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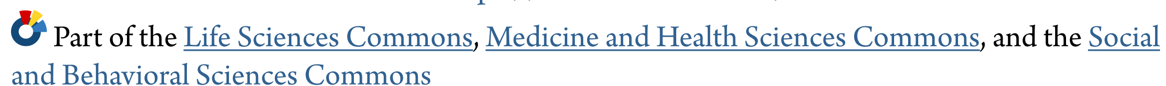
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INTRACRANIAL INFUSIONS OF NEUROTENSIN AGONISTS PRODUCE AN ANXIOLYTIC PROFILE IN A RAT ULTRASONIC VOCALIZATION MODEL

By

Floyd F. Steele III

THESIS

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ABSTRACT

INTRACRANIAL INFUSIONS OF NEUROTENSIN AGONISTS PRODUCE AN ANXIOLYTIC PROFILE IN A RAT ULTRASONIC VOCALIZATION MODEL

By

Floyd F. Steele III

Neurotensin (NT) is a peptide neurotransmitter that interacts with brain monoamine neurotransmitter systems. It has been demonstrated that neurotensin type 1 and type 2 receptor agonists influence animal models of psychological disorders and pain regulation, respectively. It has already been shown that the systemic administration of the selective neurotensin type 1 receptor agonist PD149163 can attenuate the number of fear-induced 22-kHz ultrasonic vocalizations (USVs) produced by male Wistar rats. A reduction in the number of 22-kHz USV calls is indicative of an anxiolytic effect. The current study used a USV model to evaluate the effects of PD149163 (0.1, 1.0, and 10.0 ng) and endogenous NT (0.1, 1.0, and 10.0 μg) when administered into the lateral ventricle of male Wistar rats. Both 10ng of PD149163 and 10μg of NT were shown to attenuate USV calls when administered into the lateral ventricle. PD149163 was found to have a higher potency than NT in the USV model. In addition, while 100ng of PD149163 significantly reduced USV calls, it did not reduce locomotion on an open field that was surrounded by bright lighting. These data suggest neurotensin receptor activation is a putative mechanism for novel pharmacological treatments of anxiety disorders.

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INTRODUCTION

Anxiety and Panic

Anxiety disorders are associated with attentional deficits, physical tension, restlessness, irritability, panic, phobia, and social isolation. While anxiety disorder subtypes can vary in symptomology, e.g. separation anxiety disorder, agoraphobia and specific phobia disorder, other anxiety disorders, such as Generalized Anxiety Disorder (GAD) versus Panic Disorder, differ in symptom intensity and duration (American Psychiatric Association, 2013). A comorbidity of no less than 50% has been found between anxiety disorders and major depressive disorder, and around 30% of United States citizens suffer from an anxiety disorder at least once in their lifetime (Hirschfeld, 2001).

Anxiety disorders can be comprised of acute/intense panic attack episodes which are correlated with hyperactivity of the sympathetic nervous system; this hyperactivity can result in tachycardia and hyperventilation. The current Diagnostic and Statistical Manual of Mental Disorders, DSM-5, states that panic attack symptoms can be used to predict the severity, course and treatment of all other anxiety disorders, as well as psychological disorders not classified as anxiety disorders (American Psychiatric Association, 2013). The symptoms of panic attacks can be represented using animal models of panic responding. This panic responding can be elicited by both isolatable stimuli and environmental contexts.

Molecular Basis and Pharmacological Treatments of Anxiety

There is evidence that elevated activity of the sympathetic nervous system (SNS), which is mediated by noradrenergic neurotransmission, is a product of central nervous system (CNS) chemical signaling abnormalities. It has been demonstrated that the α_2-adrenoceptor antagonist yohimbine only evokes panic attacks for individuals who have been diagnosed with panic disorder (Nutt et al., 1998). This suggests that blocking α_2-adrenergic receptors is an insufficient means of producing panic attacks. It follows that, while anxiety is sometimes treated by peripherally acting β-adrenergic antagonists, most modern anxiolytic agents target CNS systems that mediate peripheral adrenaline levels (Sinclair & Nutt, 2007).

While many CNS neurotransmitter systems have been linked to anxiety, e.g. dopaminergic D_1 and D_2 receptors, glutamatergic AMPA and NMDA receptors, and CCK_B receptors of the neuropeptide cholecystokinin, all currently marketed anxiolytic medications either modulate $GABA_A$ receptors or block the reuptake of presynaptic serotonin (Dooley & Klamt, 1993; Jessa et al., 1996; Simon et al., 1993; Nutt, 2005).Benzodiazepine (BDZ) drugs bind to the BDZ site on $GABA_A$ receptors, potentiating the inhibitory influx of chloride that is initiated by $GABA_A$ receptor agonists. Since the $GABA_A$ receptor is the primary inhibitory receptor in the CNS, BDZs such as diazepam (Valium) and alprazolam (Xanax) indirectly treat anxiety by inhibiting other neurotransmitter systems that are associated with anxiety (Sinclair & Nutt, 2007).

BDZs are often used to treat extreme cases of panic disorder, but are not the first line of treatment since, like most CNS depressants, they produce a risk of drug dependence when chronically administered (Licata & Rowlett, 2008). A withdraw

induced increase in baseline anxiety has occurred in almost a quarter of individuals who have chronically taken BDZs, and while on a BDZ, a person may become drowsy and experience motor coordination deficits (Kaplan & DuPont, 2005).

The first line of treatment for most anxiety disorders is the use of selective serotonin reuptake inhibitors (SSRIs), e.g. fluoxetine (Prozac) and sertraline (Zoloft), which are also the most common treatments for depressive disorders (Bystritsky et al., 2013). Since SSRIs increase synaptic serotonin (5-HT) levels by inhibiting reuptake of 5-HT from the synaptic cleft, brain functional abnormalities resulting in the inability of serotonergic neurons to produce and/or secrete 5-HT may be important factors in the pathology of anxiety disorders. While SSRIs act by blocking the activity of 5-HT transporter (SERT), SERT knockout mice, which display elevated synaptic 5-HT concentrations, demonstrate an anxiogenic behavioral phenotype when compared to mice that express SERT (Kalueff et al., 2007). This suggests that there are therapeutic limitations to fully blocking SERT at certain brain regions.

Pre- and postsynaptic serotonin 5-HT_{1A} receptor expression may also influence anxiety. Humans with lower densities of CNS 5-HT_{1A} receptors demonstrate more symptoms of anxiety than those expressing the average density (Condren et al., 2002). Additionally, Gross et al. (2002) showed, while raphe nucleus 5-HT_{1A} autoreceptor knockout mice don't exhibit an anxiogenic phenotype, whole brain 5-HT_{1A} receptor knockout mice do. This means that forebrain 5-HT_{1A} receptor activation can influence anxiety-like behavior independently of raphe nucleus 5-HT_{1A} activation (Gross et al., 2002). Similar to $GABA_A$ receptor activation, postsynaptic 5-HT_{1A} activation is known to inhibit both limbic and cortical glutamate transmission. Postsynaptic 5-HT_{1A} activation

enhances potassium efflux and reduces adenylyl cyclase activity in postsynaptic glutamatergic neurons (Millan, 2003). Unlike $GABA_A$ activation, 5-HT_{1A} activation is not associated with memory deficits. When on diazepam, human subjects with GAD have been shown to exhibit deficits in memory recall after a 20 minute delay; buspirone, a selective serotonin 5-HT_{1A} receptor partial agonist, does not produce these deficits (Lucki et al., 1987).

Administration of 5-HT_{1A} receptor agonists and SSRIs have been found to produce aversive effects in humans during initial treatment. The symptoms include nausea, motor deficits and depressive symptoms such as thoughts of suicide (Nutt, 2005). These aversive effects are correlated with activation of both the hypothalamo-corticotropic axis and sympathetic noradrenergic activity, suggesting that an acute increase in serotonergic neurotransmission up-regulates stress mechanisms (Millan, 2003). After the onset of treatment, anxiety symptoms often dissipate, supporting the theory that desensitization of brain 5-HT_{1A} autoreceptors and increased expression of forebrain postsynaptic 5-HT_{1A} receptors mediate the delayed efficacy of SSRIs. Le Poul et al. (1995) showed that after 3 days of daily SSRI treatment, rats demonstrated desensitization of somatodendritic 5-HT_{1A} autoreceptors in their dorsal raphe nuclei. Briones-Aranda, Rocha & Picazo (2005) found that mice expressed a greater density of amygdala 5-HT_{1A} receptors after experiencing a forced-swim procedure. SSRIs are not without additional side effects. Prolonged SSRI treatment can result in asthenia, unhealthy eating habits and sexual dysfunction (Nutt, 2005; Hirschfeld, 2003).

Subcortical Circuitry and Animal Models of Fear and Anxiety

Fear is the activation of multiple brain systems that promote defensive behavior; at healthy levels, fear responding is used to increase the fitness of a species (Fendt and Fanselow, 1999). Using rodent models, unconditioned fear can be studied by measuring a rodent's tendency to burrow, defecate, explore novel environments, interact with novel objects, hide in confined spaces, move near walls, and startle. Rodent models of anxiety are also used to study conditioned fear, e.g. conditioned freezing, fear-potentiated startle and ultrasonic vocalizations. Models of conditioned and unconditioned fear have been used to uncover some of what is currently known about the mechanisms of effective anxiolytic agents and their connection to subcortical circuitry.

There is direct evidence that the amygdala is a locus for the mediation of fear. In the fear-potentiated startle paradigm, a model that measures an increase in startle that is provoked by an external cue, potentiated startle is blocked when lesions are made in the amygdala (Hitchcock & Davis, 1986). Microinjections of the glutamatergic NMDA receptor antagonists AP-5 and AP-7 have been shown to block the acquisition of fear potentiated startle when injected into the basolateral amygdala, with AP-5 also blocking fear extinction (Campeau et al., 1992; Falls et al., 1992). Similarly, NMDA antagonism at the basolateral amygdala disrupts CS-US pairing in contextual freezing paradigms using rats (Fanselow & Kim, 1994). While these compounds blocked CS-US learning, they did not block the expression of fear-potentiated startle when they were administered after the completion of acquisition trials. The amygdala is also involved in the expression of fear. Microinjections of glutamatergic AMPA receptor antagonists block the expression of fear-potentiated startle when injected non-selectively into the amygdala; an effect that is

not produced by NMDA antagonists (Walker & Davis, 1997). Cholecystokinin CCK_B receptor activation has also been shown to increase the expression of startle when injected into the amygdala (Frankland et al., 1997).

The amygdala, while morphologically subdivided into about 6 sub-regions, is more often functionally subdivided into the basolateral complex of the amygdala (BLA) and the central complex of the amygdala (CeA). The current theory of amygdaloid fear processing is that the activated BLA, after receiving input from the thalamus and cortical structures, uses glutamatergic transmission to activate the amygdala's intercalated paracapsular islands (Royer et al., 1999). Activation of the GABAergic neurons of the lateral islands locally inhibits the medial paracapsular inhibitory GABAergic neurons (Ehrlich et al., 2009). Since the medial islands project onto the CeA, the intercalated paracapsular islands act as a feed-forward disinhibitory system that links the BLA to the CeA. This suggests that amygdala circuitry is dense with GABAergic gating mechanisms.

A diversity of GABAergic neurons has been located in the BLA as well as other parts of the amygdala. Bienvenu et al. (2012) reported that, when using a parvalbumin marker in rat BLA tissue, two functionally different GABAergic neuron subtypes were located. There are "basket" interneurons that are involved in feed-forward inhibition; they project to the soma and proximal dendrites of amygdaloid pyramidal cells. There are also interneurons that form axo-axonic synapses with BLA pyramidal cells, suggesting an additional feed-back inhibitory mechanism. There are GABAergic interneurons in the BLA that express both cannabinoid CB_1 autoreceptors and CCK_B receptors; this supports the notion that CCK_B receptors modulate the activity of GABA neurons. Microinjection

of CCK potentiates GABAergic inhibition of neurons within the amygdala by activating CCK_B receptors (Branchereau et al., 1992; McDonald & Mascagni, 2001).

Behavioral paradigms have also been used to show the importance of GABAergic transmission in fear learning. Zangrossi and Graeff (1994) demonstrated that bilateral injection of the BDZ agonist midazolam in the rat BLA increases the amount of time a rat spends exploring the open arms of an elevated plus maze; behavior predictive of anxiolytic drug effects. The $5\text{-}HT_{1A}$ full agonist 8-OH-DPAT did not increase time spent exploring the open arms in this study. It was also found that $5\text{-}HT_2$ antagonism made the animals exhibit anxiogenic behavior.

Heldt et al. (2012) found that viral disruption of glutamic acid decarboxylase 67 (GAD67) expression produces both a deficit in fear extinction and in the ability of diazepam to reduce conditioned freezing in C57BL\6 mice. The viral vectors were bilaterally microinjected into the amygdala; reduced freezing to an auditory cue correlated with fewer GAD67 enzymes within the amygdala. GAD67 metabolizes L-glutamic acid (glutamate) into GABA. BDZs show differential efficacy depending on which region of the amygdala they are administered. When midazolam is administered to the CeA, it produces a reduction in the passive avoidance of pain-eliciting stimuli (i.e. probe burying task), without reducing time spent in closed arms on the elevated-plus maze (EPM) (Pesold & Treit, 1995). Conversely, microinjections of midazolam into the BLA increases time spent on EPM open arms while having no effect on pain-related passive avoidance.

A human study conducted by Lucki et al. (1987) looked at 37 patients diagnosed with GAD. It was determined that, compared to vehicle, patients given 5 mg/kg of

diazepam were able to remember fewer terms after a 20 minute delay in the free recall task. The 5-HT_{1A} partial agonist buspirone did not produce this deficit at either 5 or 10 mg/kg. This highlights the importance of elucidating the roles of amygdala and hindbrain serotonergic systems in the modulation of anxiety. Using a trace conditioning paradigm, Zhang et al. (2013) used 1 mg/kg of the 5-HT_{2A} agonist TCB-2 to increase the rate of fear extinction. It was also confirmed that 0.5 mg/kg of MDL 11,939, a 5-HT_{2A} antagonist, can be used to delay the onset of extinction. An electrophysiological study by Hammock et al. (2009) used the patch-clamp technique to study the role of serotonergic systems in the bed nucleus of the stria terminalis (BNST), a major output from the amygdala to the thalamus. While BNST serotonergic systems consist of multiple 5-HT subtypes (e.g. 5-HT_{1A}, 5-HT_{2A}, 5-$HT_{2C,}$ etc.), it is the inhibitory effect of 5-HT that reduces anxiogenic activity associated with BNST excitation.

Vecente and Zangrossi (2012) used fluoxetine and imipramine to show that 5-HT_{2C} receptors of the BLA have a facilitating role in anxiety. 5-HT_{2C} activation led to an increase in inhibitory avoidance learning in the elevated T-maze paradigm. Strauss et al. (2013) used the elevated T-maze to show that BLA 5-HT_{1A} has the opposite effect; both 0.4 and 16 nmol of 8-OH-DPAT reduce the acquisition of both inhibitory avoidance and escape behaviors. Additionally, these concentrations produce anxiolytic effects on both the light-dark transition model and the Vogel conflict test; a greater amount of time was spent in the light compartment and more punished drinking was observed, respectively. These results were confirmed using microinjections of 0.37 nmol WAY-100635 and 10-40 nmol mitazolam to block and reverse the effects, respectively.

Dopamine (DA) also has a role in regulating anxiety and anxiety-like behavior in non-human animals. When placed in an open field, mice that are given non-selective dopamine D_1 and D_2 agonists exhibit an increase in thigmotaxis, suggesting that dopaminergic mechanisms might allow for novel anxiety treatments (Simon et al., 1994). Bartoszyk (1998) found that many dopamine D_2 receptor agonists (e.g. quinpirole, apomorphine, etc.) reduce 22-kHz ultrasonic vocalizations in male Sprague Dawley rats. Rat 22-kHz ultrasonic vocalizations (USVs) are used as a measure of negative affect and will be discussed in more detail in another section. Facilitation of dopamine D_2 autoreceptors has been demonstrated to reduce USV production in conditioned foot-stock paradigms and many of the effective dopaminergic compounds administered in Bartoszyk (1998) were selective dopamine D_2-family autoreceptor agonists (e.g. pramipexole, roxindole, 7-OH-DPAT, etc.). While a reduction in dopaminergic transmission may directly influence the expression of USVs, there is evidence that the dopamine D_2 agonist apomorphine reduces burying behavior in the Vogel conflict task and increases exploratory behavior (Hjorth et al., 1987; Talalaenko et al., 1994).

Systemic injections of dopamine D_1 agonist SKF 38393, D_1 antagonist SCH 23390, D_2 agonist quinpirole, and D_2 antagonist sulpride do not influence the acquisition of conditioned fear in fear-potentiated startle and conditioned freezing tasks (Ribeiro de Oliveira et al., 2006). After the acquisition phase of the fear-potentiated startle and conditioned freezing tasks, the dopamine D_2 agonist quinpirole (0.1 and 0.25 mg/kg) reduced startle magnitude and freezing, respectively. These doses did not decrease locomotor activity in the open field task, suggesting that dopamine D_2 receptor activity modulates the expression of both learned and unlearned fear. Contrarily, amygdaloid

microinjections of the selective dopamine D_1 antagonist SCH 23390 blocks the acquisition of fear potentiated startle, conditioned freezing and second-order conditioning (Lamont & Kokkinidis, 1998; Guarraci et al., 1999; Nader & LeDoux, 1999). While there is not an amount of SCH 23390 that has been shown to attenuate the expression of learned fear on the aforementioned models, Parez del la Mora et al. (2005) has shown that when SCH 23390 is microinjected into the BLA and intercalated islands, rats show an increase in time spent in the illuminated box of a White and Black Box test. Dopamine D_1 receptor antagonism can suppress unconditioned fear.

Intra-amygdaloid injections of dopamine D_2 receptor antagonists tend to show differential effects across animal models of fear. Ralcopride (2-8 μg) blocks the acquisition of fear potentiated startle when injected into the BLA, while smaller quantities of raclopride (0.73 and 2.4 μg) are injected into the CeA, rats exhibit an anxiogenic effect on the Shock-Probe Burying test (Greba et al., 2001). Another dopamine D_2 receptor antagonist, eticopride, has been shown to block the acquisition of freezing when a 1 μg injection is placed into the CeA (Guarraci et al., 2000). While dopamine D_2 receptor antagonism may potentiate the behavioral markers of unconditioned fear, it can attenuate the formation of conditioned fear. Bissiere et al. (2003) demonstrated that the lateral amygdala (LA) dopamine system is a gating mechanism for LA long-term potentiation (LTP). The *in vitro* conjunction of thalamic afferent presynaptic stimulation, postsynaptic LA stimulation and DA administration was found to induce LA LTP only when the LA GABAergic system was functional. When 100μM picrotoxin, a $GABA_A$ antagonist, and 100μM DA were administered into the LA,

DA did not induce LA LTP. Quinpirole, a dopamine D_2 receptor agonist, was used to facilitate the effect of the combined treatment.

Ponnusamy et al. (2005) showed, in mice, that the dopamine D_2 receptor agonist quinpirole blocked fear extinction when systemically injected prior to the extinction phase of a cued footstock paradigm. After three pairings of white noise and shock, they either injected rats with quinpirole, the dopamine D_2 receptor antagonist sulpride, or vehicle. One day after the acquisition phase, they issued the extinction phase immediately after one of the compounds was injected. Twenty-four hours after the extinction phase, Ponnusamy and colleagues administered another extinction phase in which they measured differences in the amount of freezing between the different drug conditions. While the quinpirole animals exhibited about as much freezing as they did in the prior extinction phase, the sulpride animals showed a significant decrease in freezing when compared to the vehicle animals on the second extinction day. While dopamine D_2 receptor agonism appears to suppress the expression of fear, dopamine D_2 receptor antagonism has been shown to facilitate fear extinction. Scibilia et al. (1992) used autoradiographic procedures to show that sulpride primarily binds to the CeA region of the amygdala; while dopamine D_2 receptors were located in the BLA, the CeA was found to contain a higher concentration of dopamine D_2 receptors. Additionally, sulpride has been shown to reduce freezing in a footshock-induced contextual conditioning procedure when administered into the BLA post-acquisition (de Souza Caetano, 2013).

The Neurotensin System and Monoamine Interactions

Neurotensin (NT) is a thirteen amino acid peptide neurotransmitter that was first isolated from the bovine hypothalamus (Tanganelli et al., 2012). While it regulates gastro-intestinal cellular communication, it has also been found to influence mammalian endocrine, paracrine and nervous systems. There are three known neurotensin receptor subtypes, neurotensin receptor type 1-3, but only neurotensin type 1 and type 2 receptors have been linked to overt behavior in rodent models. Neurotensin type 1 receptor (NTS1) activation has been shown to influence animal models of food intake and psychological disorders, including anxiety, depression, and schizophrenia, while neurotensin type 2 receptor (NTS2) activation has been given an analgesic profile (Boules, 2013). Both of these receptor subtypes are seven transmembrane domain G-protein coupled receptors.

NTS1 differentially activates $G_{i/o}$, G_s and $G_{q/11}$ G-protein pathways depending on the cell type and ligand that it interacts with. There is evidence that direct interactions exists between NTS1 agonists and G-proteins (Pelaprat, 2006). The affinity of NT for NTS1 has been shown to decrease when $G_{i/o}$ activity is disrupted by pertussis toxin (Gailly et al., 2000). Furthermore, pertussis toxin reduces NT induced $GTP_{\gamma}S$ binding for rat NTS1 receptors, again demonstrating a preference for NT/ $G_{i/o}$ interactions (Najimi et al., 2002). While less is known about NTS2/G-protein interactions, it has been hypothesized that NTS2 receptors also differentially activate multiple G-protein pathways (Pelaprat, 2006).

Endogenous NT has nearly fifteen times greater affinity for NTS1 than it does for NTS2. NTS2 was first found to be activated by the synthetic histamine H_1 receptor

antagonist levocabastine, which has no affinity for NTS1 (Vincent et al., 1999). The synthetic compound SR48692 has been shown to selectively antagonize NTS1 activation. The binding sites for SR48692 overlap with NT binding sites, suggesting that SR48692 acts as a competitive antagonist for NT at NTS1 (Labbe-Jullie et al., 1998). NTS2 expressing COS cells, which are derived from monkey kidney tissue, have a ligand-receptor binding profile almost complimentary to NTS1. In this cell line, SR48692 administration at NTS2 produces intracellular Ca^{2+} mobilization. While NT, neuromedin N and levocabastine do not antagonize SR48692 induced Ca^{2+} mobilization, they have been shown to act as both agonists and antagonists for other SR48692 activated NTS2 second messenger pathways (Pelaprat, 2006).

NTS1 exists in the CNS on both pre- and post-synaptic neurotensinergic neurons (Tanganelli, 2012). neurotensinergic systems have been found to co-localize with both amino acid and monoamine neurotransmitter systems in brain regions such as the ventral tegmental area, substantia nigra, striatum, nucleus accumbens, raphe nucleus, hypothalamus, and amygdala (Boudin et al., 1996). This suggests that neurotensinergic/monoaminergic interactions could be integral to the mechanisms by which NTS1 activation facilitates behavior. While little is currently known about neurotensinergic/noradrenergic interactions, there is interaction data concerning the other monoamines.

Dilts et al. (1996) used both tryptophan hydroxylase and 5-hydroxytrytophan (5-HTP) levels to demonstrate that NT attenuates sound stress-induced increases in 5-HT levels. Prior to a sound stress test, rats received an i.p. injection of NSD 1015, an aromatic amino acid decarboxylase inhibitor which prevents 5-HTP from degrading into

5-HT, and an i.c.v. injection of NT. NT significantly reduced both tryptophan hydroxylase, which converts tryptophan into 5-HTP, and 5-HTP levels when compared to sham brain tissue after the sound stress test was administered. The decrease in 5-HT levels does not imply that NT decreases serotonergic activity. Jolas and Aghajanian (1996) demonstrated that application of NT and its 8-13 amino acid fragment produced excitatory activity at the dorsal raphe nucleus; this increased activity was blocked by SR48692.

NT may also be linked to serotonergic activity in the limbic system. Shugalev et al. (2008) demonstrated that the 5-HT_{1A} receptor agonist 8-OH-DPAT shares a profile with NT in a passive avoidance task. When 8-OH-DPAT or NT are administered into the rat substantia nigra, shorter latencies are observed before the animals move onto a shock grid that has been associated with aversive shock. Inversely, both 8-OH-DPAT and NT produce greater latencies prior to crossing when they are administered into the dorsal cervical nucleus. The decrease in passive avoidance learning observed in animals treated with NT in the substantia nigra coincided with increases in 5-HT and its metabolite, 5-hydroxyindoleacetic acid, in their caudate nuclei. It is still not known whether these similar behavioral profiles are representing similar physiological pathways, but there is a high probability that both NT and 5-HT facilitate dopaminergic transmission within the rat midbrain.

There is an abundance of direct evidence that NT and DA systems interact. Mice that are knockouts for NTS1 show greater d-amphetamine induced locomotion than their wild type counterparts while expressing less mRNA for the dopamine D_1 receptor (Liang et al., 2010). The decrease in dopamine D_1 receptor expression coincided with a reduced

affinity of the dopamine D_2 receptor family antagonist raclopride for D_2. Relationships between midbrain dopamine D_2 receptors and NTS1 expression have also been evaluated using electrophysiological techniques. Amano et al. (2008) demonstrated that synapses between the LA and the BLA for tissue extracted from NTS1 knockout mice were prone to express LTP at currents that did not produce LTP in wild type synapses. They also showed that the pharmacological blockade of NTS1 via SR48692 eliminated LTP in knockout preparations. The dopamine D_2 receptor antagonist sulpride also eliminated LTP in NTS1 knockout brain tissue while the dopamine D_2 agonist quinpirole produced LTP for wild type preparations. These findings allude to the possibility that neurotensinergic activity modulates dopaminergic activity, which is consistent with our current understanding of NTS_1/D_2 interactions.

Fawaz et al. (2009) used a combination of electrophysiological and pharmacological techniques to elucidate relationships between NTS_1 and D_2 autoreceptors on presynaptic DA neurons in the nucleus accumbens (NAc) shell. Tetanus, or constant, stimulation at the NAc shell produces increases in extracellular DA levels. When the selective dopamine D_2 autoreceptor agonist quinpirole was applied, *in vitro*, electrical stimulation-induced increases in DA were inhibited. Contrarily, both the dopamine D_2 antagonist sulpride and the NT 8-13 fragment, the active fragment, facilitated DA release. It was theorized that the similar effects of sulpride and NT 8-13 at electrically stimulated DA neurons are due to presynaptic dopamine D_2 receptor modulation by both sulpride and NT 8-13. The effects of NT 8-13 could be masked by prior sulpride treatment.

There is also evidence that NT modulates the affinity that striatal dopamine D_2 receptors have for DA (von Euler & Fuxe, 1987). These NTS_1/D_2 interactions are shown to take place at the presynaptic terminals of glutamate projections from the cortex to the limbic system and at postsynaptic terminals of NAc GABA neurons, which inhibit neurons in the ventral pallidum (Tanganelli, 1994; Ferraro et al., 2000). These interactions also occur both pre- and post-synaptically within the basal ganglia (Antonelli et al., 2007). NT appears to facilitate amino acid neurotransmission by antagonizing the dopamine D_2 receptor, but the glutamatergic NMDA receptor is also a candidate for direct interactions with NT systems. Ferraro et al. (2011) have hypothesized that increases in NT induced cortical glutamate might be a result of NT up-regulating postsynaptic NMDA receptor activity on glutamate neurons.

There is an abundance of data suggesting that NTS_1 and D_2 form complexes at plasma membranes. Using confocal microscopy techniques, Borroto-Escuela et al. (2013) were able to visualize three different homologies of NTS_1/D_2 heteromers within the HEK293T cell line. These heteromers consisted of co-localized NTS_1 and D_2 binding sites. The two major homologies indentified were either at pre- or postsynaptic terminals. There are possibly many interactions taking place between NTS_1 and D_2 at these heteromers. The NTS agonist JMV-449 blocks the ability of quinpirole to activate the dopamine D_2 receptor $G_{i/o}$ G-protein cascade, which eventually leads do decreased cAMP response element-binding protein (CREB) activity at a neuron's nucleus (Borroto-Escuela et al., 2013).

JMV-449 also facilitated quinpirole induced mitogen-activated protein kinase (MAPK) activity. The NTS_1/ $G_{q/11}$ G-protein pathway could have protein kinase C both

desensitizing the D_2 receptor and facilitating MAPK activity (Thibault et al., 2011). Borroto-Escuela et al. (2013) proposed that a synergistic effect might occur when MAPK activity is influenced by both the $NTS_1/G_{q/11}$ and $D_2/G_{i/o}$ pathways. It is still uncertain whether the reduced affinity of the dopamine D_2 receptor homologies for D_2 agonists is due to cytoplasmic interactions between the receptor sites or more direct interactions at the cell membrane. Regardless, NTS_1 agonists appear to produce conformational changes in D_2 which reduces its ability to bind to both DA and synthetic D_2 ligands (Koschatzky et al., 2011). Since the effects NTS agonists are homologous to dopamine D_2 receptor antagonists and endogenous NT modulates glutamate, GABA and 5-HT transmission, the neurotensin NTS_1 receptor has become a novel target for the pharmacological treatment of psychological disorders.

Neurotensin Type 1 Receptor Agonists in Animal Behavioral Models

NT and NT analogs produce effects in a multitude of animal models involving rodents. In open field tasks, both NT and NT analogues produce a reduction in movement. I.c.v. injections of NT at concentrations of 1, 5, and 10µg will produce a decrease in locomotion at the perimeter of a novel open field, but will also increase the time a rat spends within the center of a novel open field (Elliot et al., 1986). When organisms stay near the center of an 'open' environment, they are believed to be demonstrating anxiolytic, or anti-anxiety, behavior. NTS1 agonist-induced attenuation of locomotor activity has also been demonstrated by the brain penetrant selective NTS1 agonist PD149163. Vadnie et al. (2014) gave systemic injections of PD149163 to C57BL/6J mice, which exhibited hypolocomotion at doses as low as 0.1 mg/kg. While

NTS1 agonists have been shown to reverse amphetamine-induced hyperlocomotion in rats, Norman et al. (2008) found that after 7 days of repeated i.c.v. administration of either NT or PD149163, the compounds would not reverse amphetamine-induced hyperlocomotion; this means that they exhibited behavioral tolerance. Amphetamine-induced hyperlocomotion was potentiated by either NT or PD149163 administration after 21 days of repeated administration.

Neurotensinergic activity influences food intake in rats. NT administered unilaterally at the rat paraventricular hypothalamus produces dose-dependent reductions in food intake (Stanley et al., 1983). This reduction in food intake has been linked to NTS1-leptin interactions. Kim et al. (2008) hypothesized that these interactions are integral to leptin's appetite suppressant efficacy. They found that NTS1 knockout mice do not exhibit profound reductions in food intake after an acute 5µg i.c.v. administration of leptin. PD149163 also demonstrates appetite suppressant effects which do not exhibit tolerance (Feifel et al., 2010). Subcutaneously administered PD149163 suppresses appetite in rats containing leptin and in ob/ob mice that are leptin deficient. This demonstrates that while NTS1 activation facilitates leptin activity, the converse relationship has not been demonstrated. The appetite suppressant effects are found to coincide with hypothermia (Feifel et al., 2010).

CNS NT activity has been associated with antinociception in rats. On the hot plate paradigm, i.c.v. injections of NT into the rat CeA demonstrate an ED_{50} at 2.4µg while i.c.v. injections into the lateral ventricle demonstrate an ED_{50} of 93.2µg (Kalivas et al., 1982). Additionally, many derivatives of the NT 8-13 fragment are effective on multiple animal models of analgesia (Hughes et al., 2010).

These antinociceptive effects may be mediated by interactions between NT systems and the pariaqueductal grey (PAG). Projections from the PAG to the rostral ventromedial medulla are potentiated when NT is administered at the PAG (Li et al., 2001). While NT does produce antinociception, it is believed that these effects are mostly mediated through NTS2. Intrathecal injections of levocabastine and the selective NTS2 agonist JMV-431 produce antinociception in rats after receiving chronic constriction injury (Tetreault et al., 2013). Levocabastine and JMV-431 have also been shown to reduce pain in the plantar-formalin injection model of nonciception (Roussy et al., 2009).

NTS1 agonists appear to influence many models of learning. Using the novel object discrimination task, Azmi et al. (2006) showed that 3μg of PD149163 significantly increased the time a rat spent exploring a novel object. This concentration was able to reverse the disruptive effects of scopolamine and was blocked by the non-selective NTS antagonist SR142948A. PD149163 has been shown to improve social discrimination in the Brattleboro rat strain, a strain which demonstrates learning deficits in models of schizophrenia, while also improving delaying non-match to sample performance in the Brown Norway strain, a strain of rats exhibiting many age-related learning deficits (Feifel et al., 2009; Keiser et al., 2014). Rowe et al. (2006) found direct associations between the number of NT binding sites throughout the rat brain and performance in the Morris water maze (MWM) spatial memory task. Injections of NT into the CeA reduce the escape latency for rats navigating a MWM (Laszlo et al., 2010). This demonstrates that the CeA NT system has a role in spatial memory. Additionally, hundred and 250ng bilateral CeA injections of NT produce conditioned place preference in rats, an effect that is blocked by SR48692 at the 100ng concentration (Laszlo et al., 2010).

NTS1 activation has major implications for the treatment of psychological disorders. Binder et al. (2002) showed that NTS1 antagonism produces a disruption in the latent inhibition of cue conditioned foot-shock induce freezing behavior. Since latent inhibition, or the tendency of animals to ignore initially irrelevant stimuli, disruption is a model of learning deficits in patients with schizophrenia, NTS1 agonists are putative treatments of psychotic disorders. PD149163 blocks the psychotic profile of the 5-HT_{2A} agonist DOI in the rat pre-pulse inhibition task (Feifel, Melendez & Schilling, 2003). In the conditioned avoidance paradigm, a task designed to elucidate the sensory-gating effects of anti-psychotic drugs, it has been demonstrated that, unlike the typical anti-psychotic haloperidol, PD149163 produces a significant shift from conditioned avoidance to conditioned escape without producing catalepsy at effective doses (Holly, Ebrecht & Prus, 2011).

NTS1 activation has also been linked to reductions in anxiogenic behavior. Shilling & Feifel (2008) were able to attenuate fear-potentiated startle in rats after subcutaneous injections of 1 mg/kg PD149163. This dose did not produce significant reductions in baseline startle. In contexts that produce mild shock, differences in freezing can be shown between NTS1 knockout mice and their wild type counterparts (Yamada et al., 2010). These differences, however, are masked when a greater current is applied. Prus, Hillhouse & LaCrosse (2014) demonstrated that PD149163 produces reductions in rat 22-kHz ultrasonic vocalizations at doses as low as 0.3 mg/kg. Tasks which measure ultrasonic vocalization, while still rare, are reliable models of rodent affective states.

Ultrasonic Vocalization as an Indicator of Anxiety

Rat ultrasonic vocalizations (USVs) are a product of laryngeal muscle contractions that are mediated by the hindbrain (Brudzynski, 2009). The constant frequency 22-kHz type calls rats produce are indicative of a 'negative' emotional state while the frequency modulated 50-kHz calls are indicative of a 'positive' emotional state (Brudzynski, 2013). 22-kHz calls are believed to function as alarm calls and their emission correlates with many defensive behaviors such as conditioned freezing, staying in the closed arms of an EPM, and the burying of aversive objects (Molewijk et al., 1995; Borta, Wohr, & Schwarting, 2006). The 22-kHz calls are also emitted when a rat has its attention directed towards a predator; the frequency of the call is directly proportional to a rat's proximity to its predator (Litvin, Blanchard, & Blanchard, 2007). This means that 22-kHz USVs might act as stimuli that predict aversive stimuli, in general. Unlike 22-kHz USVs, 50-kHz USVs are emitted when a rat is undergoing appetitive behaviors such as sexual intercourse and cocaine self-administration (Wohr & Schwarting, 2012).

It has become a well-established fact that one of the functions of these vocalizations is communication. USVs are emitted at times when rat colonies work as a group; that is, like a contagious behavior, the entire colony unanimously exhibits avoidance or approach behavior (Brudzynski, 2013). This implies that USVs may act as socially transmitted predictors for either appetitive or aversive stimuli. When prerecorded 22-kHz and 50-kHz USVs are played back to rats that are not initially producing calls, those rats begin to produce the same calls that are emitted and acquire avoidance or approach behavior (Sadananda, Wohr, & Schwarting, 2008). Expression of c-fos shows that playback of 50-kHz USVs evokes activation of regions associated with positive

affect, such as the ventral tegmental area, while 22-kHz USVs are linked to activation of the amygdala and PAG; which have been linked to negative affect. 50-kHz calls are mediated by mesolimbic dopaminergic activity while 22-kHz calls are mediated by mesolimbic cholinergic activity. All neural activity that correlates with USV production is downstream from the rostral projections of different ventral tegmental area sub-nuclei (Brudzynski, 2013).

22-kHz USVs appear to be indicative of anticipatory fear rather than immediate fear responding. Jelen, Soltysik & Zagrodzka (2003) monitored 22-kHz USV emissions in rats that were both trained under a cue that predicts a mild shock and a safety cue, that when presented at the end of the non-safety cue, predicted that no shock would occur. While trained rats would produce constant vocalization, the non-safety cue produced reductions in calls while the safety cue reversed this effect. This might imply that 22-kHz USVs are indicative of fear states that are products of a rat's inability to predict the occurrence of aversive stimuli within a given environment. As implied, 22-kHz USVs can be conditioned to aversive stimuli. When lesions are made to the CeA, only conditioned 22-kHz USV production is abolished while 22-kHz calls are produced in the presence of an aversive stimulus (Choi & Brown, 2003). 22-kHz calls can also be used to elucidate the aversive effects of drugs. Burgdorf et al. (2001) gave rats either lithium chloride or vehicle prior to putting them into a distinct chamber. Rats began to produce 22-kHz USV calls in the chamber that was paired with lithium chloride without producing calls in the chamber that was paired with vehicle.

22-kHz USV calls appear to be indicative of negative affect in contextual fear conditioning models. Molewijk et al. (1995) generated an anxiolytic profile for a

conditioned foot-shock induced USV paradigm. On day 1, rats were trained to produce USVs in an operant chamber via a 7min training session which consisted of 6 pseudo-randomly distributed 0.8mA shocks from the chamber's shock-grid. These rats were then given a pre-test session, on day 2, which consisted of them being put in the chamber for 10min while their USVs were recorded. Rats that met the baseline USV criterion were subsequently tested, on day 3, using the same procedure as day 2 with the exception of drug administration.

It was determined that this model is sensitive to compounds that are known to have anxiolytic profiles. Alprazolam, a BDZ site positive modulator, SSRIs and 5-HT_{1A} receptor agonists reduced the number of recorded 22-kHz USV calls (Molewijk et al., 1995). This effect was also demonstrated by the 5-HT/NA reuptake inhibitor imipramine and α_2-adrenorecptor agonists, which inhibit noradringeric activity. This paradigm is also sensitive to the dopamine D_2 receptor antagonist haloperidol, but only at doses that disrupt motor behaviors (Molewijk et al., 1995; Prus, Hillhouse & LaCrosse, 2014). For most compounds that produce anxiolytic effects on this USV model, they do not produce anxiolytic effects at doses that disrupt locomotion on an open field.

RATIONALE

Prus, Hillhouse & LaCrosse (2014) have already shown that acute administration of the selective NTS1 agonist PD149163 attenuates 22-kHz USVs in male Wistar rats trained under the conditioned foot-shock model used by Molewijk et al. (1995). The aim of the current project was to extend these findings by testing the CNS effects of PD149163 and NT in the conditioned foot-shock induced USV model. I.c.v. administration of PD149163 determines if its effects are centrally mediated; there is a chance that the effect that was produced by subcutaneous administration of PD149163 was a result of peripheral neurotensinergic activity only. Since the full NT peptide is too large to pass through the blood brain barrier, the effect of subcutaneous NT has not been tested in this USV model. I.c.v. administration of NT allows for a profiling of the USV related anxiolytic properties of endogenous NT within the conditioned foot-shock paradigm. Given that there are relationships between NT and monoamine systems and that anxiety-like behavior can be influenced by NT and monoamine systems, these findings might aid in the development of putative compounds for the pharmacological treatment of anxiety disorders.

METHODS

MATERIALS

Subjects

Twenty male Wistar rats where purchased for this study (Charles River Laboratories, Portage, MI). Ten subjects were purchased for Experiment 1 while 10 were purchased for Experiment 2. All rats were housed in an animal room that is part of Northern Michigan University's Neuropsychopharmacology Laboratory. This room is set to an automatic light/dark cycle in which the lights turn on at 8:00 a.m. and turn off at 8:00 p.m., regulating their sleep cycle. Subjects were housed in individual changes and, since their arrival; their weights were measured a few times a week. Once they reached approximately 300 grams, they underwent a pre-training/testing procedure which would determine their eligibility for drug testing (described below). All procedures administered to the subjects were approved by the Institutional Animal Care and Use Committee (IACUC) (see Appendix A).

Anesthetics/Analgesic

Pentobarbital and chloral hydrate were purchased from Sigma-Aldrich as agents in an anesthetic solution. To create this solution, 810 mg of pentobarbital, 4.3 g of chloral hydrate, and 2.12 mg of magnesium sulfate were dissolved in a 100 mL solution containing 29% propylene glycol and 14% ethanol. This anesthetic was injected into the intraperitoneal cavity prior to the surgical procedure. Penicillin G, purchased from Butler

Schein Animal Health (Dublin, OH), was subcutaneously injected immediately after sedation in order to reduce the likelihood of surgical or post-surgical infection. Buprenorphine, a partial agonist at mu-opioid receptors, was used as a post-operative analgesic; for the two days following surgery. It was administered subcutaneously every morning and evening at a concentration of 0.05 mg/kg.

Test Compounds

PD149163 was generously provided by the NIMH Drug Repository (Bethesda, MD) and NT was purchased from AnaSpec (Fremont, CA). For easier distribution throughout the CNS, these compounds were prepared in a phosphate buffering solution (PBS) that was purchased from Sigma-Aldrich (St. Louis, MO). Ten mL of 1.2 mM calcium chloride was added to this solution in order to make it homologous to cerebrospinal fluid. PD149163 was prepared at 0.0001, 0.001, 0.01, and 0.1μg/2μL concentrations. NT was prepared at concentrations of 0.1, 1.0, and 10.0μg/2μL.

Surgical Equipment

Twenty-six gage guide cannulae (C315G-SPC) were purchased from PlasticsOne (Roanoke, VA); extending 3 mm below the pedestal. A stereotaxic surgical device was used to position anesthetized subjects during the surgery (Stoelting Co., Wood Dale, IL). An autoclave made by Inotech (Dietikon, CH) was used to sterilize all surgical tools immediately prior to surgery and an electric razor was used to remove hair from the surgical zone before operating. A dental drill was used to produce three 1 mm diameter holes in the skull after surgical scissors and cotton swabs were used to remove dermal

and periosteum tissue from the surgical zone. Two 1.2 by 3 mm^2 screws and acrylic cement were used to mount the cannulae to the skull. After surgery, heated pads and towels were used to prevent hypothermia. Thirty-three gage dummy cannulae (C315DC-SPC) were purchased from PlasticsOne for placement into guide cannulae between testing sessions.

Microinjection Equipment

Thirty-three gage injection cannulae (C315I-SPC) were purchased from PlasticsOne (Roanoke, VA) for placement inside the guide cannula during the i.c.v. injection procedure. These cannulae extended 2 mm passed the guide cannulae once inserted. Teflon tubing, with an interior diameter of 0.12 mm, (Bioanalytical Systems, Inc., Lafayette, IN) was used to transport injected fluid from a syringe pump to an injection cannula. The syringe pump (MD-1001) (Bioanalytical Systems, Inc.) pushed solution out of a 1 mL syringe, through the Teflon tubing, at a rate of 2μL per minute

USV Apparatus

A commercially built 30.5 by 24.1 by 21.0 cm ultrasonic vocalization chamber was used for all experimental procedures (Med Associates, St. Albans, VT) (see Appendix B). This chamber is equipped with a USV detector (ANL-937-1) that records USVs within a 20-30 kHz frequency band. It binned USVs produced at each frequency throughout a spectrum of sound pressure. Based on previous literature (Brudzynksi, 2013), USVs recorded under 30 dB or USVs that were less than 0.3 seconds in duration were excluded from analysis. An electrical amplifier was used to run a 0.8 mA current

throughout the chamber's grid floor. These mild shocks were 8 seconds in duration. Two signal light were used to illuminated the chamber during the entirety of all training and testing sessions. All shocks, lighting and USV recording was controlled using MED PC 4 software (Med Associates, St. Albans, VT).

Open Field Apparatus

Subjects were tested on a circular open field approximately 117.5 cm in diameter. This open field doubles as a Barnes maze, so there were 12 open holes lining the perimeter of the field placed at equidistant intervals (see Appendix B). The Barnes maze escape box was removed while the Barnes maze was used as an open field. Bright lights and a camera (Panasonic, Newark, NJ) were mounted above the open field. Locomotion was analyzed using Ethovision 7 video tracking software (Noldus Information Technology, Wageningen, NL).

PROCEDURES

Outline of USV Procedure

The USV procedure is differentiated into two distinct phases. The first phase is a pre-training/testing procedure followed by a stereotaxic surgical procedure, which prepares the subjects for i.c.v. injection. The second phase occurs one week after the surgical procedure and consists of retraining followed by drug testing. The drug testing procedure involves the recording of USVs after i.c.v. injections of NTS agonists. This USV procedure is a form of contextual fear-conditioning which has its subjects associate

foot-shocks with the chamber they are shocked in. This makes them produce conditioned USVs, which are used as a measure of anxiety-like behavior.

Pre-operative Training and Testing

The pre-training/testing procedures were modified from those described by Molewijk et al. (1995). Pre-training/testing consisted of three consecutive days. On days 1 and 2, each rat was put in a shock chamber and was administered 6 pseudo-randomly distributed shocks within a 7 minute session. On day 3, each rat was put in the shock-associated chamber and given a 2 minute 'reminder' session which involved the administration of 1 shock towards the middle of the session. Thirty minutes later, 22-kHz USVs were recorded in the shock-associated chamber over a 10 minute testing session; no shock was implemented during the testing session. Any subjects that produced fewer than 80 calls during the pre-testing session were excluded from the surgical procedure and further testing.

Surgical Procedure

Anesthetized rats had their heads shaved and were mounted to the ear and incisor bars of a stereotaxic surgical device. The dermal layer of tissue surrounding the top of the skull was removed using stainless steel surgical scissors. Smaller scissors and cotton swabs were used to cut and remove the remaining periosteum that surrounded the skull. Once the skull was exposed, 95% ethanol was applied, using a cotton swab, to all regions of the skull. The location of bregma was then determined.

A hole was drilled at: Anterior/Posterior = -1.0 mm, Medial/Lateral = +2.0 mm from bregma. The guide cannula was lowered 2 mm below the hole. During testing, an injection cannula would be inserted that projects 2 mm beyond the tip of the guide cannula and into the lateral ventricle (stereotaxic coordinates were provided by the Rat Brain Atlas, Paxinos and Watson, 1986). Two more holes were drilled into the skull and screws were used to attach acrylic cement to the skull.

USV Testing Procedure

The testing procedure was conducted over a course of 6 consecutive days. Day one consisted of the training session mentioned in above sections. On day two, the subjects were placed in the experimental chamber for 2 minutes, receiving one shock (i.e., a reminder shock) after approximately the first minute. After the 'reminder' shock trial, they were taken out of the box and given the i.c.v. injection.

Prior to perfusion, the dummy cannula was removed and was replaced by an injection cannula. Once the injector was secure, the syringe pump was turned on and a 2 minute timer was started. The syringe pump pushes compounds into the brain at 2μL per minute. The pump was turned off after the first minute, having allowed 2μL of solution to enter the lateral ventricle. The injection cannula was left in the injection site for an additional minute to allow for any remaining solution to disperse from the tip of the injector. The animal was then placed back into its home cage.

Thirty minutes after injection, the subject was put back into the chamber for a 10 minute test trial. In Experiment 1, the day 2 procedure was continued for 4 consecutive days. On day 2, PBS was administered to all subjects for the first baseline measure. On

days 3, 4, and 5, PD149163 was given to all subjects at 0.1, 1.0, and 10ng amounts, respectively. The 6th day was a second baseline testing day, which consisted of the same set of procedures as day 2. In Experiment 2, all procedures were identical to Experiment 1 with the exception that, instead of PD149163, NT was administered on days 3, 4, and 5, at 0.1, 1.0, and 10.0μg, respectively. Cross testing was also done after the second baseline test session: in Experiment 1, 10μg of NT was injected on day 7, and in Experiment 2, 10ng of PD149163, PBS and 100ng of PD149163 were injected on days 7, 8, and 9, respectively.

Outline of Locomotor Procedure

Subjects in Experiment 2 were tested on an illuminated open field after PD149163 cross-confirmation data was collected.

Locomotion Testing Procedure

All subjects were habituated to the open field over a 10 minute session one day prior to the first testing session. On the first testing day, subjects were monitored on the open field for 10 min after a PBS injection, which occurred 30 min prior to testing. Day 2 only differed from Day 1 in that 0.1μg of PD149163 was administered instead of PBS. The procedure on Day 3 was identical to the Day 1 procedure. All microinjection procedures were identical to the protocol described in the USV experiments.

HISTOLOGY

To determine probe placement accuracy, rats were euthanized with at least 100 mg/kg pentobarbital (i.p.). A small amount of ink was inserted into the injection site

using an injection cannula, Teflon tubing and a 1 mL syringe. This treatment makes it easier assess cannulation accuracy. The subject's brains were subsequently extracted and placed into scintillation vials filled with formalin. After 48 hours of sitting in formalin, the brains were sectioned to verify the placement of injection cannulae. Subjects with inaccurate cannula placement were excluded from the data analysis (see Appendix B).

DATA ANALYSIS

During all 10 minute USV recording sessions, the number of 22-kHz calls emitted between 0.3 and 4 seconds in duration were recorded in MED PC 4. Descriptive data was expressed using mean number of calls per session +/- standard error of the mean. First and second baseline testing values were compared using a paired t-test and the average baseline was calculated. Analysis of Variance (ANOVA) and Dunnett's multiple comparison tests were used to test for significant differences in the number of USVs between average baseline and treatment conditions.

During all 10 minute locomotion recording sessions, the distance traveled was measured in centimeters. Descriptive data was expressed using the mean distance traveled per session +/- standard error of the mean. First and second baseline testing values were compared using a paired samples t-test and the mean baseline was calculated. A paired samples t-test was used to test for significant differences in the distance traveled between the mean baseline and treatment condition. Statistical analysis of all experimental data was run using Graph Pad Prism 6 software (GraphPad Software, Inc., La Jolla, CA).

RESULTS

USV PROCEDURE

Experiment 1

Figure 1 represents a comparison of the mean values for first and second PBS baselines in Experiment 1. There was no statistically significant difference between the first and second baseline values, $t = 0.12$, $p > 0.05$. Figure 2 represents a comparison of the mean values of the average baseline, which was calculated from the first and second baselines, and three PD149163 treatment conditions: 0.1, 1.0, & 10.0ng. PD149163 administration produced a decrease in USV production, $F(3,6) = 17.33$, $p < 0.01$. Ten ng of PD149163 significantly reduced the number of vocalizations when compared to vehicle, $p < 0.001$. Figure 3 represents a comparison of mean value for the second baseline and the mean number of vocalizations after administration of NT. NT produced a significant decrease in USVs at the 10μg concentration, $t = 3.29$, $p < 0.05$.

Experiment 2

Figure 4 represents a comparison of the mean values for first and second PBS baselines in Experiment 2. There was no statistically significant difference between the first and second baseline values, $t = 0.95$, $p > 0.05$. Figure 5 represents a comparison of the mean values of the average baseline, which was calculated from the first and second baselines, and three NT treatment conditions: 0.1, 1.0, & 10.0μg. NT administration produced a decrease in USV production, $F(3,4) = 8.20$, $p < 0.05$. Ten μg of NT

significantly reduced the number of USVs when compared to vehicle, $p < 0.05$. Figure 6 represents a comparison between the mean values of a third baseline, 10ng of PD149163, and 100ng of PD149163. There was a statistically significant decrease in USVs produced by PD149163, $F(2,3) = 7.39$, $p < 0.05$. PD149163 produced a significant decrease in vocalizations at the 100ng concentration, $p < 0.05$.

LOCOMOTOR PROCEDURE

Figure 7 represents a comparison of the mean values for first and second PBS baseline distances traveled. There was a statistically significant difference between the first and second baseline distances, $t = 6.05$, $p < 0.01$. There was a significantly shorter distance traveled during the second baseline. Figure 8 represents a comparison of the mean values of the average baseline distance traveled and a 100ng PD149163 treatment condition. There was no statistical difference between the average baseline and 100ng PD149163 conditions, $t = 0.03$, $p > 0.05$.

Figure 1: Experiment 1: First and Second PBS Baseline Comparison

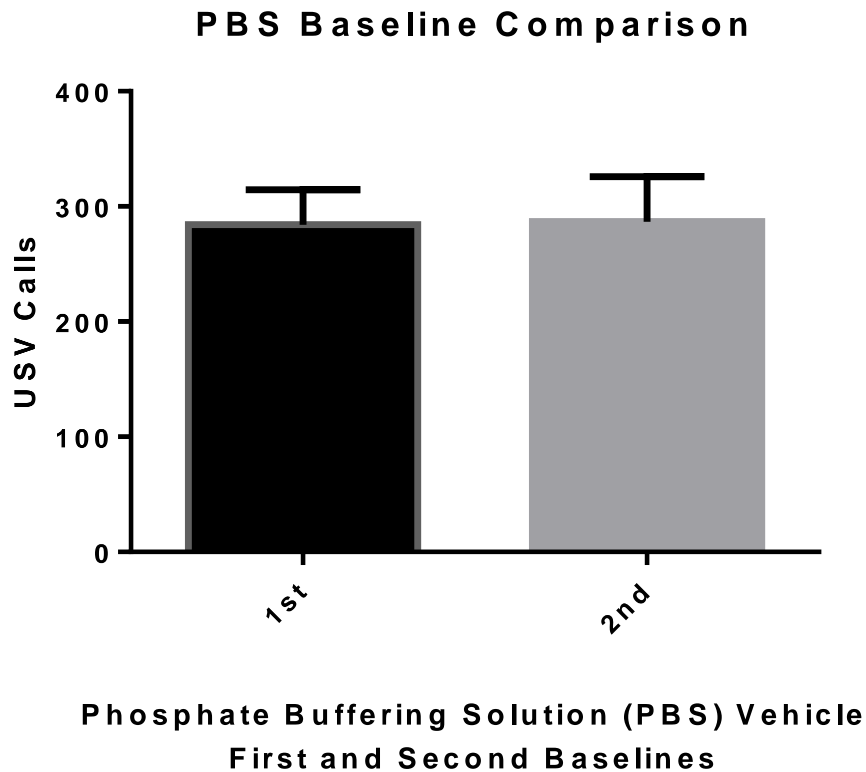

Figure 1 represents a comparison of USV production for the 1st and 2nd PBS baselines in Experiment 1. USV calls refer to the number of 22 kHz calls recorded during the 10 min testing session. 1st and 2nd refer to the assessments before and after testing PD149163. Data are shown as means (+/- standard error of the mean). N = 7

Figure 2: Experiment 1: PD149163 Treatment Curve

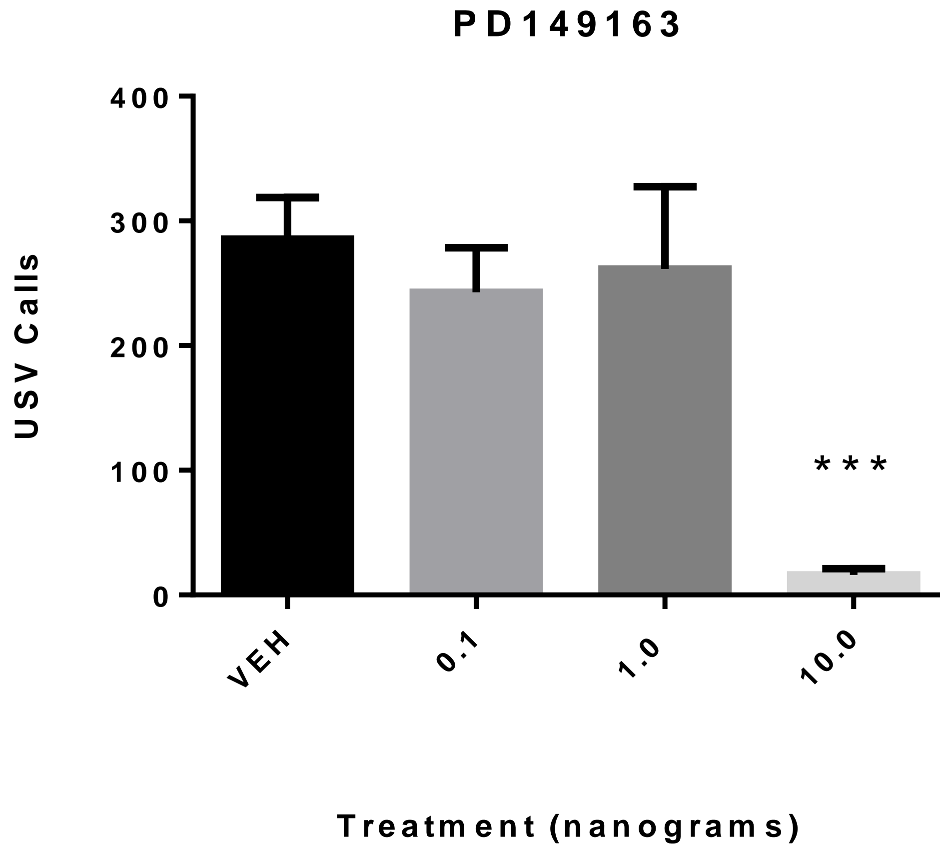

Figure 2 represents a comparison of USV production between the average baseline, which was calculated from the 1st and 2nd PBS baselines, and three PD149163 treatment conditions: 0.1, 1.0, & 10.0ng. ***$p < 0.001$ versus vehicle. VEH = vehicle (PBS). Data are shown as means (+/- standard error of the mean). N = 7

Figure 3: Experiment 1: NT Cross-testing

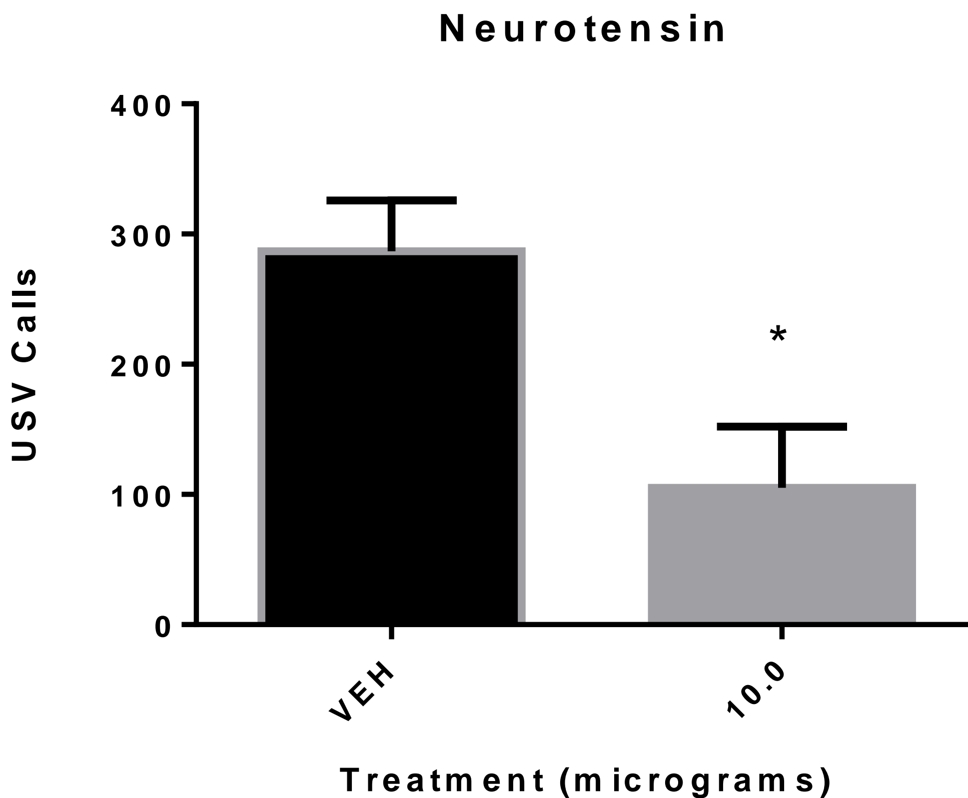

Figure 3 represents a comparison of USV production for the 2nd baseline and 10µg NT. *p<0.05 versus vehicle. VEH = vehicle (PBS). Data are shown as means (+/- standard error of the mean). N = 7

Figure 4: Experiment 2: First and Second PBS Baseline Comparison

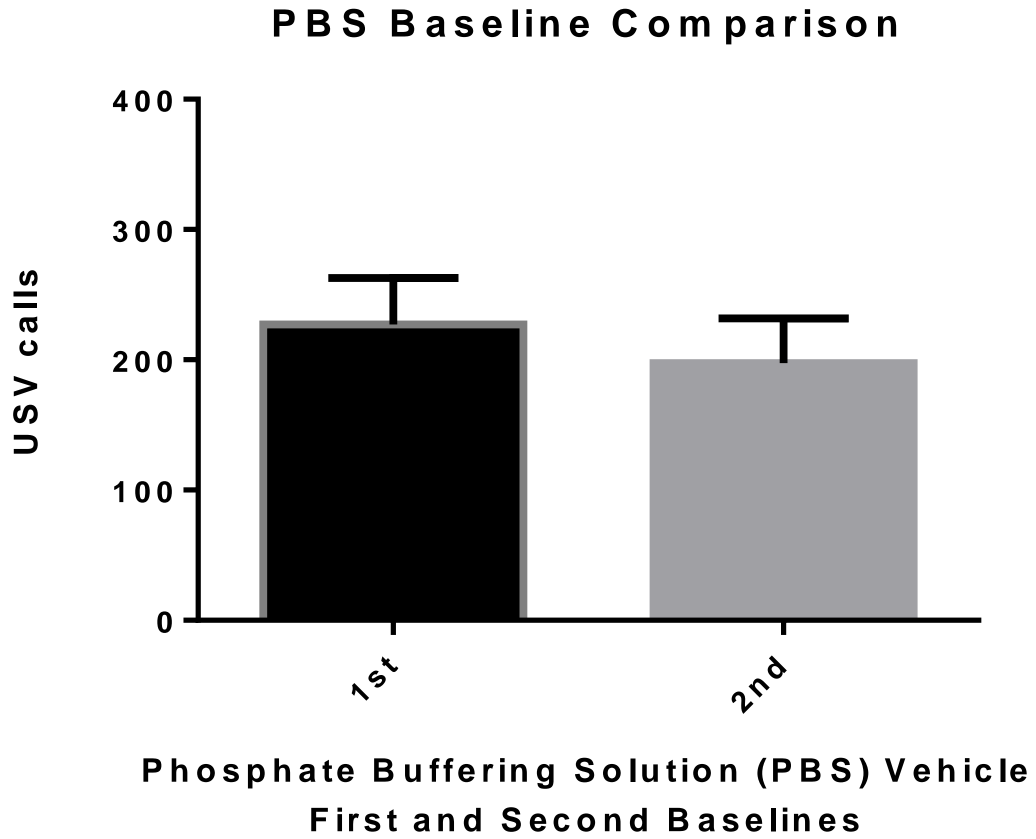

Figure 4 represents a comparison of USV production for the 1st and 2nd PBS baselines in Experiment 2. 1st and 2nd refer to the assessments before and after testing NT. Data are shown as means (+/- standard error of the mean). N = 5

Figure 5: Experiment 2: NT Treatment Curve

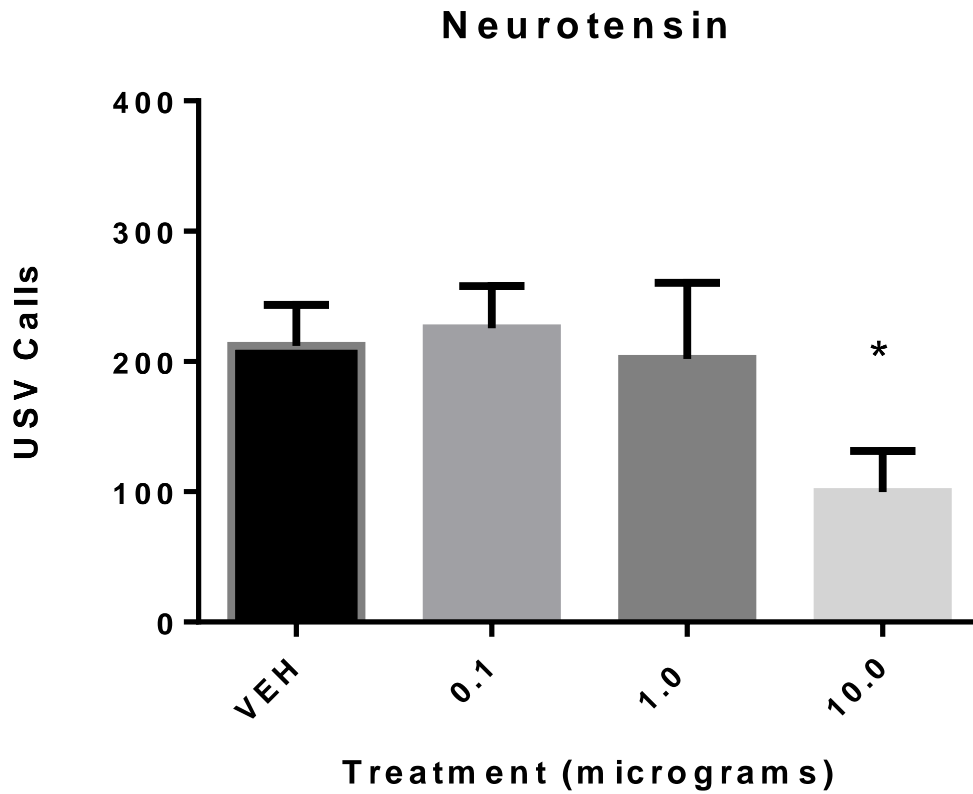

Figure 5 represents a comparison of USV production between the average baseline, which was calculated from the 1st and 2nd PBS baselines, and three NT treatment conditions: 0.1, 1.0, & 10.0µg. *$p < 0.05$ versus vehicle. VEH = vehicle (PBS). Data are shown as means (+/- standard error of the mean). N = 5

Figure 6: Experiment 2: PD149163 Cross-testing

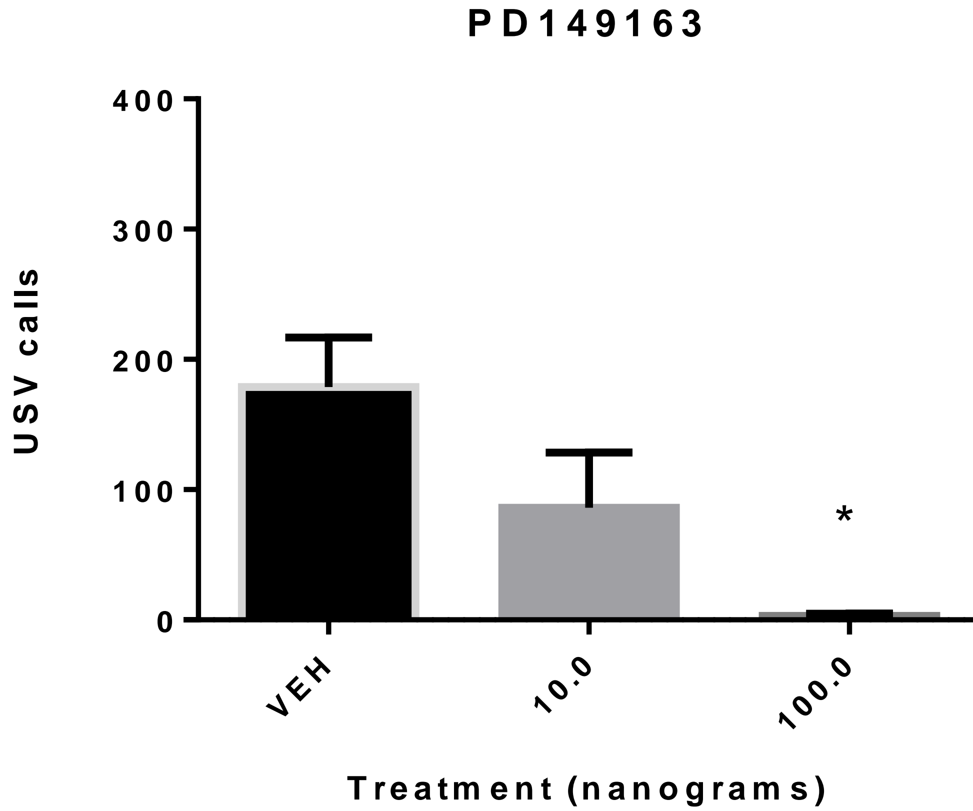

Figure 6 represents a comparison of USV production for the 3rd baseline, 10ng and 100ng PD149163. *p<0.05 versus vehicle. VEH = vehicle (PBS). Data are shown as means (+/- standard error of the mean). N = 4

Figure 7: Open Field Experiment: First and Second PBS Baseline Comparison

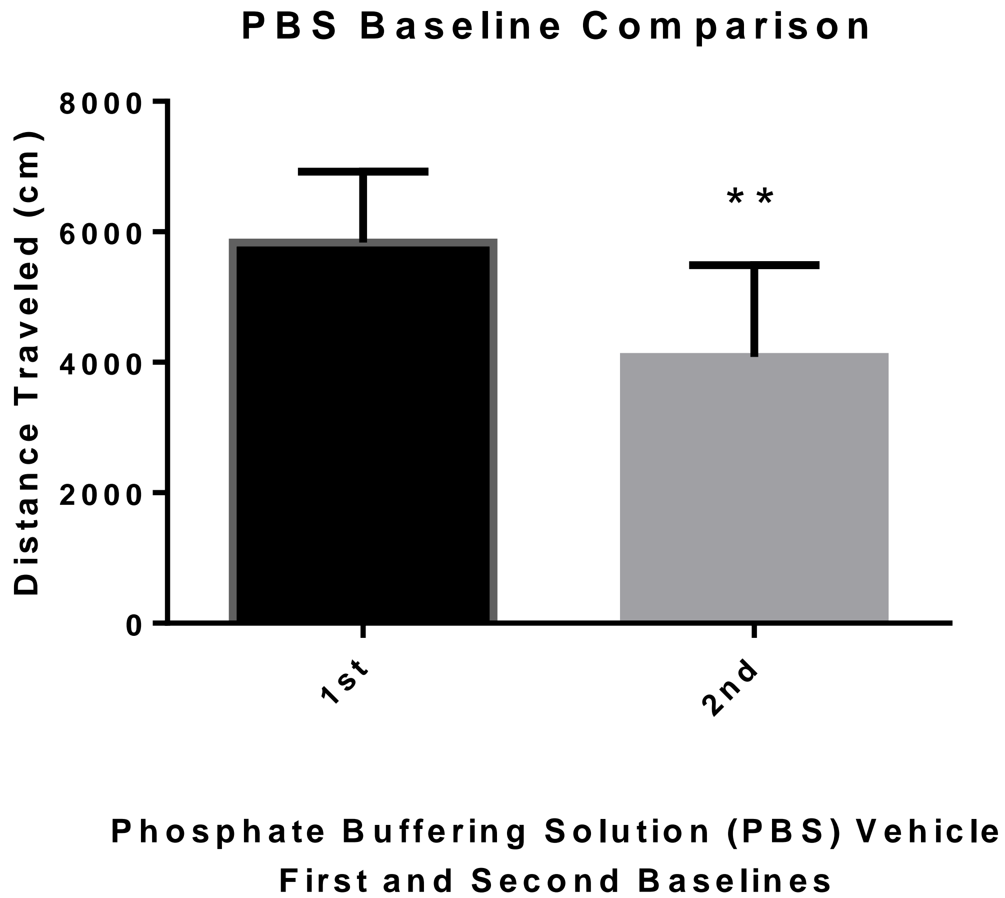

Figure 7 represents a comparison of distance traveled for the 1st and 2nd PBS baselines in the open field experiment. There was a significantly shorter distance traveled during the second baseline. **$p < 0.01$ versus baseline. Data are shown as means (+/- standard error of the mean). N = 4

Figure 8: Open Field Experiment: PD149163 Treatment

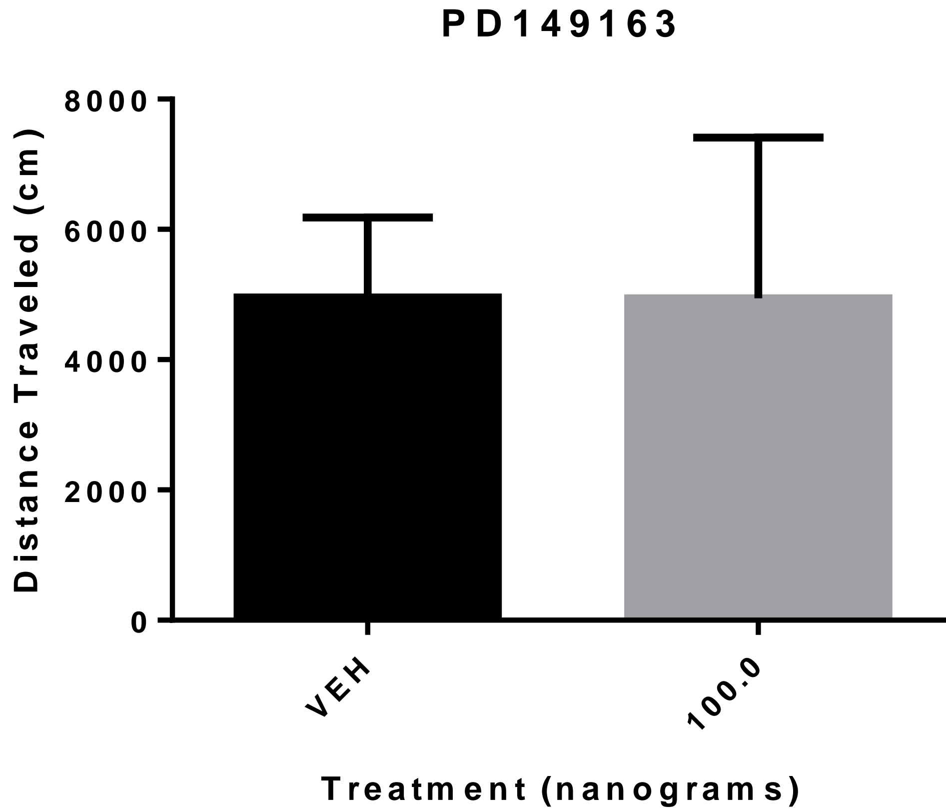

Figure 8 represents a comparison of distance traveled for the average baseline distance traveled and a 100ng PD149163 treatment condition. VEH = vehicle (PBS). Data are shown as means (+/- standard error of the mean). N = 4

DISSCUSION

Ten and 100 ng concentrations of PD149163 produced significant reductions in 22-kHz USV calls in the conditioned foot-shock paradigm. This is the first time the intracranial effects of PD149163 in this USV model have been reported. These findings extend the findings of Prus, Hillhouse, & LaCrosse (2014), who demonstrated that subcutaneous injections of PD149163 can attenuate USV calls. The current project shows that central NTS1 activation might be a primary mediator during the effects demonstrated by systemic administration of PD149163. The maximum efficacy of PD149163 between systemic and intracerebroventricular administration methods does not differ; both administration methods produce robust effects at larger concentrations. The treatment curve illustrated by Figure 2 is similar in form to dose-response curves produced by systemic administration of PD149163 using the USV paradigm (Prus, Hillhouse & LaCrosse, 2014). These graphs suggest that PD149163 administration may produce a narrow range of concentration-dependent changes in USV production, although a monotonic curve might have been produced if a half-log concentration was also administered (i.e. 3.2 ng PD149163).

Ten μg of neurotensin (NT) reduced USV production in this USV model. This was the first time NT has been tested in this paradigm. The intracerebroventricular injection method was required to test the potential effects of NT; large peptides, such as NT, cannot pass through the blood brain barrier. While NT did not produce effects that were as profound as the effects demonstrated by PD149163 (Figures 2 and 5), the effect demonstrated by NT suggests that the effects of PD149163 are mediated by neurotensin

receptor activation. The brain neurotensin system may have specific relationships with brain mechanisms associated with conditioned 22-kHz USV production. Likewise, there could be relationships between brain neurotensin and general 22-kHz USV production. Since a microdialysis procedure has demonstrated that NT administration increases prefrontal cortex acetylcholine levels, there is a low likelihood that NT administration decreases a rat's 'ability' to produce USVs (Petkova-Kirova et al., 2008). The production of 22-kHz USVs is, in part, mediated by cholinergic projections from the basal forebrain to the prefrontal cortex (Brudzynski, 2009).

Cross-testing was implemented in Experiments 1 and 2. Subjects in Experiment 1 demonstrated an effect on the USV task at 10µg NT while having a history of PD149163 exposure (Figure 3). This effect was similar to the effect of 10µg of NT in Experiment 2. Subjects in Experiment 2 demonstrated an effect on the USV task at not 10ng, but 100ng, while having a history of NT exposure (Figure 6). The effect of 100ng of PD149163 in Experiment 2 was similar to the effect of 10ng of PD149163 in Experiment 1. The cross-testing demonstrated similar results between Experiments 1 and 2.

Experiments 1 and 2 demonstrated that PD149163 administration has a 100-1000 fold greater potency than NT in the conditioned foot-shock induced USV paradigm. This difference in potency cannot be explained by merely accounting for the respective affinities of PD149163 and NT for the NTS1 receptor. NT has been demonstrated to have affinity for NTS1 of K_i = 0.25 nM in mouse brain tissue (Pettibone et al., 2002), while Petrie et al. (2004) found that PD149163 has an affinity for NTS1 of K_i = 159 nM in rat brain tissue (see Petrie et al. (2004) for a table illustrating PD149163's highly selective binding assay). The difference in NTS1 affinity could account for differential efficacy,

but one must be skeptical about making comparisons across species and laboratories. A difference in receptor selectivity could also account for differential efficacy. PD149163 only activates the NTS1 receptor while NT activates NTS1 and can act as an antagonist at NTS2 (Pelaprat, 2006). As described in the introduction, there is a possibility that NT analogs, such as PD149163, and endogenous NT have differing affinities for NTS1-coupled G-proteins. This means that PD149163 and neurotensin could be activating distinct second messenger systems within NTS1 expressing neurons. Skrzydelski et al. (2003) found evidence that while NT can activate $G_{q/11}$, G_s and $G_{i/o}$ pathways, the NT 8-13 analog EISAI-1 orientates the receptor towards its C-terminus, EISAI-1 bound NTS1 then preferring the G_s and $G_{i/o}$, but not $G_{q/11}$, pathways; $G_{q/11}$ is bound to the third intracellular loop of NTS1.

One hundred ng of administered PD149163 did not reduce locomotion on a brightly illuminated Barnes maze/open field. This was demonstrated in the same subjects that exhibited a significant reduction in 22-kHz USVs at 100ng of administered PD149163. This suggests that the reduction in USVs produced by the 100ng concentration is not due to a non-specific reduction in bodily movement. In the Azmi et al. (2006) novel object discrimination experiment mentioned in the introduction, 3µg of PD149163 in the lateral ventricle did not produce an overall reduction in rat exploratory behavior. Vadnie et al. (2014) and Prus et al. (unpublished) have shown that mice and rats, respectively, produce reductions in locomotion after systemic administration of PD149163. Rats will demonstrate a reduction in locomotion on a brightly illuminated open field 30 min after systemic administration of not 0.1, but 1.0 mg/kg, of PD149163 (Prus et al., unpublished). Systemic administration of PD149163 at 0.1 mg/kg is sub-

effective in the USV preparation while 1.0 mg/kg produces a profound effect (Prus, Hillhouse & LaCrosse, 2014). Additionally, the reduction in rat fear-potentiated startle that was observed at a 1.0 mg/kg dose of PD149163 in Shilling & Feifel (2008) coincided with a reduction in baseline startle, suggesting that the drug effect in the fear-potentiated startle treatment may have been influenced by a general disruption of behavior. The discrepancies between systemic and i.c.v. motor data suggests that the general reduction in behavior observed after systemic administration of PD149163 is primarily influenced by the PNS.

It can be argued that this USV model used presents effects that are non-specific to anxiolytic-like behavior, but it is inarguably a more specific measure than the traditional quantification of 'freezing'. Freezing is a behavior that, by its general definition, is susceptible to drug-induced response inhibition. McNish, Gewirtz & Davis (1997) defined freezing as 'the mean activity before training minus the mean activity after training'. This notion of 'activity' is vague and can be though equivocal to locomotion. Atsak et al. (2011) explicitly defines freezing in terms of locomotion: 'locomotor activity of witnesses (subjects) is sampled as 5 minute time-bins and the percentage change in locomotion was calculated by subtracting the locomotor activity measured in the first 5 minutes (taken as a baseline) from the locomotor activity sampled in the subsequent 5 minute time-bins'.

While tasks that measure freezing should not be discredited, measuring 22-kHz ultrasonic vocalizations allows for a more precise accurate quantification of fear-related behavior. The measurement of discrete 22-kHz ultrasonic vocalizations, exhibiting a set of identifiable acoustic parameters (Brudzynski, 2013), does not produce the ambiguity

that freezing measurements produce. There also exist discrepancies between the methods of observing freezing between laboratories. In Atsak et al. (2011), Noldus Ethovision software was used to analyze freezing while spectators looking at surveillance footage scored freezing in McNish, Gewirtz & Davis (1997). USVs within the 18-32 kHz frequency band have not been associated with any behaviors that are non-defensive while 'no-locomotion' can be as indicative of sedation as it is defensive, or fear-induced, states in rodent models.

Alternatively, a model of conditioned-fear that avoids the issue of non-specific reductions in responding is the conditioned emotional response (CER) task. This task measures increased responding, compared to vehicle, in the presence of a conditioned aversive stimulus as indicative of an anxiolytic effect. Future evaluation of neurotensin agonists using the CER task might better elucidate the anxiolytic profile of the neurotenisn system, but as stated in the introduction, Neurotensinergic compounds produce appetite suppression. This means that the reinforcer used in the CER task might have to be a reinforcer other than food. so it Before the anxiolytic-like effects of NTS1 agonists are fully allocated to NTS1 activation, more NTS1 agonists as well as selective NTS2 and NTS3 agonists need to have their putative anxiolytic properties evaluated using animal models of anxiety. Furthermore, NTS antagonists, both selective and non-selective, need to be tested in conjunction with NTS agonists to determine if the actions of these Neurotensinergic compounds are mediated through a Neurotensinergic system. The brain neurotensin system is a potential target for the treatment of anxiety-related disorders.

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APPENDIX A

SIGNATURE PAGE

IACUC #: 220 **PROPOSAL TITLE (From cover page):** Assessment of neurotensin effects on 22-kHz vocalizations in rats

X. ACKNOWLEDGEMENT BY PRINCIPLE INVESTIGATOR

I acknowledge responsibility for this project. I have read the Northern Michigan University Principles for the Care and Use of Laboratory Animals and certify that this project will be conducted in compliance with those principles. I assure that I will obtain Institutional Animal Care and Use Committee approval prior to significant changes in the protocol. I assure that this project does not unnecessarily duplicate previous research or instructional projects. I assure that students, staff and faculty on the project are qualified or will be trained to conduct the project in a humane, safe, and scientific manner.

Signature: ______________________ 06/20/2013
Principal Investigator Date

XI. APPROVAL OF SCIENTIFIC MERIT (to be completed by the Department Head)
Before it is initiated, this project must be reviewed and approved on the basis of its scientific merit.

☐ Review conducted by external agency.
☐ Governmental Agency: Please specify the reviewing agency or board Federal agency (e.g., NIH, NSF, USDA, etc.) and evidence of approval

☐ Nongovernmental agency (e.g., University review, Other specify):

☐ Departmental Review: I assure that this project has been reviewed and approved for scientific or instructional merit by:

☐ Expert reviewer (Name)

☐ Departmental Committee Review (Committee Name and Chairperson):

☐ Other (Describe):

Signature: ______________________ 06/24/2013
Department Head Date

XII. REVIEWED AND APPROVED BY IACUC REVIEWERS

Signature: ______________________ 06/24/2013
Institutional Animal Care and Use Committee Chair Date

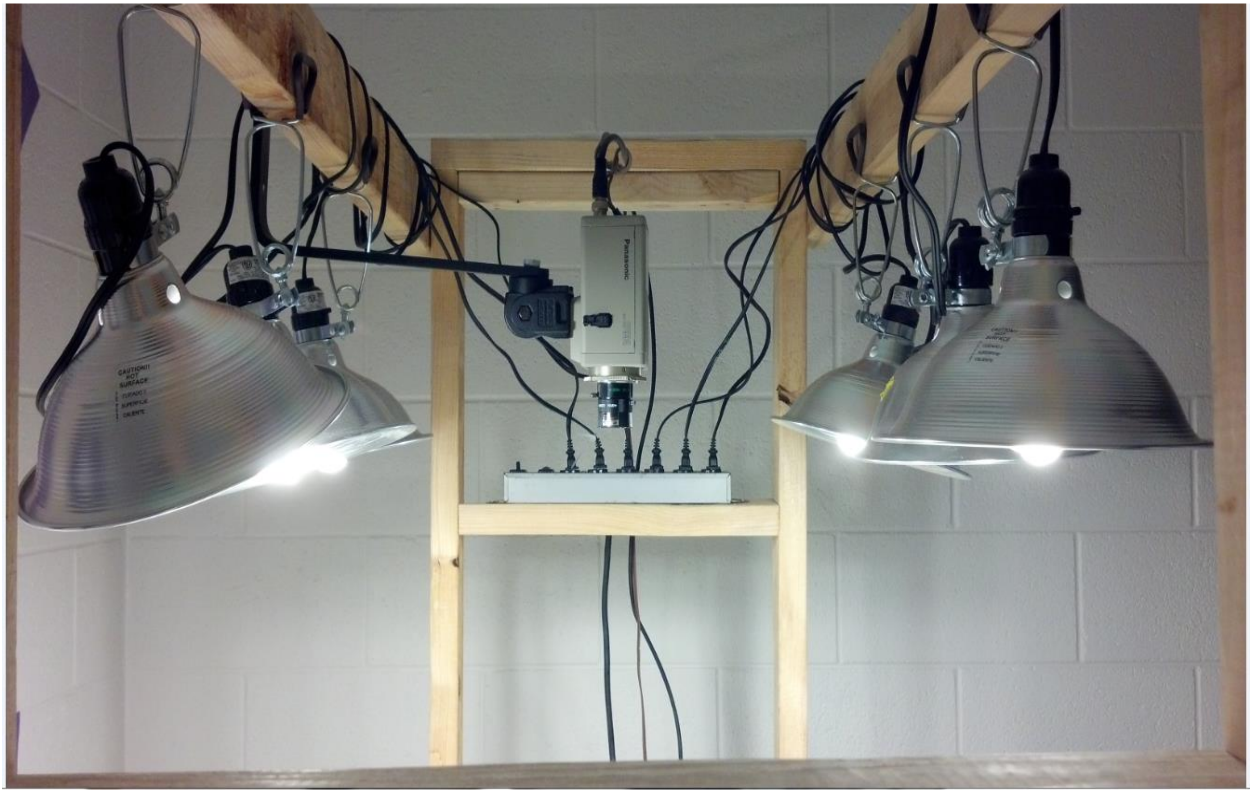
Signature: ______________________ 06/24/2013
Institutional Animal Care and Use Officer Date

IACUC Application - Revised September 16, 2010 11

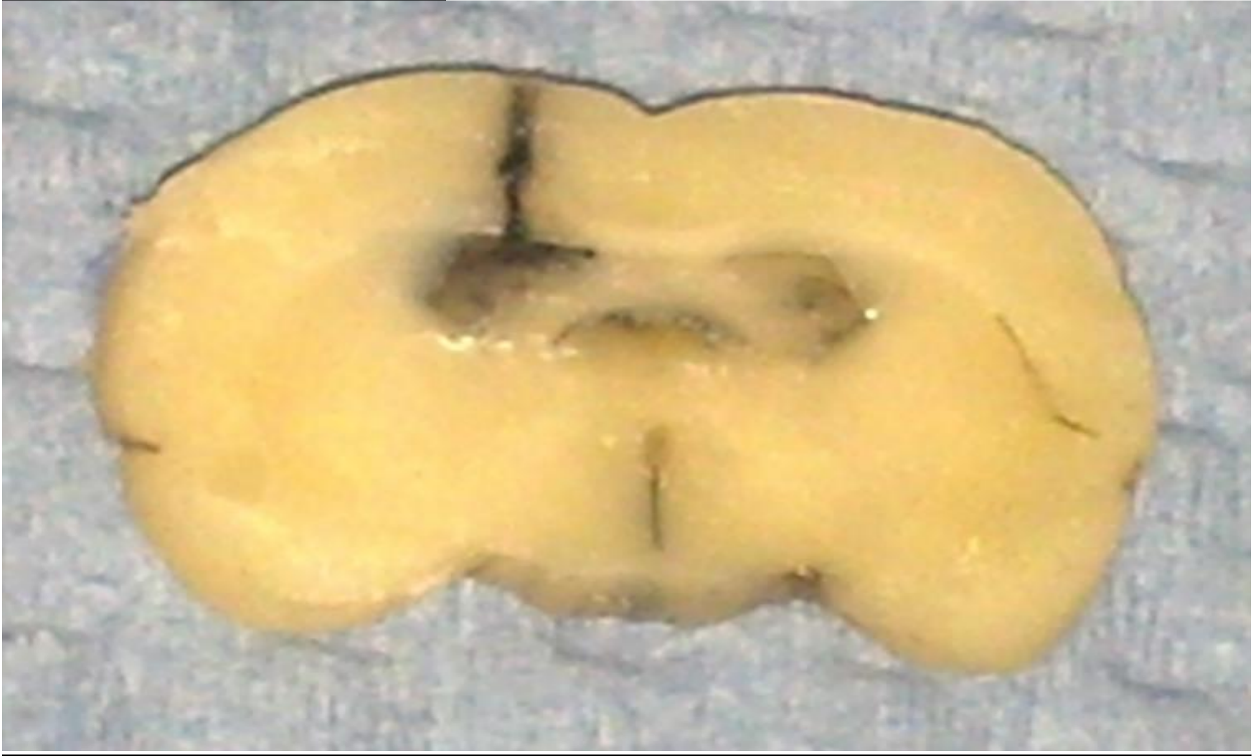
APPENDIX B

USV Apparatus

Open Field Apparatus



Successful Probe Placement



Unsuccessful Probe Placement