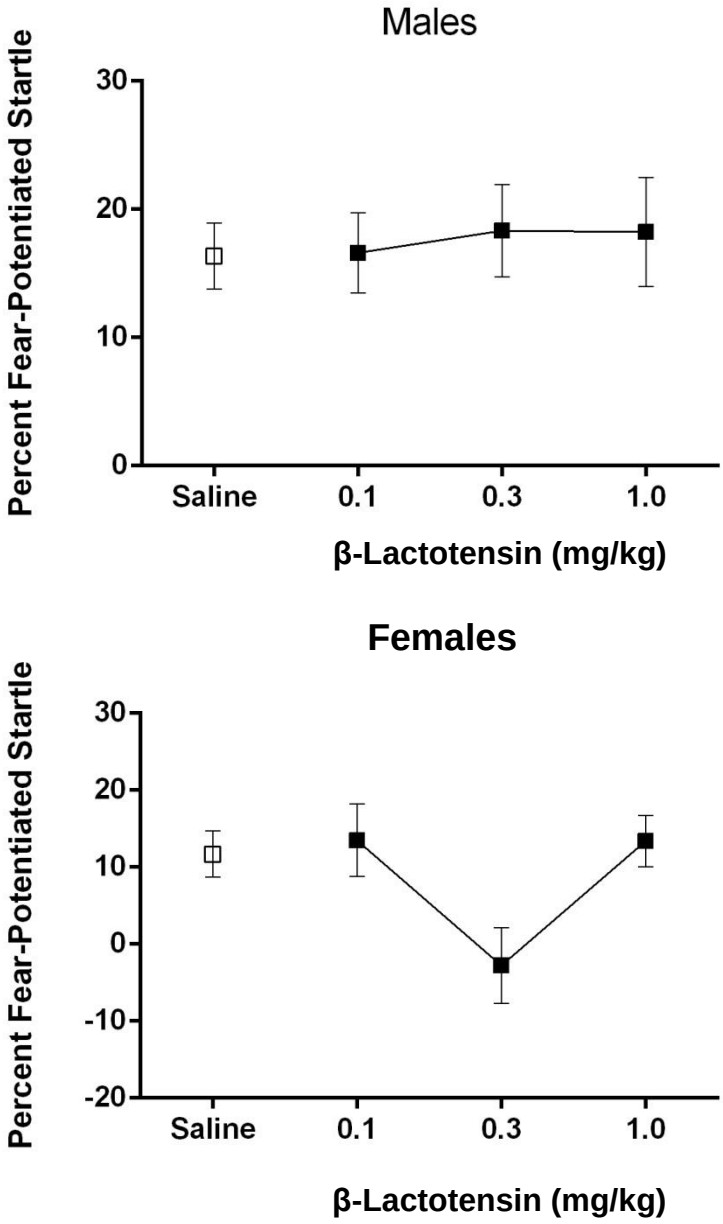

Figure 12: The effect of β-Lactotensin administration on percent fear-potentiated startle in male (top) and female (bottom) mice. Data are expressed as mean +/- SEM, N=15.

β-Lactotensin Statle Magnitude: Male Light-Noise and Noise-only

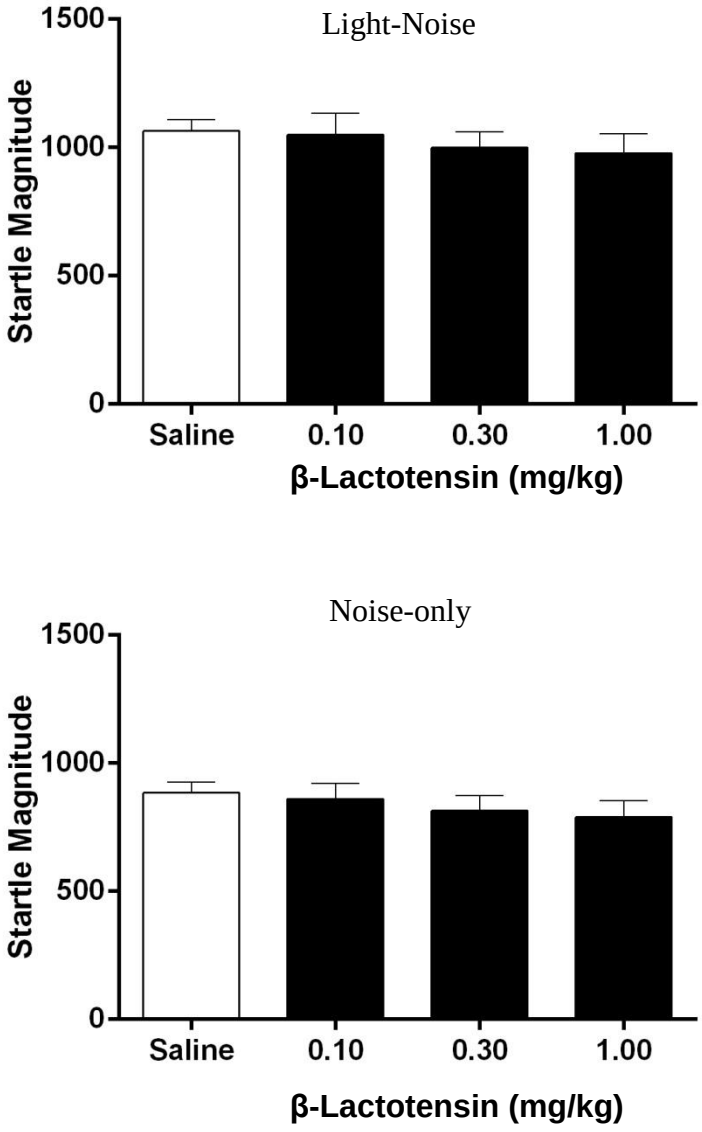

Figure 13: The light-noise (top) and noise only (bottom) startle magnitude during β-Lactotensin administration compared to saline in male mice. Data are expressed as mean +/- SEM, N=15.

β-Lactotensin Statle Magnitude: Female Light-Noise and Noise-only

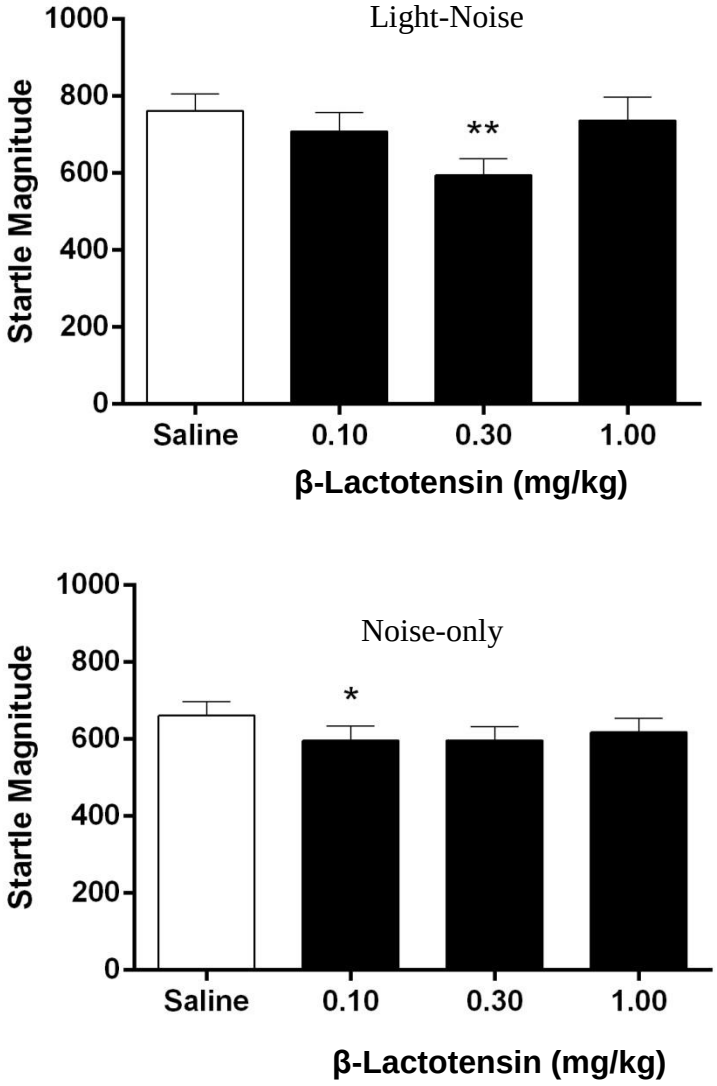

Figure 14: The light-noise (top) and noise only (bottom) startle magnitude during β-Lactotensin administration compared to saline in female mice. *p<0.05 & **p<0.01 versus saline. Data are expressed as mean +/- SEM, N=15.

β-lactotensin: Total Distance Traveled

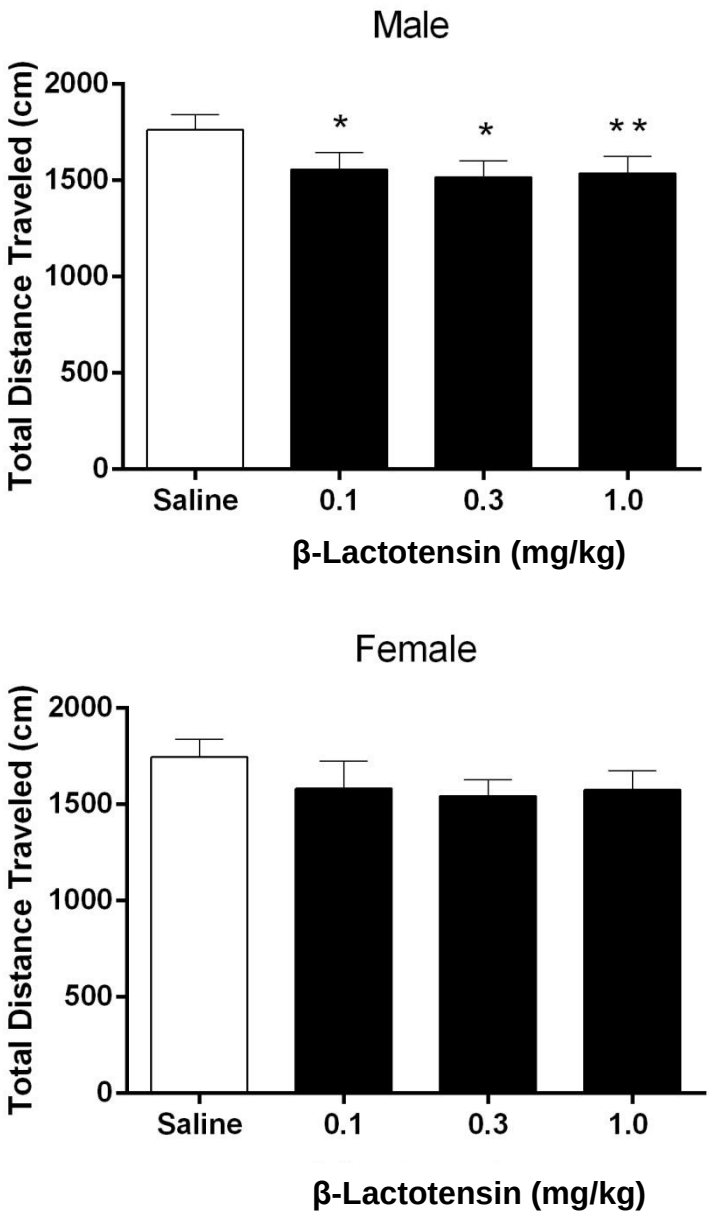

Figure 15: The effects of β-lactotensin on the total distance traveled (cm) in the open field apparatus in male (top) and female (bottom) mice. *p<0.05 & **<0.01 versus saline. Data are expressed as mean +/- SEM, N=15.

β-lactotensin: Time Spent in the Center

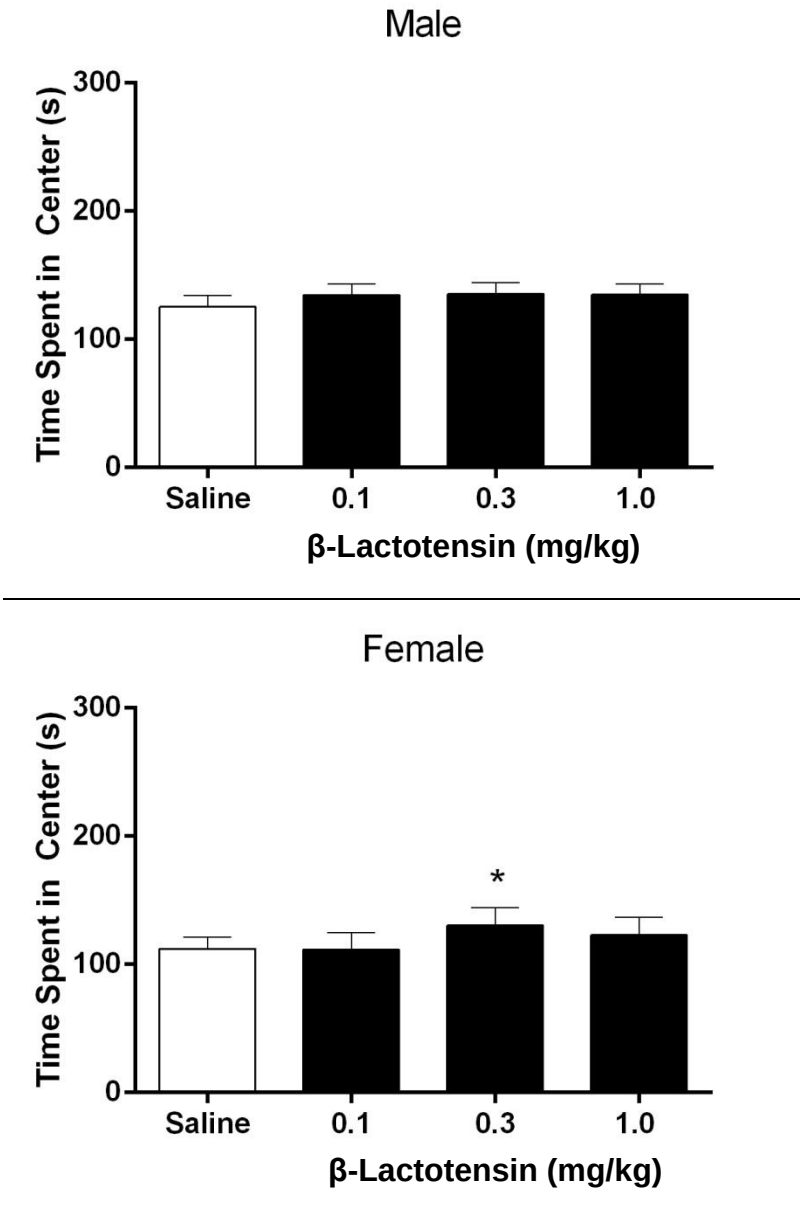

Figure 16: The effects of β-Lactotensin on the total time (s) spent in the center of the open field apparatus compared to saline in male (top) and female (bottom) mice. *$p<0.05$ versus saline. Data are expressed as mean +/- SEM, N=15.

β-lactotensin: Total Entries and Exits of the Center

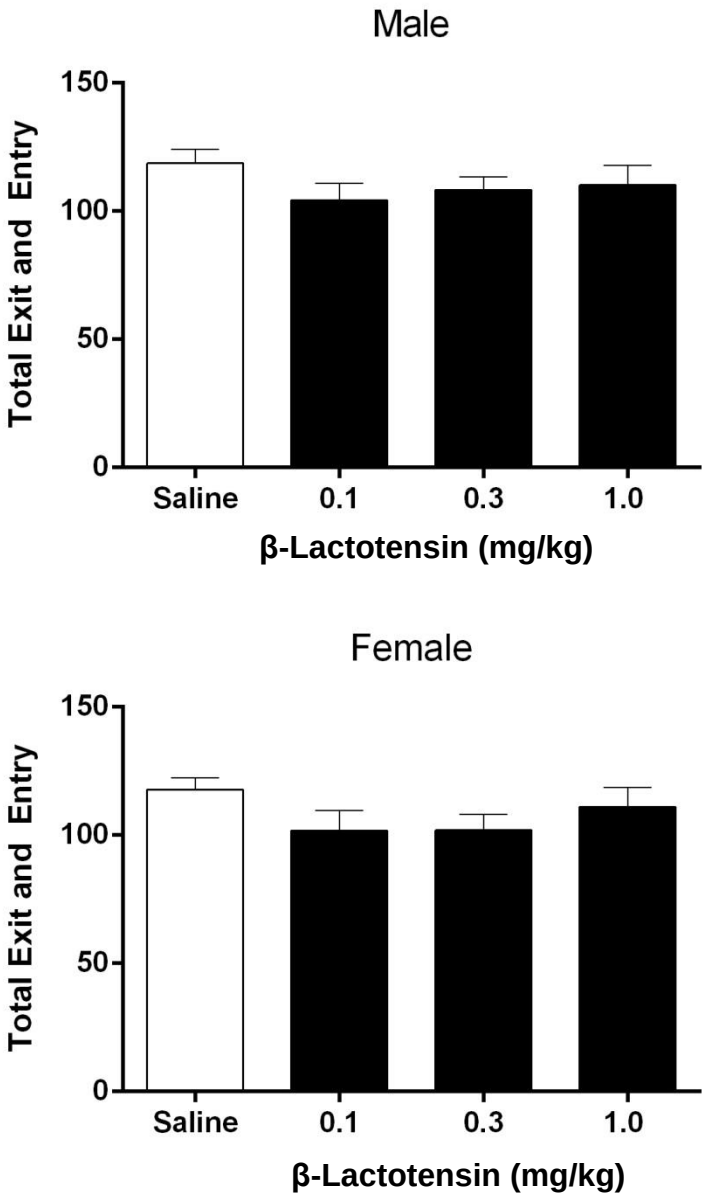

Figure 17: The effects of β-Lactotensin on the total exits and entries of the center of the open field apparatus compared to saline in male (top) and female (bottom) mice. Data are expressed as mean +/- SEM, N=15.

Buspirone

There was no statistical difference in FPS between saline before (M=19.10, SEM=2.43) and after (M=21.88, SEM=4.15) testing buspirone in male mice, t(14)=0.50, p=0.63. There was no statistical difference in FPS between saline before (M=21.18, SEM=4.12) and after (M=21.7, SEM=4.23) testing buspirone in female mice, t(14)=0.02, p=0.98.

The light-noise startle magnitude was compared to the noise-only startle magnitude for saline (i.e., the mean of saline values before and after testing buspirone) in the male buspirone group. There was a statistically significant increase in the startle magnitude in the light-noise (M=1067, SEM=61.24) condition compared to the noise-only (M=852.6, SEM=52.36) condition for males, t(29)=8.41, p<0.0001 (Figure 18 top). The light-noise and noise-only startle magnitude for saline was also compared in the female buspirone group. There was also a statistically significant increase in the startle magnitude in the light-noise (M=718.6, SEM=41.82) condition compared to the noise only (M=553.7, SEM=30.02) condition for female, t(29)=7.02, p<0.0001 (Figure 18 bottom).

A two-way mixed factor ANOVA for FPS between sex and dose of buspirone revealed no statistically significant effect of dose, F(3,84)=0.71, p=0.55, sex, F(1,28)=0.15, p=0.70, and interaction, F(3,84)=0.41, p=0.74 (Figure 19). Figure 20 (top) shows the FPS for saline or buspirone administration to male mice. Buspirone administration did not significantly alter the FPS in male mice, F(1.89, 26.47)=1.41,

p=0.26. Figure 20 (bottom) shows the FPS for saline or buspirone administration to female mice. Buspirone administration did not significantly altered the FPS, $F(2.75,38.51)=0.14$, $p=0.92$, in female mice.

A two-way mixed factor ANOVA for startle magnitude between sex and light-noise trials revealed a significant effect for dose, $F(3,84)= 10.12$, $p<0.0001$, sex, $F(1,28)= 18.65$, $p=0.0002$, but no interaction, $F(3,84)= 1.139$, $p=0.3382$ (data not shown). Further analysis showed saline, 1.0, 2.5 and 5.0 mg/kg to be significantly decreased in females compared to males. A two-way mixed factor ANOVA for startle magnitude between sex and noise-only trials revealed a significant effect for dose, $F(3,84)= 7.538$, $p=0.0002$, sex, $F(1,28)= 15.74$, $p=0.0003$, but no interaction, $F(3,84)= 0.4127$, $p=0.7443$ (data not shown). Further analysis showed saline, 1.0, 2.5 and 5.0 mg/kg to be significantly decreased in females compared to males. A one-way repeated measure ANOVA for startle magnitude during the light-noise trials for buspirone was significantly different in male mice across doses, $F(2.28, 31.86)=3.62$, $p=0.03$ (Figure 21 top). This was due to a significant decrease in startle magnitude at the dose of 5.0 mg/kg compared to saline. Startle magnitude during the noise-only trials for buspirone was not significantly different in male mice across doses; $F(2.79, 39.01)=1.71$, $p=0.18$ (Figure 21 bottom). Startle magnitude during the light-noise trials for buspirone was significantly different in female mice across doses; $F(2.43, 34.06)=9.32$, $p=0.0003$ (Figure 22 top). This was due to a significant decrease in startle magnitude at the 2.5 and 5.0 mg/kg doses compared to saline. Startle magnitude during the noise-only trials for buspirone was significantly different in female mice across doses; $F(2.45, 34.26)=11.61$, $p<0.0001$ (Figure 22

bottom). This was due to a significant decrease in startle magnitude at the 1.0, 2.5 and 5.0 mg/kg doses compared to saline.

A two-way mixed factor ANOVA for total distance traveled (cm) between sex and dose of buspirone revealed a statistically significant effect of dose, $F(5,140)=43.75$, $p<0.0001$, but neither sex, $F(1,28)=0.06$, $p=0.81$, nor the interaction, $F(5,140)=0.89$, $p=0.49$. Figure 23 (top) shows the total distance traveled (cm) after saline or buspirone administration to male mice. Buspirone administration significantly altered the total distance traveled in male mice, $F(3.19, 44.59)=27.77$, $p<0.0001$. This was due to a significant decrease in total distance traveled at the 1.0, 2.5 and 5.0 mg/kg doses in male mice. Figure 23 (bottom) shows the total distance traveled (cm) after saline or buspirone administration to female mice. Buspirone administration significantly altered the total distance traveled in female mice, $F(2.77, 38.83)=17.49$, $p<0.0001$. This was due to a significant decrease in total distance traveled at the 1.0, 2.5, and 5.0 mg/kg doses in female mice.

A two-way mixed factor ANOVA for total time (sec) spent in center between sex and dose of buspirone revealed a statistically significant effect of dose, $F(5,140)=7.94$, $p<0.0001$, but neithor sex, $F(1,28)=1.07$, $p=0.31$, not the interaction, $F(5,140)=0.89$, $p=0.49$. Figure 24 (top) shows the total time spent in center (sec) after saline or buspirone administration to male mice. Buspirone administration significantly altered the total time spent in center, $F(3.14, 43.90)=8.92$, $p<0.0001$, in male mice. This is due a significant increase in time spent in the center for the 1.0, 2.5, and 5.0 mg/kg doses in male mice. The total number of entries and exits of the center was significantly altered in male mice, $F(3.41, 47.67)=16.62$, $p<0.0001$ (Figure 25 top). This was due to a

significant decrease at the 1.0, 2.5 and 5.0 mg/kg doses. Figure 24 (bottom) shows the total time spent in center (sec) after saline or buspirone administration to female mice. Buspirone administration did not significantly altered the time spent in center, $F(2.94, 41.17)=2.15$, $p=0.11$, in female mice. The total number of entries and exits of the center was significantly altered in female mice, $F(2.54, 35.54)=5.84$, $p=0.0037$ (Figure 25 bottom). This was due to a significant decrease at the 1.0 and 5.0 mg/kg doses.

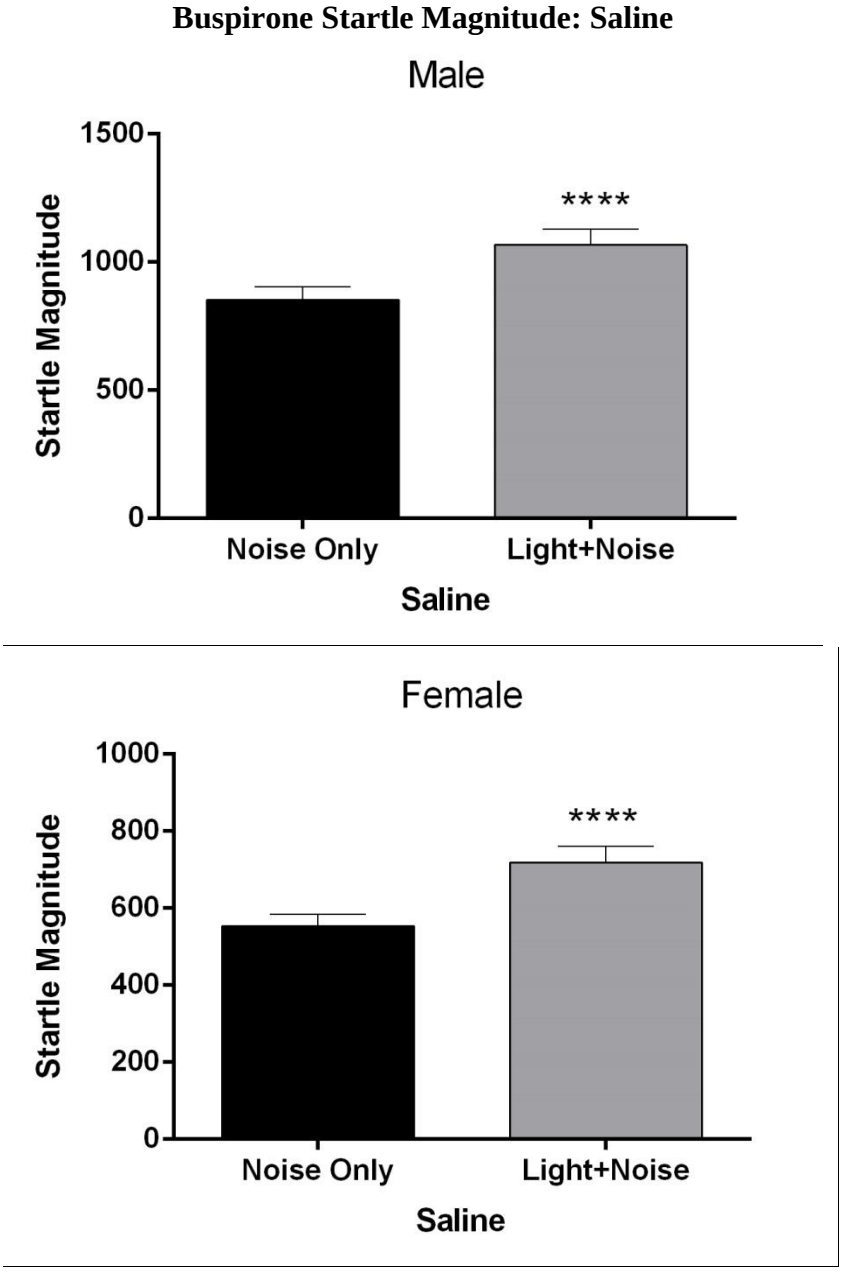

Figure 18: The light-noise and noise-only startle magnitude during saline administration for the buspirone group in male (top) and female (bottom) mice. ****$p<0.0001$ light+noise versus noise-only. Data are expressed as mean +/- SEM, N=15.

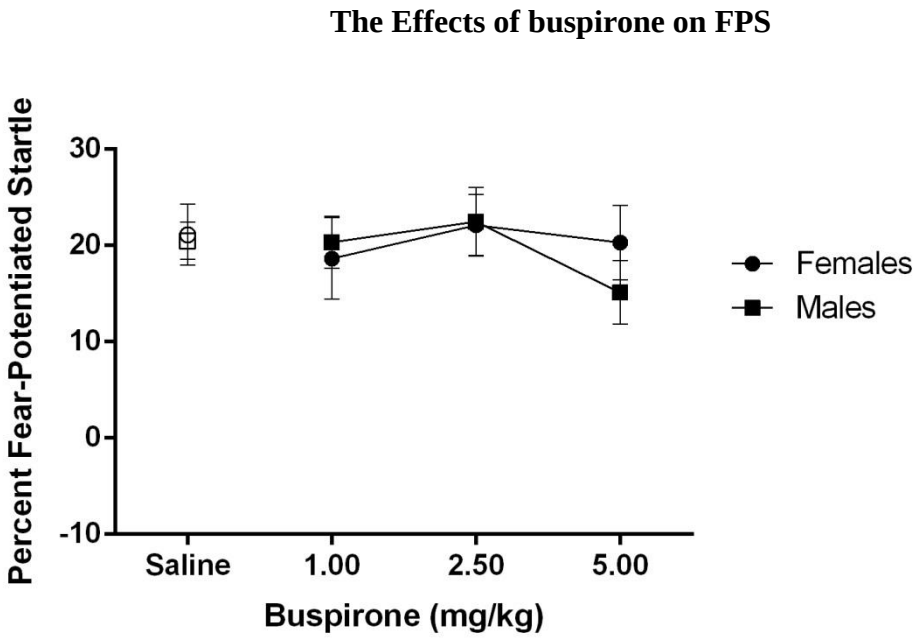

Figure 16: The effect of buspirone administration on FPS in male (square) and female (circle) mice. Data are expressed as mean +/- SEM, N=15.

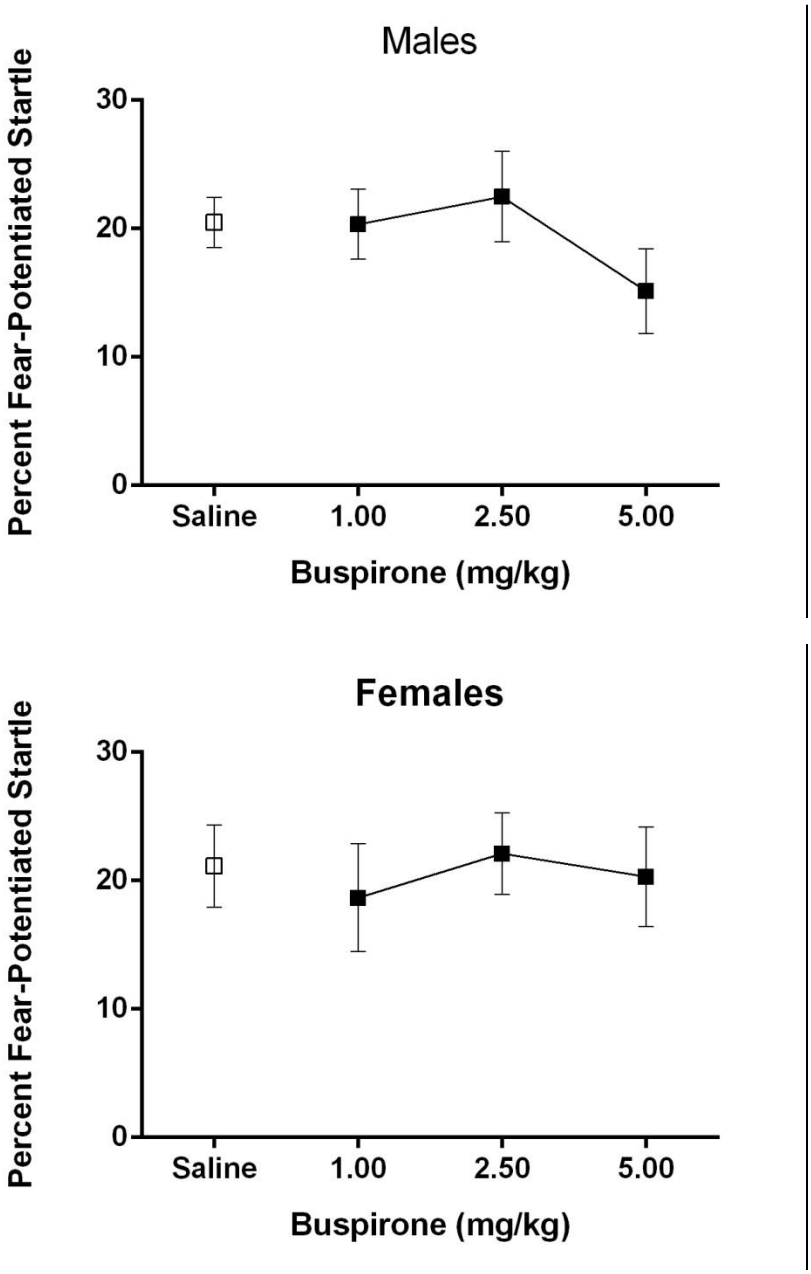

Figure 17: The effect of buspirone administration on percent fear-potentiated startle in male (top) and female (bottom) mice. Data are expressed as mean +/- SEM, N=15.

Buspirone Startle Magnitude: Male Light-Noise and Noise-only

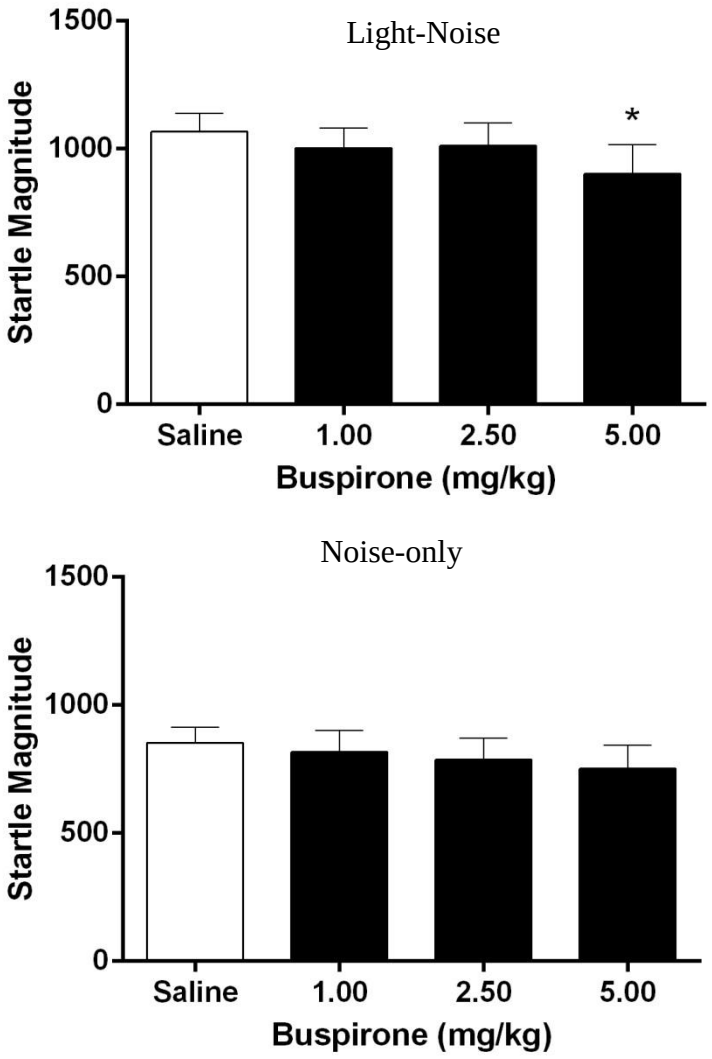

Figure 18: The light-noise (top) and noise only (bottom) startle magnitude during buspirone administration compared to saline in male mice. *p<0.05 versus saline. Data are expressed as mean +/- SEM, N=15.

Buspirone Startle Magnitude: Female Light-Noise and Noise-only

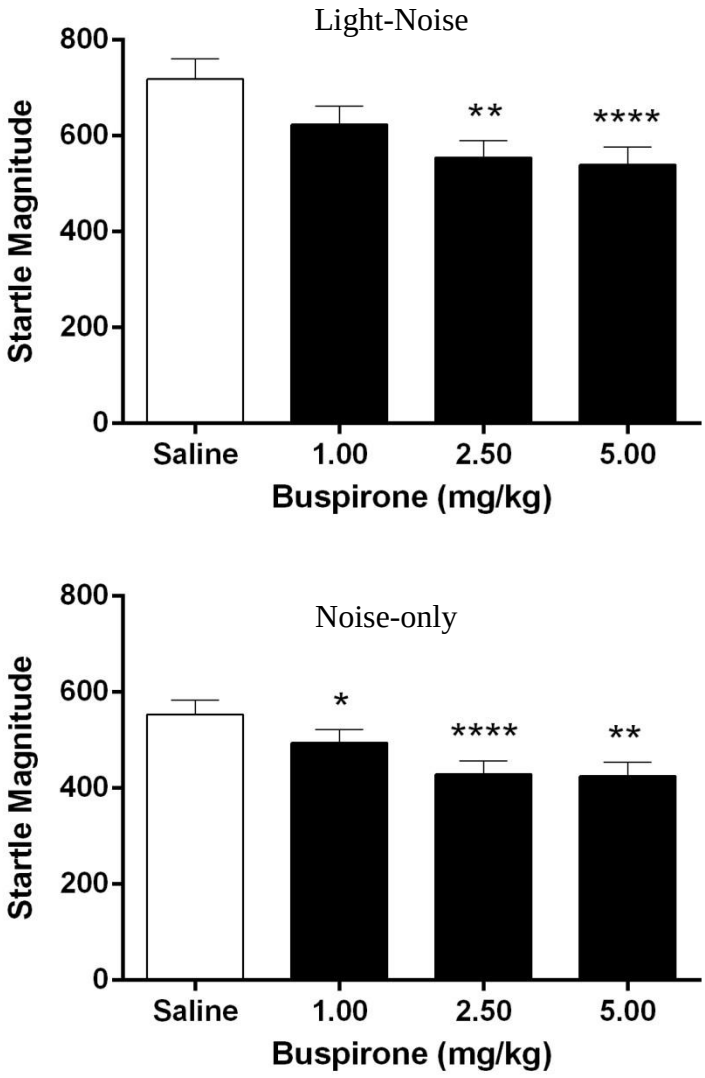

Figure 19: The light-noise (top) and noise only (bottom) startle magnitude during β-Lactotensin administration compared to saline in female mice. *p<0.05, **p<0.01 & ****p<0.0001 versus saline. Data are expressed as mean +/- SEM, N=15.

Buspirone: Total Distance Traveled

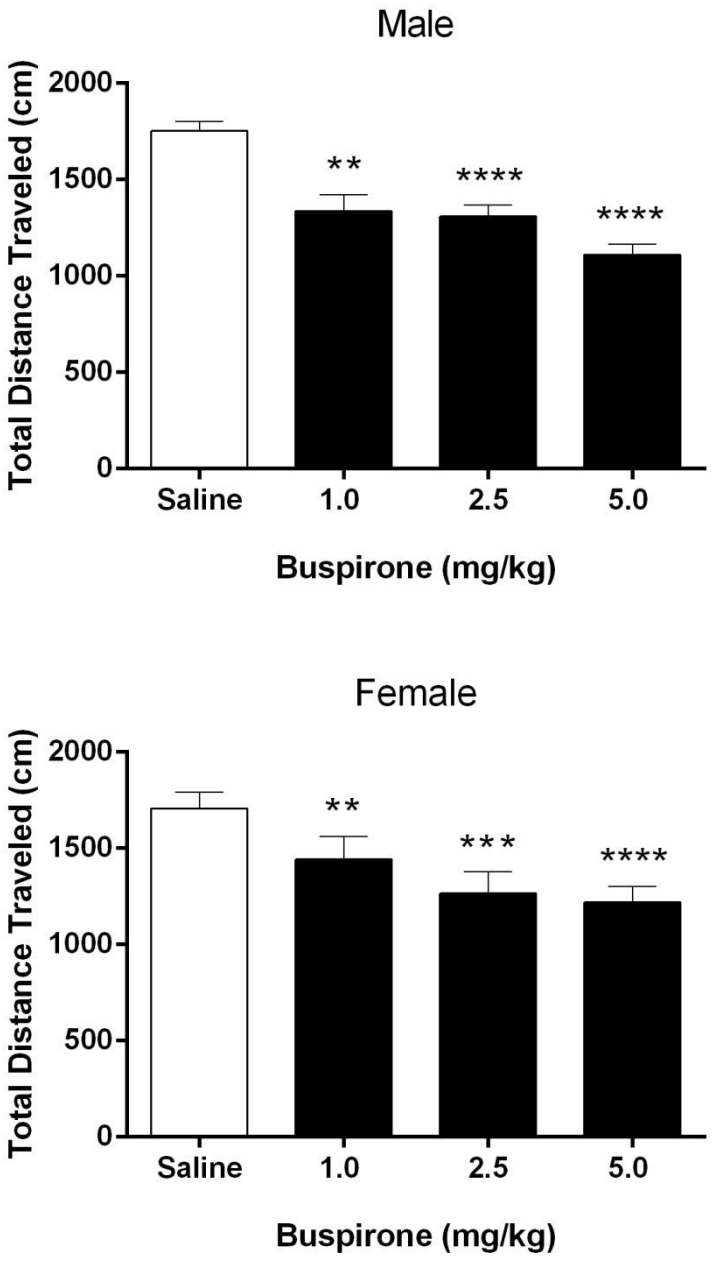

Figure 20: The effects of buspirone on the total distance traveled in male (top) and female (bottom) mice. **p<0.01, ***p<0.001 & ****p<0.0001 versus saline. Data are expressed as mean +/- SEM, N=15.

Buspirone: Time Spent in the Center

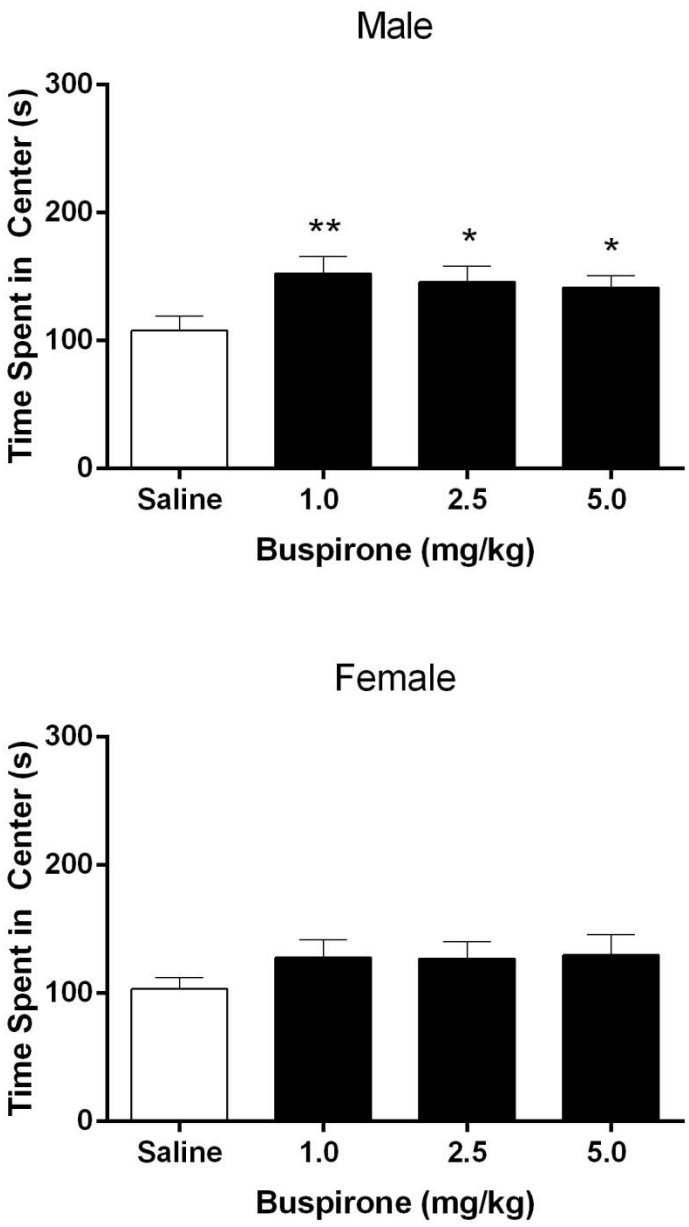

Figure 21: The effects of buspirone on the total time (s) spent in the center of the open field apparatus in male (top) and female (bottom) mice. *$p<0.05$ & **$p<0.01$ versus saline. Data are expressed as mean +/- SEM, N=15.

Buspirone: Total Entires and Exits of the Center

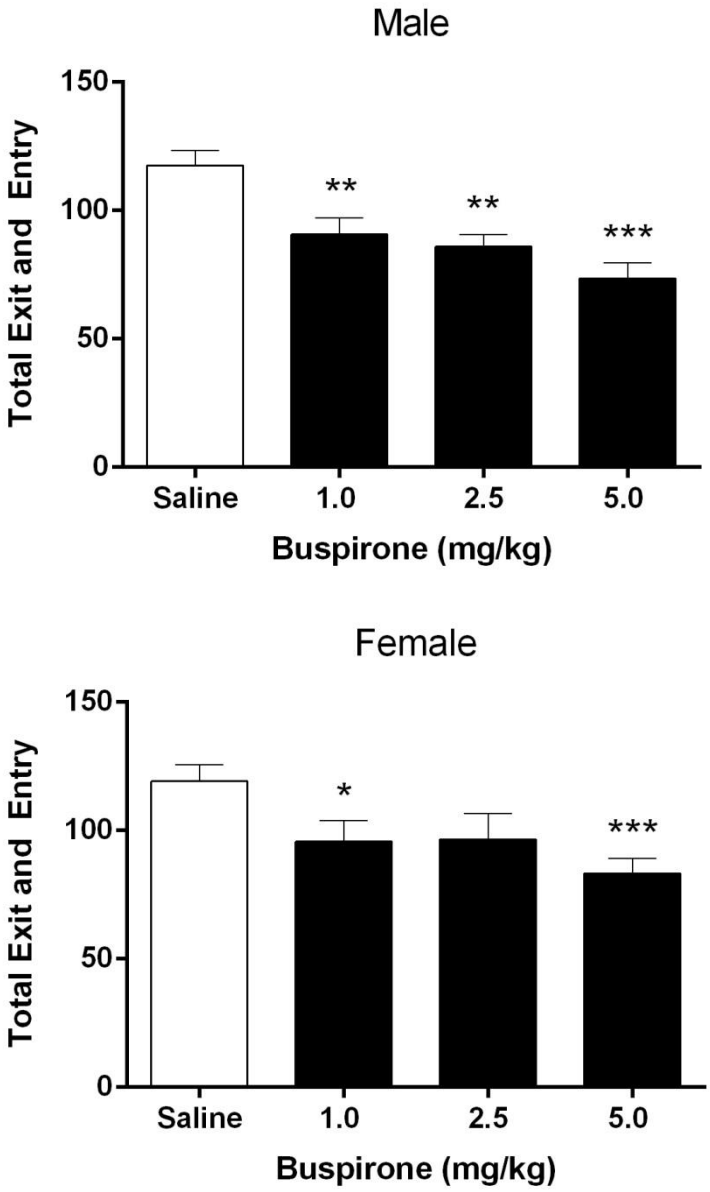

Figure 22: The effects of buspirone on the total exits and entries of the center of the open field apparatus compared to saline in male (top) and female (bottom) mice. *p<0.05, **p<0.01 & ***p<0.001 versus saline. Data are expressed as mean +/- SEM, N=15.

DISCUSSION

This was the first study to examine PD149163 and β-Lactotensin in male and female mice using a fear-potentiated startle paradigm. The present study demonstrated the differential effects of PD149163, a NTS_1 agonist, β-Lactotensin, a NTS_2 agonist, and buspirone, an anxiolytic and partial 5-HT_{1A} agonist, on FPS, startle magnitude, in male and female mice. PD149163 did not decrease, but rather increased FPS in male mice. Female mice, however, showed a decrease in FPS at the highest dose of PD149163. β-Lactotensin, at the doses tested, did not statistically increase or decrease FPS, however there was a significant decrease in female mice at the 0.3 mg/kg dose compared to male mice. Finally, there were no significant differences found in percent FPS using buspirone.

We examined the effect of multiple treatments in male and female mice, by testing saline before and after drug treatment and found no significant decrease in FPS for any group. This indicates that habituation did not occur over time, and suggests that any decreases in FPS occurred due to treatment. This may have been due to the training session 24 hours prior to each test session and gives support for a repeated measures design in order to study FPS. Winslow et al. (2007) also used a within subjects to study FPS in monkeys. Rhesus monkeys developed a persistent increase of the startle response when the CS was on during test sessions. A training session was completed prior to each test session also.

A significant increase in FPS in the PD14963 male group was found at the 0.1 mg/kg dose. We further examine this effect and looked at the differences between the light-noise and noise-only startle magnitude. When comparing the different trials, the light-noise trials decreased in startle magnitude but not enough to be considered different from saline and the noise-only trials decreased enough to be considered different from saline. This may indicate that PD149163 did not have an effect (or as strong of an effect) on the cue light, but decreased the sensitivity of the subject more during the noise only trials. The increase in FPS is contradictory to previous research. Shilling & Feifel (2008) found a decrease in FPS following administration of PD149163 in rats. Although PD149163 has been shown to decrease total distance traveled in mice, the decrease in locomotor activity was not thought to be a factor for the increase in FPS in male mice (Vadnie et al., 2014). In fact, one would hypothesize to see a decrease in FPS if locomotor activity also decreased. Time spent in the center and entries and exits of the center was not affected, therefore the subject was not trying to avoid the center which would be an indicator of an anxiolytic effect. Given the decrease in locomotor data, and no effect on time spent in the center and total entries and exits of the center, one would predict a decrease in FPS, however the opposite was found.

Females expressed a decrease in FPS after a dose of 1.0 mg/kg of PD149163. Both the light-noise and noise-only startle magnitude were decreased. The startle magnitudes were similar to that of a dummy weight in the chamber, meaning that the animals were not startling as much when the noise was produced regardless of the light being on or off. Female locomotor activity and entries and exits of the center were

decreased at the high dose. A possible reason for why the females showed a decrease in the FPS was due to a decrease in locomotor activity.

NTS_1 receptor knockout male mice traveled less and spent less time in the center of an open field compared to wild-type controls (Fitzpatick et al., 2012). While the male mice FPS data are contradictory to previous research, the female mice showed similar effects found by administration of PD149163. A decrease in FPS and startle magnitude following administration of PD149163 was previously found in rats (Shilling & Feifel, 2008). Vadnie et al. (2014) found a decrease in locomotor activity following injections of PD149163 in mice. Our study further supports that PD149163, at higher doses, disrupts general behavior.

β-Lactotensin decreased females FPS when compared to males at the 0.3 mg/kg dose. We further examined this effect by looking at the startle magnitudes for light-noise and noise-only trials. The light-noise startle magnitude was significantly decreased after administration of the 0.3 mg/kg dose of β-Lactotensin, while the noise-only startle magnitude was not affected. The locomotor activity, time spent in the center, and number of entries and exits of the center did not increase or decrease, therefore locomotor inhibition alone cannot explain the decrease in FPS at the 0.3 mg/kg dose. Baseline acoustic startle was not different between NTS_1 and NTS_2 knockout and wild-type mice, and showed that different drugs affected pre-pulse inhibition differently in NTS_1 and NTS_2 knockout mice (Oliveros et al., 2010). This lends support to continue studying the differences between NTS_1 and NTS_2 receptor agonists and antagonists.

Previous research indicated that buspirone blocked FPS in rats (Kehne, Cassella, and Davis, 1988; Risbrough et al., 2003). Our study found buspirone did not affect FPS in either male or female mice. This could be due to the way the subjects were trained. Moderate shocks produced enhanced startle amplitudes, while higher intensity shocks produced lower startle amplitudes (Walker et al., 1997). In rats, 0.4 mA produced the biggest difference between light-noise and noise-only conditions with a decrease in startle amplitude and FPS at higher intensities. Our study used 0.3 mA and pilot data showed an increase of a 30-40 percent FPS (unpublished).

Male and female rat NT mRNA expression and NT immunoreactivity is similar until the fifth week of postnatal life. This is when the sexual dimorphism of NT expression is established due to the presence sex hormone levels (Bello et al., 2004). NT mRNA expression and NT immunoreactivity in female rats are different than males, and the levels fluctuate during the estrous cycle (Kinkead et al., 2000). Further, estrogen has been shown to enhance NT/neuromedin gene expression (Watters & Dorsa, 1998). NT immunoreactive levels oscillate during the estrous cycle and are high during diestrus and low during estrous (Bello et al., 1999). Hiroi and Neumaier (2005) showed that injections of estrogen in ovariectomized female rats increased fear potentiated startle when compared to ovariectomized females without injections of estrogen. Perhaps the estrous cycle had an interaction with the drugs. Future research may want to control the estrous cycle by using ovariectomized female mice.

NTS_1 expression has been found in a variety of human tumors; Ewing's sarcoma, meningioma, astrocytoma, medullablastoma, and medullary thyroid cancers had the highest incidence percent (above 25 percent) (Reubi, Waser Schaer, and Laissue, 1999).

NTS_1 agonists may simulate tumor growth in lung, pancreatic, colon, prostate, and breast cancer, and NTS_1 antagonists may inhibit tumor growth in these cancers (For review see: Carraway & Plona, 2006). Further support shows that SR48692, a neurotensin receptor antagonist, inhibits the growth of small cell lung cancer cells (Moody, Chiles, Casibang, Moody, Chan, and Davis, 2001). While NTS_1 is associated with progressing tumor and cancer growth, the NTS_2 receptor has not been implicated in cancer progression (Leyton, Garcia-Marin, Jensen, and Moody, 2002). With the decrease at the 0.3 mg/kg dose of β-Lactotensin, further research may want to examine the effects of a more selective NTS_2 receptor agonist or antagonist may have on anxiety.

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Appendix A

PD149163: Subject	Sex	Saline Before	Low Dose	Medium Dose	High Dose	Saline After
FPS1	Male	12/30/15	1/5/16	1/12/16	1/19/16	1/26/16
FPS2	Male	12/30/15	1/5/16	1/12/16	1/19/16	1/26/16
FPS3	Male	12/30/15	1/5/16	1/12/16	1/19/16	1/26/16
FPS4	Mae	12/30/15	1/5/16	1/12/16	1/19/16	1/26/16
FPS5	Male	12/30/15	1/19/16	1/5/16	1/12/16	1/26/16
FPS6	Male	12/30/15	1/19/16	1/5/16	1/12/16	1/26/16
FPS7	Male	12/30/15	1/12/16	1/19/16	1/5/16	1/26/16
FPS8	Male	12/30/15	1/12/16	1/19/16	1/5/16	1/26/16
FPS24	Female	12/30/15	1/5/16	1/12/16	1/19/16	1/26/16
FPS25	Female	12/30/15	1/5/16	1/12/16	1/19/16	1/26/16
FPS26	Female	12/30/15	1/5/16	1/12/16	1/19/16	1/26/16
FPS27	Female	12/30/15	1/5/16	1/12/16	1/19/16	1/26/16
FPS28	Female	12/30/15	1/19/16	1/5/16	1/12/16	1/26/16
FPS29	Female	12/30/15	1/19/16	1/5/16	1/12/16	1/26/16
FPS30	Female	12/30/15	1/12/16	1/19/16	1/5/16	1/26/16
FPS31	Female	12/30/15	1/12/16	1/19/16	1/5/16	1/26/16
Subject	Sex	Saline Before	Low Dose	Medium Dose	High Dose	Saline After
FPS55	Male	2/16/16	2/25/16	3/2/16	3/8/16	3/15/16
FPS56	Male	2/16/16	2/25/16	3/2/16	3/8/16	3/15/16
FPS57	Male	2/16/16	2/25/16	3/2/16	3/8/16	3/15/16
FPS58	Mae	2/16/16	2/25/16	3/2/16	3/8/16	3/15/16
FPS59	Male	2/16/16	3/8/16	2/25/16	3/2/16	3/15/16
FPS60	Male	2/16/16	3/8/16	2/25/16	3/2/16	3/15/16
FPS61	Male	2/16/16	3/2/16	3/8/16	2/25/16	3/15/16
FPS77	Female	2/16/16	3/2/16	3/8/16	2/25/16	3/15/16
FPS78	Female	2/16/16	2/25/16	3/2/16	3/8/16	3/15/16
FPS79	Female	2/16/16	2/25/16	3/2/16	3/8/16	3/15/16
FPS80	Female	2/16/16	3/8/16	2/25/16	3/2/16	3/15/16
FPS81	Female	2/16/16	3/8/16	2/25/16	3/2/16	3/15/16
FPS82	Female	2/16/16	3/2/16	3/8/16	2/25/16	3/15/16
FPS83	Female	2/16/16	3/2/16	3/8/16	2/25/16	3/15/16
β-Lactotensin: Subject	Sex	Saline Before	Low Dose	Medium Dose	High Dose	Saline After
FPS9	Male	1/1/16	1/7/16	1/14/16	1/21/16	1/28/16
FPS10	Male	1/1/16	1/7/16	1/14/16	1/21/16	1/28/16
FPS11	Male	1/1/16	1/7/16	1/14/16	1/21/16	1/28/16
FPS12	Mae	1/1/16	1/7/16	1/14/16	1/21/16	1/28/16

FPS13	Male	1/1/16	1/21/16	1/7/16	1/14/16	1/28/16
FPS14	Male	1/1/16	1/21/16	1/7/16	1/14/16	1/28/16
FPS15	Male	1/1/16	1/14/16	1/21/16	1/7/16	1/28/16
FPS16	Male	1/1/16	1/14/16	1/21/16	1/7/16	1/28/16
FPS31	Female	1/1/16	1/7/16	1/14/16	1/21/16	1/28/16
FPS32	Female	1/1/16	1/7/16	1/14/16	1/21/16	1/28/16
FPS33	Female	1/1/16	1/7/16	1/14/16	1/21/16	1/28/16
FPS34	Female	1/1/16	1/7/16	1/14/16	1/21/16	1/28/16
FPS35	Female	1/1/16	1/1/16	1/7/16	1/14/16	1/28/16
FPS36	Female	1/1/16	1/21/16	1/7/16	1/14/16	1/28/16
FPS37	Female	1/1/16	1/14/16	1/21/16	1/7/16	1/28/16
FPS38	Female	1/1/16	1/14/16	1/21/16	1/7/16	1/28/16
β-Lactotensin: Subject	Sex	Saline Before	Low Dose	Medium Dose	High Dose	Saline After
FPS62	Male	2/18/16	2/27/16	3/4/16	3/10/16	3/17/16
FPS63	Male	2/18/16	2/27/16	3/4/16	3/10/16	3/17/16
FPS64	Male	2/18/16	2/27/16	3/4/16	3/10/16	3/17/16
FPS65	Male	2/18/16	2/27/16	3/4/16	3/10/16	3/17/16
FPS66	Male	2/18/16	3/10/16	2/27/16	3/4/16	3/17/16
FPS67	Male	2/18/16	3/10/16	2/27/16	3/4/16	3/17/16
FPS68	Male	2/18/16	3/4/16	3/10/16	2/27/16	3/17/16
FPS84	Female	2/18/16	3/4/16	3/10/16	2/27/16	3/17/16
FPS85	Female	2/18/16	2/27/16	3/4/16	3/10/16	3/17/16
FPS86	Female	2/18/16	2/27/16	3/4/16	3/10/16	3/17/16
FPS87	Female	2/18/16	3/10/16	2/27/16	3/4/16	3/17/16
FPS88	Female	2/18/16	3/10/16	2/27/16	3/4/16	3/17/16
FPS89	Female	2/18/16	3/4/16	3/10/16	2/27/16	3/17/16
FPS90	Female	2/18/16	3/4/16	3/10/16	2/27/16	3/17/16
Buspirone: Subject	Sex	Saline Before	Low Dose	Medium Dose	High Dose	Saline After
FPS17	Male	1/3/16	1/9/16	1/16/16	1/23/16	1/30/16
FPS18	Male	1/3/16	1/9/16	1/16/16	1/23/16	1/30/16
FPS19	Male	1/3/16	1/23/16	1/9/16	1/16/16	1/30/16
FPS20	Male	1/3/16	1/23/16	1/9/16	1/16/16	1/30/16
FPS21	Male	1/3/16	1/16/16	1/23/16	1/9/16	1/30/16
FPS22	Male	1/3/16	1/16/16	1/23/16	1/9/16	1/30/16
FPS23	Male	1/3/16	1/9/16	1/16/16	1/23/16	1/30/16
FPS40	Female	1/3/16	1/9/16	1/16/16	1/23/16	1/30/16
FPS41	Female	1/3/16	1/9/16	1/16/16	1/23/16	1/30/16
FPS42	Female	1/3/16	1/9/16	1/16/16	1/23/16	1/30/16
FPS43	Female	1/3/16	1/23/16	1/9/16	1/16/16	1/30/16
FPS44	Female	1/3/16	1/23/16	1/9/16	1/16/16	1/30/16
FPS45	Female	1/3/16	1/16/16	1/23/16	1/9/16	1/30/16
FPS46	Female	1/3/16	1/16/16	1/23/16	1/9/16	1/30/16

Buspirone: Subject	Sex	Saline Before	Low Dose	Medium Dose	High Dose	Saline After
FPS47	Male	2/23/16	2/29/16	3/6/16	3/12/16	3/19/16
FPS48	Male	2/23/16	2/29/16	3/6/16	3/12/16	3/19/16
FPS49	Male	2/23/16	2/29/16	3/6/16	3/12/16	3/19/16
FPS50	Male	2/23/16	2/29/16	3/6/16	3/12/16	3/19/16
FPS51	Male	2/23/16	3/12/16	2/29/16	3/6/16	3/19/16
FPS52	Male	2/23/16	3/12/16	2/29/16	3/6/16	3/19/16
FPS53	Male	2/23/16	3/6/16	3/12/16	2/29/16	3/19/16
FPS54	Male	2/23/16	3/6/16	3/12/16	2/29/16	3/19/16
FPS69	Female	2/23/16	2/29/16	3/6/16	3/12/16	3/19/16
FPS70	Female	2/23/16	2/29/16	3/6/16	3/12/16	3/19/16
FPS71	Female	2/23/16	2/29/16	3/6/16	3/12/16	3/19/16
FPS72	Female	2/23/16	2/29/16	3/6/16	3/12/16	3/19/16
FPS73	Female	2/23/16	3/12/16	2/29/16	3/6/16	3/19/16
FPS74	Female	2/23/16	3/12/16	2/29/16	3/6/16	3/19/16
FPS75	Female	2/23/16	3/6/16	3/12/16	2/29/16	3/19/16
FPS76	Female	2/23/16	3/6/16	3/12/16	2/29/16	3/19/16

Application to Use Vertebrate Animals in Research, Testing or Instruction

Northern Michigan University

Project Title (If using external funds, enter the title used on the grant application): The Effects of Neurotensin on the Expression of Fear-Potentiated Startle in Mice

Shaded area for IACUC use only.

Application Number: 254 Amended
Date Application Received: November 2, 2015
☒ Approved ☐ Denied on November 9, 2015

General Instructions
Please check the IACUC website to ensure you are using the current version of the form. All parts of this form must be submitted electronically to the Institutional Animal Care and Use Committee (email: IACUC@nmu.edu) and the relevant Department Head or other departmental designee. Review of this application will commence upon receiving the electronic application, but the project may not begin until all required approval signatures are obtained via Right Signature. Please contact the IACUC chair (email: IACUCChr@nmu.edu) if you have any questions.

Review Dates:
Designated Member Review of applications (appropriate for USDA Use Categories B and C) will be completed within two weeks after receipt of the electronic application.
Full Committee Review of applications will take place on the last Friday of every month. Applications for Full Committee Review must be electronically received by the first Friday of the month. Full Committee Review is required for applications that fall under USDA Use Categories D and E. Applications that fall under USDA Use Categories B and C will receive Full Committee Review if requested by an IACUC member. Detailed procedures on the IACUC review processes are located at the IACUC website.

I. Principal Investigator (Must be a faculty member or Department Head): Adam Prus
Co- Investigator: Mark Vanden Avond
Department: Psychology
Phone number: x2941

II. Funding Sources/Course Information and Dates

If the proposed work is for a course, please include the number of the course and title of the course

Assessment of fear potentiated startle in mice

Funding Sources (External & Internal, if applicable) Internal?

Additional Funding Pending (click on the correct box)? ☒Yes ☐No

Project/Course Start Date: January 5, 2015
End Date (three year maximum): 1/5/2017

This application is (check one~~)~~ ☒~~New~~ ☒ **Modification of an application currently approved by the** —

Institutional Animal Care and Use Committee (a new protocol must be submitted after three years)