ANTIDEPRESSANT EFFECTS OF THE NTS1 AGONIST PD149163 IN THE FORCED SWIM TEST

Lawrence Carey IV
lcarey@nmu.edu

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ANTIDEPRESSANT EFFECTS OF THE NTS₁ AGONIST PD149163 IN THE FORCED SWIM TEST

By

Lawrence Carey IV

THESIS

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This thesis by Lawrence Carey 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.

____________________________________________________________
Committee Chair: Dr. Adam J. Prus
Date

____________________________________________________________
First Reader: Dr. Paul T. Andronis
Date

____________________________________________________________
Second Reader: Dr. Erich N. Ottem
Date

____________________________________________________________
Department Head: Dr. Paul T. Andronis
Date

____________________________________________________________
Dr. Brian D. Cherry
Date
Assistant Provost of Graduate Education and Research
ABSTRACT

ANTIDEPRESSANT EFFECTS OF THE NTS₁ RECEPTOR AGONIST PD149163 IN THE
FORCED SWIM TEST

By

Lawrence Michael Carey IV

Neurotensin is a neuropeptide that influences monoaminergic neurotransmission in areas of the brain involved in the pathophysiology of depression. The forced swim test is a commonly used screening model for putative antidepressant medications. Drugs that have antidepressant effects in humans reliably decrease the time animals spend in an immobile posture in the forced swim test without increasing general locomotor activity as measured in an open field test. The present study sought to examine the effects of the neurotensin NTS₁ receptor agonist PD149163 and the tricyclic antidepressant drug imipramine on immobility in the forced swim test and on locomotor activity in an open field in mice. PD149163 decreased the total time spent immobile at doses of 0.1 mg/kg and 1.0 mg/kg, without increasing overall locomotor behavior in the open field test. Imipramine produced a reduction in immobility at a dose of 10.0 mg/kg, but not at 1.0 mg/kg, without increasing locomotor activity. These results indicate that since drugs that target the neurotensin system display antidepressant properties in rodent models of depression they may represent a novel mechanism for treatment of depressive symptoms in humans.
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INTRODUCTION

Depression is a serious medical condition that is estimated to affect roughly 10-25% of women, and 5-12% of men (American Psychiatric Association, 2013). Depression may occur at any age, but the average age of onset occurs during the mid twenties (American Psychiatric Association, 2013). The time-course and duration of symptoms is variable as some people will only experience isolated episodes of depression, while others will have recurring episodes throughout their lives. Those who develop more than one episode of depression are at a higher risk of developing subsequent episodes later in life. Approximately 60% of individuals who experience one major depressive episode will experience a second episode later in life. Approximately 70% of people who do develop a second episode will experience a third depressive episode, and among those who experience a third episode, 90% will experience further problems with depression (American Psychiatric Association, 2013). Several factors may influence the development of depression, including alterations in several of the brain’s monoaminergic neurotransmitter systems, genetics, substance abuse, and a variety of other psychiatric conditions. The World Health Organization has estimated that depression will constitute the second leading cause of illness-related disability by 2020 (Murray & Lopez, 1997). On a global scale, medications to treat depression are the third highest selling class of drug (Celada et al., 2004), most of which target one or more of the brain’s monoaminergic neurotransmitter systems. However, currently available antidepressant medications do not adequately treat the disorder, and patient
responsiveness to treatment is poor. Various studies have reported values ranging from fewer than 50% to less than 28% of patients being effectively treated with one medication alone (Kocsis, 2000, Trivedi, 2006, Fava, 2000). Subsequently, physicians are required to prescribe a cocktail of medications. Furthermore, in the patients who did respond to antidepressant treatment in these studies, symptom improvement was observable only after 10-14 weeks. The protracted latency to the onset of therapeutic effects, as well as the staggeringly low response rates, indicates the need for novel pharmacotherapies for the treatment of depression.

The Monoamine hypothesis of depression

Much of what we know about the biological basis of depression has been inferred from studying the effects that antidepressant drugs exert in the brain. Because most drugs that are effective at combating the symptoms of depression in humans alter neurotransmission of one or more of the monoamine neurotransmitter systems, to date, much of the research into the etiology of the disorder has focused on a monoamine-based hypothesis of dysfunction. In the mid-1960’s depression began to gain recognition as a medical disorder with organic causes. The monoamine hypothesis of depression states that the symptoms of depression are caused by deficiencies in monoamine neurotransmission, and by correcting these imbalances, the symptoms may be ameliorated (Schildkraut, 1965).

The monoamines are a family of structurally related neurotransmitters that include the catecholamines dopamine, norepinephrine, and epinephrine, and the indolamine...
serotonin. Although these neurotransmitters differ widely in their distribution and types of receptors they act upon, they share several characteristics that are of interest to the etiology and treatment of depression. The monoamines are packaged into vesicles by the vesicular monoamine transporter, of which two distinct subtypes exist. Vesicular monoamine transporter type 1 (VMAT1) is primarily located in the periphery, while vesicular monoamine transporter type 2 (VMAT2) is located in the central nervous system (Masson et al., 1999). The packaging into vesicles is a crucial step in monoamine neurotransmission, as it maintains the concentration gradient of monoamines by aiding in their uptake from the extracellular space. This process also serves to protect them from leakage and premature metabolism in the presynaptic terminal (Masson et al., 1999). Alterations in VMAT2 may play a role in the genesis of depressive symptoms, as well as an individual’s responsiveness to treatment (Valevski, 2010).

The monoamines also share similar mechanisms of degradation. Two different enzymes are involved in the metabolism of monoamines: monoamine oxidase (MAO), and catechol O-methyltransferase (COMT). MAO metabolizes dopamine, serotonin, and norepinephrine, while COMT primarily metabolizes dopamine and norepinephrine. MAO regulates monoamine neurotransmission by degrading monoamines, and in normally functioning individuals MAO acts to preserve homeostasis of neurotransmitter levels in the brain. When levels of monoamines increase, increases in MAO are also observed. Likewise, when brain levels of monoamines decrease, MAO levels also decrease (Schwartz, 2013). Therefore, dysfunctions in MAO levels indicate one possible avenue to explain monoaminergic deficiencies observed in depression (Schwartz, 2013). COMT-mediated degradation of dopamine may also play an important role in the genesis of
depressive symptoms, as individuals with altered levels of COMT activity have been found to be at an increased risk for development of depression (Anytpa et al., 2013). While the monoamines share several regulatory mechanisms regulating their release and degradation, their specific receptor signal transduction mechanisms and neural regions innervated by the individual neurotransmitters differ greatly. For these reasons, I will consider each of the monoamine’s specific contributions to the generation of depressive symptoms separately.

The role of dopamine in depression

Dopamine belongs to the catecholamine family of the monoamine neurotransmitters and acts on five different subtypes of metabotropic receptors. These five subtypes of receptors are designated the D$_1$, D$_2$, D$_3$, D$_4$, and D$_5$ receptors. Two separate families of dopamine receptors can be drawn from these five subtypes of receptors, the D$_1$-like family, which includes the D$_1$ and D$_5$ receptors, and the D$_2$-like family, which includes the D$_2$, D$_3$ and D$_4$ receptors (Vallone et al., 2000).

The two dopamine receptor families differ in the downstream effects they exert intracellulary upon activation. The D$_1$-like family is generally considered to have stimulatory effects on the cells that express them, while the D$_2$-like family is generally considered to have inhibitory effects (Vallone et al., 2000). All dopamine receptors exert their effects on cellular functioning through activation of G proteins coupled to the receptors. The D$_1$-like family act as positive regulators of cyclic AMP by directly activating adenylyl cyclase, which in turn activates protein kinase A (PKA) (Jackson &
PKA phosphorylates different cytoplasmic and nuclear proteins, leading to alterations in gene expression, cellular metabolism, and various other modulatory effects on ion channel functioning (Choi et al., 1993). The D$_2$-like family exerts their inhibitory effects by inhibiting adenylyl cyclase activity (Vallone et al., 2000).

Dopamine is synthesized by neurons in three discrete brain regions, which project to four major targets. Dopamine neurons in the ventral tegmental area project to cortical areas via the mesocortical pathway, and the limbic system via the mesolimbic pathway. Dopamine neurons in the substantia nigra pars compacta project to the striatum via the nigrostriatal pathway. Small populations of dopamine neurons in the hypothalamus project to the pituitary gland via the tuberoinfundibular pathway.

Some of the functions that are interrupted in depression- such as motivation, psychomotor behavior and feelings of pleasure- are all processes regulated, in part, by dopamine neurotransmission. Furthermore, not all individuals are responsive to medications that modulate serotonin and/or norepinephrine neurotransmission. Therefore, deficits in these dopamine projection streams may play a role in the genesis of depressive symptoms. The psychomotor symptoms observed in depression may in fact be due to alterations in dopaminergic neurotransmission to brain regions associated with motor behavior, as upregulation of D$_2$ receptors have been observed in the basal ganglia and cerebellum in depressed individuals (D’haenen et al., 1994). Upregulation of the dopamine reuptake transporter in the striatum have also been observed in depressed
individuals, which may contribute to low synaptic levels of dopamine (Laasonen-Balk et al., 1999).

The symptom of anhedonia experienced by people with depression may also be linked to dopaminergic neurotransmission. In rodent models of depression, such as the effort expenditure behavioral model, which tests an animal’s willingness to work for a reward, reductions in dopaminergic neurotransmission to the nucleus accumbens correlate with reductions in efforts to obtain rewards (Salamone et al., 1999 & Neil et al., 2002). In this task, reductions in the effort an animal is willing to expend to obtain a reward is used as an index of anhedonia, or a decrease in the hedonic value of a reward, which is used as a correlate to the human symptoms of anhedonia. Administration of tricyclic antidepressants, or the selective serotonin reuptake inhibitor fluoxetine have been shown to increase dopaminergic neurotransmission to the nucleus accumbens (Ichikawa & Meltzer 1995). In the chronic mild stress model of depression- where animals are exposed to mild stressors such as periods of food and water deprivation, changes in the lighting or temperature conditions in their home cages- decreases in \( D_2 \) and \( D_3 \) receptor binding have been observed in the nucleus accumbens, an effect which is reversed by chronic administration of antidepressant medication (Papp et al., 1994). Rodents exposed to chronic mild stress conditions also display reductions in the locomotor and reinforcing effects of the \( D_2 \) & \( D_3 \) receptor agonist quinpirole (Wilner et al., 1992).

In the forced swim test, which is a rodent screening model for novel antidepressants, animals forced to swim in an inescapable environment rapidly develop
an immobile posture. The time an animal spends immobile in used as an indicator of
distress in this task, which can be reduced by administration of D2 & D3 receptor agonists,
dopamine/norepinephrine reuptake inhibitors, and tricyclic antidepressants. These effects
can be blocked by D2 & D3 receptor antagonists (Basso et al., 2005 & Borsini et al.,
1990). Studies designed to test for alterations in dopamine levels in human subjects have
revealed lower cerebrospinal fluid concentrations of homovanillic acid, a dopamine
metabolite, in depressed patients compared to controls. Once dopamine is released it is
metabolized into homovanillic acid via MAO and/or COMT. Lower levels of metabolites
circulating in cerebral spinal fluid are thought to correlate to decreases in
neurotransmitter release. These findings indicate since these depressed individuals have
lower levels of dopamine metabolism occurring as evidenced by their decreased levels of
homovanillic acid, they may also have deficiencies in dopaminergic neurotransmission.
Lower levels of circulating dopamine may therefore be correlated with depression
(Mendels et al., 1972).

The ability of an antidepressant drug to increase dopaminergic neurotransmission
has been shown to correlate with therapeutic efficacy (Rampello et al., 1991). Further
involvement of the dopamine reward system in the genesis of anhedonic symptoms of
depression have been drawn from studies designed to measure the reward responsiveness
of non-medicated depressed patients in response to administration of amphetamine
(Dunlop & Nemeroff, 2007). Non-medicated, severely depressed patients had a
heightened sensitivity to the rewarding effects of amphetamine administration versus
controls. These findings caused investigators to believe that to compensate for a possible
decrease in dopaminergic neurotransmission, up-regulation of dopamine receptors
coupled with a down-regulation of dopamine transporters may have occurred, resulting in the elevated sensitivity of depressed subjects to amphetamine (Dunlop & Nemeroff, 2007).

This hypothesis has been supported through the use of functional magnetic resonance imaging. Following amphetamine administration, non-medicated depressed individuals displayed greater levels of behavioral responsiveness, and differences in activity in the ventrolateral prefrontal cortex, orbitofrontal cortex, caudate nucleus and putamen when compared to healthy controls (Tremblay et al., 2005). Postmortem findings in depressed individuals who had committed suicide found reductions in dopamine transporter expression, and increases in D$_2$ & D$_3$ receptors in the central and basal amygdalar nuclei when compared to controls, while D$_1$ receptor expression did not differ between depressed individuals and controls (Klimek et al., 2002). The exact role of the dopaminergic system in the genesis of depressive symptoms remains under investigation. However, the dopamine plays a pivotal role in non-pathological behaviors such as attention, reward, pleasure, motivation, and movement. Since the core symptoms of depression involve dysfunctions in many of these processes, it is likely that the dopamine system contributes in some way to the pathophysiology of depression.

*The role of serotonin in depression*

The synapses that serotonin- (5-HT) producing neurons of the raphe system make with other brain regions may play a role in the genesis of depressive symptoms. For example, serotonergic neurons originating in the median raphe project to areas of the
limbic system, making them likely to modulate anxiety, stress, motivational, and reward based behaviors. Serotonergic neurons originating in the dorsal raphe nuclei project to areas of the basal ganglia and the substantia nigra, which provides modulatory input to the basal ganglia, indicating a potential for modulatory effects on psychomotor behavior (Frazer & Hensler, 1994). The diversity of serotonergic signaling is immense, as at least 15 serotonin receptor subtypes have been identified (Nichols & Nichols, 2008). The serotonin receptors can be classified into several families including the 5-HT$_1$ and 5-HT$_2$ receptor families. The other serotonin receptors are designated 5-HT$_3$, 5-HT$_4$, 5-HT$_5$, 5-HT$_6$ and 5-HT$_7$. All of the serotonin receptors, with the exception of the 5-HT$_3$ receptor, are metabotropic receptors (Nichols & Nichols, 2008).

The 5-HT$_1$ family are located both pre- and post-synaptically and mediate their inhibitory effects via coupling of the receptor to a Gi/o protein, which leads to an inhibition of adenylyl cyclase, preventing the accumulation of cyclic AMP, and thus reducing activation of the PKA and its intracellular targets (Nichols & Nichols, 2008).

Of the 5-HT$_1$ receptor family, the 5-HT$_{1A}$ and 5-HT$_{1B}$ receptors seem to play the largest role in the onset of depression. The 5-HT$_{1A}$ receptor is present on serotonergic cell bodies in the dorsal and median raphe where it mainly functions as a somatodendritic autoreceptor, decreasing the firing rate of serotonin releasing neurons by activation of inward rectifying potassium channels and creating sustained levels of hyperpolarization (Aghajanian et al., 1995). As for the neural targets serotonin neurons project to, including the frontal cortex, amygdala, hippocampus, septum and entorhinal cortex, 5-HT$_{1A}$ receptors exist mainly postsynaptically, although this difference in receptor expression
may still have a modulatory effect on serotonin release via feedback loops to the raphe system (Hensler, 2003).

The 5-HT$_{1A}$ receptor seems to play a role in the response to antidepressant drug administration. Initially, administration of a selective serotonin reuptake inhibitor increases serotonin concentrations in the raphe nuclei, but due to the inhibitory nature of the 5-HT$_{1A}$ autoreceptor, the drug initially causes decreases in the firing rate of midbrain raphe nuclei. After some time however, these receptors are thought to undergo functional desensitization allowing for a disinhibition of raphe serotonergic neurons (Nichols & Nichols, 2008).

By administering a 5-HT$_{1A}$ antagonist, it is theorized that the relatively long latency period for the onset of therapeutic efficacy in response to antidepressant drug administration can be shortened, a hypothesis which has been supported in animal models of depression (Artigas, 1993). Mice with a genetic deletion of the serotonin 5-HT$_{1A}$ receptor also display depressive- and anxiety-like behaviors in a variety of behavioral assessments including the forced swim test, elevated plus maze, and open field tests (Ramboz et al., 1998).

The 5-HT$_{1B}$ receptor mimics some of the effects of the 5-HT$_{1A}$ receptor in response to selective serotonin reuptake inhibitors. The 5-HT$_{1B}$ receptor is selectively down-regulated in the raphe nuclei following treatment with the selective serotonin reuptake inhibitor fluoxetine, while expression in areas in the terminal field of serotonin releasing neurons remains unchanged (Neumaier et al., 1996). Similar to the 5-HT$_{1A}$ receptor, the 5-HT$_{1B}$ receptor may also undergo functional desensitization in response to
chronic antidepressant administration, suggesting the possibility that antagonizing the 5-HT\textsubscript{1B} receptor may also be a potential target to decrease the latency to the onset of therapeutic effects of antidepressant medications.

The 5-HT\textsubscript{2} receptor family also has a few receptor subtypes that are pertinent to the pathophysiology of depression. The 5-HT\textsubscript{2} receptor family are metabotropic receptors coupled to several different members of the G\textsubscript{q} family of G proteins (Nichols & Nichols, 2008). Activation of G\textsubscript{q} proteins achieves general stimulatory effects via activation of the PKC signaling pathway, which gives rise to increased intracellular calcium levels via modulation of ion channels or release from the endoplasmic reticulum. These effects can ultimately result in a diverse array of intracellular changes to cells.

Many antidepressant medications target 5-HT\textsubscript{2A} receptors, particularly in the prefrontal cortex (Celada et al., 2004). 5-HT\textsubscript{2A} receptors are highly expressed in the neocortex, and their actions there are thought to mediate aspects of executive functioning that are often impaired in people with depression. Many clinically effective antidepressant medications block the actions mediated by 5-HT\textsubscript{2A} receptors, and with chronic treatment, downregulation of 5-HT\textsubscript{2A} receptors has been observed (Marek et al., 2003). In the differential reinforcement of low rate responding at 72 second schedules behavioral model, in which animals must learn to wait a period of 72 seconds between responses in order to receive rewards, blockade of 5-HT\textsubscript{2A} receptors in the prefrontal cortex augments the antidepressant effects of selective serotonin reuptake inhibitors. These effects are thought to be mediated by postsynaptic 5-HT\textsubscript{2A} receptors, as the observed effects did not involve a presynaptic increase in the release of serotonin (Marek
et al., 2001). The medial prefrontal cortex sends projections to many areas associated with the different symptoms of depression, such as the nucleus accumbens, the amygdala, limbic structures, the hypothalamus, and other prefrontal regions. Changes in prefrontal functioning may, therefore, influence the development of anhedonic symptoms: anxiety and altered stress responses, mood disturbances and motivational problems, alterations in endocrine function and changes in hunger, appetite, and sleep behavior. Additionally, serotonergic modulation of the connections to these brain regions may lead to impairments in memory and other cognitive functions (Groenewegen & Uylings, 2000).

The 5-HT$_{2C}$ receptor is also of interest in determining the underlying pathophysiology of depression. 5-HT$_{2C}$ receptors are highly expressed in the amygdala, and the application of 5-HT$_{2C}$ agonists produces patterns of neuronal activation in the amygdala, hippocampus and hypothalamus indicative of anxiety behavioral states (Hackler et al., 2007). Via action at the amygdala and hypothalamus, the 5-HT$_{2C}$ receptor may be involved in modulation of the hypothalamic-pituitary-adrenal axis, and may play a role in the elevations in cortisol observed in depressed individuals (Heisler et al., 2007).

Two more noteworthy serotonin receptors may also play a role in the genesis of depressive symptoms. Both are coupled to the G$_s$ family of G proteins, which have an overall stimulatory effects on expressing cells by driving cAMP production via stimulation of adenylyl cyclase, which, in turn, activates the PKA signaling pathway (Nichols & Nichols, 2008). The 5-HT$_6$ receptor is expressed throughout the striatum, nucleus accumbens, cortex, olfactory tubercle, hippocampus, thalamus, amygdala, hypothalamus, and cerebellum (Nichols & Nichols, 2008). The 5-HT$_6$ receptor alters
dopaminergic and acetylcholine levels, an effect that has the potential to enhance the anhedonic and cognitive symptoms seen in depression (Nichols & Nichols, 2008). The 5-HT₆ receptor may also play a role in the effects of antidepressant medications. While antagonism of the 5HT6 receptor blocks the antidepressant effects of selective serotonin reuptake inhibitors, agonists at the 5HT6 receptor have shown promise as antidepressant agents in animal models (Svenningsson et al., 2007, & Wesolowska et al., 2007).

There is interest in the potential role the serotonin 5-HT₇ receptor may play in the pathophysiology of depression. Although a lack of specific agonists for the receptor have limited efforts in determining the exact function of this receptor, knockout studies in 5-HT₇ deficient mice have demonstrated a decrease in immobility in the forced swim test, a behavioral index which is intended to measure antidepressant activity in humans (Guscott et al., 2005). Administration of 5-HT₇ antagonists also promotes the effects of antidepressant medications in the forced swim test in wild type mice (Wesolowska et al., 2007).

The role of norepinephrine in depression

There are three general types of noradrenergic adrenoceptors. β adrenoceptors are generally stimulatory in nature via coupling to Gs-type G proteins; α₁ adrenoceptors are also stimulatory in nature via coupling to Gq-type G proteins, while α₂ adrenoceptors exert inhibitory effects through their coupling to Gi-type G proteins. α₁ adrenoceptors may play a facilitatory role in serotonergic neurotransmission, as application of α₁
antagonists cause a decrease in serotoninergic neuronal firing in the raphe nuclei (Barabaran & Aghajanian, 1980). Presynaptically, $\alpha_2$ receptors serve mainly to modulate the release of both serotonin and norepinephrine. As either autoreceptors, when acting on adrenergic neurons, or heteroreceptors- receptors that modulate the function of a cell via neurotransmitter released from adjacent neurons- when acting on serotonergic neurons.

Consistent with the adaptations observed in receptor sensitivity and distribution studies for the other monoamine neurotransmitters in response to chronic antidepressant administration, and in animal models of sustained depressive symptoms, adaptations also occur in the noradrenergic system. Desensitization and downregulation of expression of $\beta$ adrenoceptors has been observed following chronic administration of the tricyclic antidepressant desipramine, and also following electroconvulsive therapy (Heal et al., 1989). Upregulation of $\beta$ adrenoceptors has also been observed in depressed individuals, and a decrease in $\beta$ adrenoceptor expression is considered a clinical marker for antidepressant drug efficacy (Leonard, 1997). Changes in $\alpha_1$ adrenoceptor expression have also been reported, but in contrast to $\beta$ adrenoceptors, $\alpha_1$ adrenoceptors seem to be up-regulated in response to antidepressant drug administration. Results of radioligand binding assays have demonstrated an increase in $\alpha_1$ binding following repeated administration of the tetracyclic antidepressant mirtazapine, leading investigators to believe that increases in the responsiveness and number of $\alpha_1$ adrenoceptors may have taken place (Rogoz, et al., 2002). These results have been duplicated using the norepinephrine reuptake inhibitor reboxetine (Rogoz & Kolasiewicz, 2001).
$\alpha_2$ adrenoceptors are thought to undergo a functional desensitization in response to chronic antidepressant administration, a process that may account for the latency in the onset of therapeutic efficacy in antidepressant drugs. As heteroreceptors on serotonergic neurons, $\alpha_2$ adrenoceptors are thought to decrease the release of serotonin. As autoreceptors, $\alpha_2$ adrenoceptors decrease the release of norepinephrine. Thus, by modifying the receptors expression or efficacy, increases in adrenergic and serotonergic neurotransmission may be achieved (Dennis et al., 1987).

Evidence for this desensitization hypothesis is drawn from studies using a combination of chronic antidepressant treatment combined with $\alpha_2$ agonists to measure decreases in the behavioral effects of $\alpha_2$ agonism. Following 3-4 weeks of treatment with the tricyclic antidepressant desipramine, decreases in the behavioral responses to $\alpha_2$ agonism, including antinociceptive effects, and durations in the loss of righting reflexes were observed. These results were reversible by administration of $\alpha_1$ antagonists (Guo et al., 1998), and indicate that $\alpha_1$ receptors may play a modulatory role in antidepressant induced hyporesponsiveness to $\alpha_2$ agonists.

Further evidence for the desensitization hypothesis has come from findings that norepinephrine concentrations are significantly elevated following chronic treatment with desipramine, an effect which lasts following cessation of drug treatment. This stimulatory effect on norepinephrine release was further potentiated following a challenge administration of desipramine 48 hours after drug treatment had ceased (Sachetti et al., 2001). To test whether these effects were specific to the $\alpha_2$ receptor, the $\alpha_2$ agonist clonidine was administered and resulted in decreases in noradrenergic neurotransmission
in control animals with no change observed in desipramine-treated animals (Sachetti et al., 2001). These findings indicate that the facilitatory effects desipramine exerts on noradrenergic neurotransmission may be due to a lasting desensitization specifically at \( \alpha_2 \) adrenergic receptors.

Changes in the expression of \( \alpha_2 \) receptors have been demonstrated in humans as well. Treatment with tricyclic antidepressant drugs attenuates the hypotension, sedation, and reduction in norepinephrine turnover in response to clonidine administration in humans as well as animals (Charney 1981 & 1983), an effect that did not occur following treatment with the tetracyclic antidepressant mianserin (Charney et al., 1984). Furthermore, a review of postmortem studies of suicide victims not receiving antidepressant medication found increases in \( \alpha_2 \) expression in the prefrontal cortex of 31-40% of individuals observed (Garcia-Sevilla et al., 1999). These findings, taken together, may indicate that while in some instances \( \alpha_2 \) desensitization may play a role in the efficacy of antidepressant medications such as the tricyclics, in other antidepressant medications like the tetracyclics, it may not be necessary to achieve therapeutic efficacy. These results may be further interpreted as an indication of the diversity in the pathophysiology of depression, which is exemplified by the fact that not everyone reacts the same way to certain classes of antidepressant medication. Medications with one mechanism of action (inhibition of serotonin reuptake) may produce great improvements in symptoms in one patient, while providing no relief whatsoever in another.
**Pharmacotherapies for depression**

Most of the currently available medications used in the treatment of depression in humans target one or more of the brain’s monoaminergic neurotransmitter systems. Typically, these medications aim to increase levels of monoamines in the brain. A variety of medications with varying mechanisms of action are currently available to physicians to assist patients in the management of depressive symptoms. These include: (1) the selective serotonin reuptake inhibitors, which increase the availability of serotonin synaptically via blockade of serotonin reuptake transporters, (2) mixed serotonin/norepinephrine reuptake inhibitors, which increase the availability of serotonin and norepinephrine via blockade of their reuptake, (3) monoamine oxidase inhibitors, which prevent the degradation of monoamines by monoamine oxidase, (4) dopamine/norepinephrine reuptake inhibitors, which increase synaptic concentrations of dopamine and norepinephrine via blockade of dopamine and norepinephrine reuptake, (5) and the tricyclic antidepressants, which act as serotonin/norepinephrine reuptake inhibitors and antagonists at a range of receptors.

The tricyclics were among the first available treatment strategies for depression, with imipramine being the first clinically available tricyclic antidepressant in the early 1950’s (Lopez-Munoz et al., 2009). There is some variation among the specific affinities between tricyclic antidepressants, but they were originally developed as anti-histaminergic compounds and most commonly act as antagonists at the histamine $H_1$ receptor (Lopez-Munoz, 2004). The tricyclic imipramine acts as a potent inhibitor of serotonin reuptake, an inhibitor of norepinephrine reuptake, and as an antagonist at $H_1$
receptors, α₁ adrenoceptors, cholinergic muscarinic receptors, and 5-HT₂A receptors (Gillman, 2007). This binding profile may account for both the therapeutic efficacy of imipramine and some of the unpleasant side effects associated with the use of tricyclic antidepressants. For instance, although H₁ antagonism may play a role in the therapeutic efficacy of imipramine, it may also contribute to the sedation and weight gain observed following treatment (Kroeze et al., 2003). Antagonism at the 5-HT₂A receptor has been hypothesized to play a role in improving the observed dysfunctions in sleep observed in depressed individuals (Thase, 2006). Antagonism at the α₁ receptor is presumed to play a role in the development of postural hypotension developed following administration of tricyclics like imipramine (Smith, 2001). The development of anti-muscarinic side effects like dry mouth, blurred vision, urinary retention, tachycardia, constipation and impairments in memory arise from antagonism of muscarinic receptors (Gillman, 2007). These anti-muscarinic properties are especially worrisome for the elderly, where therapeutic doses have been noted to produce delirium (Gillman, 2007).

In addition to these adverse effects, responsiveness to antidepressant therapy varies on an individual basis. These variations depend largely on the severity of symptoms. Patients with mild to moderate depression seem to respond less favorably to antidepressant treatment (Zimmerman et al., 2002). Patient compliance may also play a role in the ineffectiveness of antidepressant medications. Due to the long onset of action of these medications and the presence of adverse side effects at therapeutic dosages, it has been estimated that as few as 30% of patients take their medications as prescribed (Weich et al., 2007 & Bockting et al., 2008). The combination of adverse effects and the long latency (10-14 weeks) to the onset of therapeutic actions seen in antidepressant
medications highlight the need for alternative mechanisms of action for the treatment of depression.

The neurotensin system

Neurotensin is a neuropeptide signaling molecule first isolated in the bovine hypothalamus in 1973 by Robert Carraway and Susan E. Leeman. The full peptide molecule consists of 13 amino acid residues, of which segments 8-13 are linked to the biological effects of neurotensin (Lambert et al., 1995). There are currently three identified neurotensin NTS receptors within the central nervous system, designated the NTS₁, NTS₂ and NTS₃ receptors.

The three receptors differ significantly in their affinities for the neurotensin peptide. The NTS₁ receptor binds neurotensin with high affinity and is insensitive to the H₁ receptor antagonist levocabastine (Tanaka, et al., 1990), while the NTS₂ receptor has low affinity for neurotensin itself, but binds levocabastine with high affinity (Vincent et al., 1999). Neurotensin acts as an agonist at the NTS₁ receptor, and upon binding to the NTS₁ receptor, neurotensin causes a coupling of the agonist bound receptor to a G₉-type G-protein, and subsequent activation of an associated signal cascade. It is unclear precisely what action neurotensin binding to the NTS₂ receptor produces, as species-specific differences exist in the post-binding events associated with the NTS₂ receptor (Vincent et al., 1999). Clones of human NTS₂ receptors transfected into Chinese hamster ovaries or *Xenopus* oocytes are antagonized by application of neurotensin or
levocabastine, while both of these compounds act as agonists at the NTS\textsubscript{2} receptor in mouse or rat tissue preparations (Vincent et al., 1999).

The NTS\textsubscript{1} & NTS\textsubscript{2} receptors are both G-protein coupled receptors, while the NTS\textsubscript{3} receptor is unique, as it is not coupled to a G-protein. The neurotensin 3 receptor has a low affinity for neurotensin and shares a 100% amino acid homology with the gp95/sortilin complex previously isolated from human tissue (Mazella et al., 1998). The physiological relevance of this receptor is unclear, as no signaling mechanisms have been linked to this binding site (Tyler-McMahon, 2000).

The neurotensin peptide plays a role in a diverse set of functions in both the central and peripheral nervous system. In the periphery, neurotensin acts as a paracrine and endocrine peptide with notable effects in the gastrointestinal tract, cardiovascular system, and in the proliferation of normal and cancerous cell growth (Tyler-McMahon et al., 2000). In the central nervous system, administration of neurotensin produces hypothermia, reduces nociception, and has modulatory effects on monoaminergic neurotransmission and effects on endocrine transmission, stimulating the release of corticotrophin releasing factor, galanin, enkephalin, cholecystokinin and growth hormone–releasing hormone (Tyler-McMahon, 2000). However, due to the large size of the full peptide, and the fact that it is degraded rapidly via protease metabolism following systemic administration, the full peptide form of neurotensin is does not cross the blood brain barrier. However, only the final 6 amino acids (8-13) of the neurotensin peptide are needed for the full physiological effects of the neurotensin peptide, a finding which has allowed for the development of brain penetrant analogs of the 8-13 segment of the
neurotensin peptide (Tyler-McMahon, 2000). NTS₁ agonists produce all the physiological and behavioral responses associated with neurotensinergic neurotransmission. Therefore, until further knowledge can be gained regarding the biological relevance of the NTS₂ receptor within and across species, the effects of manipulating the neurotensin system are assumed to be mediated by the NTS₁ receptor.

*The NTS₁ receptor*

As the NTS₁ receptor is the only neurotensin receptor that binds neurotensin with high affinity, it is assumed to play the most important role in the physiological actions of the neurotensin peptide. NTS₁ activation mediates its excitatory effects following agonist binding through a preferential coupling to G_{q₁₁} G-proteins, leading to activation of the protein kinase C pathway through activation of phospholipase C and phosphatidylinositol 4,5-biphosphate with inositol triphosphate and Ca^{2+} acting as second messengers (Richard et al., 2001). The agonist bound NTS₁ receptor may also bind to G_s G-proteins in certain circumstances, as increases in cAMP production have been found following agonist application in Chinese hamster ovary tissue preparations (Yamada et al., 1994). Activation of the NTS₁ receptors in these tissue preparations also results in a prolonged activation of mitogen-activated protein kinases and the growth associated gene krox-24. These effects were eliminated following application of the NTS₁ antagonist SR 48692 (Poinot-Chazel, et al., 1996).

Once an agonist binds to the NTS₁ receptor several proposed mechanisms mediate the physiological effects of the agonist-bound receptor. First, upon binding internalization
of the agonist/receptor complex may function as a transcriptional regulator. Second, allosteric interactions between the NTS$_1$ receptor and the D$_2$ receptor may occur, and binding of an agonist to the NTS$_1$ receptor may lower the affinity of the D$_2$ receptor to bind ligands. Third, NTS$_1$ receptor activation may alter neuronal excitability, and following agonist binding may alter second messenger signal cascades and ion channel functioning through it’s coupling to G-Proteins (Binder et al., 2001, St-Gelais et al., 2006).

**Distribution of the NTS$_1$ receptor**

The NTS$_1$ receptor is widely distributed throughout many areas of the brain. A comprehensive mapping of the location of NTS$_1$ receptors in the rat brain using immunohistochemical labeling comparatively verified with application of 125-Iodine labeled neurotensin was performed by Boudin et al. (1996). In the telencephalon, NTS$_1$ receptor immunoreactivity was discovered on pyramidal cell bodies within layers II-III & V, and on dendrites in layer IV in the frontal and parietal cortices. In the anterior cingulate, endopiriform and insular cortices, NTS$_1$ receptors were detected mainly on axon terminals within layer IV. In the perirhinal cortex, labeling was present on axon terminals in layers I-III & VI, with punctate labeling present on cell bodies in layers IV &V. Entorhinal cortical labeling displayed a weak and scattered immunoreactive signal across cell bodies. In the retrosplenial cortex labeling occurred primarily in terminal regions in layer I, while layers II & III displayed labeling on both cell bodies and axon terminals. Within the caudate-putamen, NTS$_1$ receptors were identified on axon
terminals and cell bodies, and on cell bodies and axons within the nucleus accumbens. Within the olfactory tubercle, labeling was concentrated on dendrites and cell bodies within the granule cells of the islands of Calleja. In the basal forebrain NTS$_1$ receptors were located on cell bodies within the medial septum, diagonal band of Broca, and were densely labeled on dendrites and cell bodies within the magnocellular preoptic nucleus, substantia innominate, and globus pallidus. Axonal labeling with sparse labeling of cell bodies was present within the lateral septum and the bed nucleus of the stria terminalis. Labeling within the amygdala differed between the substructures therein, with cell bodies, dendrites and axon terminals present on the posterior cortical nucleus, neuropil labeling within the basomedial and lateral amygdalar nuclei, and axonal labeling within the central nucleus. In the hippocampus, NTS$_1$ receptors were found in the presubiculum, parasubiculum, and subiculum on cell bodies, dendrites, and axon terminals. In the CA1, CA2, and CA3 regions, as well as the pyramidal and granule cell layers labeling was detected on cell bodies. Within the diencephalon, several thalamic nuclei also displayed an immunoreactive signal. Labeling was strongest on cell bodies within the anterior dorsal thalamic nucleus, though labeling was also present on beaded fibers within the paraventricular thalamic nucleus, cell bodies and neuropil in the reticular nucleus, and cell bodies and dendrites within the nucleus of the optic tract. In the hypothalamus, densely labeled axon terminals were identified within the medial and lateral nuclei, and densely labeled cell bodies and processes were identified in the suprachiasmatic nucleus. In the periventricular nucleus, the parvocellular part of the anterior paraventricular nucleus, as well as the lateral mammillary nucleus, NTS$_1$ receptors were identified on axons. In the zona incerta, NTS$_1$ receptors were located on cell bodies in the ventral
portion, while dorsally they were present mainly on dendrites. Scattered labeling was also
observed across the medial and lateral habenula. In the mesencephalon, densely labeled
cell bodies and dendrites were identified in the substantia nigra pars compacta, ventral
tegmental area and other associated midline structures including the interfascicular
nucleus and the nucleus of the raphe linearis caudalis. Immunoreactivity was also
detected on cell bodies within the substantia nigra pars reticulata and pars lateralis. NTS₁
receptors were also detected on cell bodies and dendrites within the periaqueductal grey,
dorsal raphe, median raphe, and laterodorsal tegmental nuclei. Axonal labeling was also
identified in the laterodorsal tegmentum and locus coeruleus. Within the brainstem,
immunoreactivity was observed on cell bodies and neuropil throughout the pontine
nuclei, with heavily labeled cell bodies identified within the reticulotegmental nucleus,
and densely labeled processes around the medial lemniscus and medical longitudinal
fasciculus. In the medulla, NTS₁ receptors were detected on cell bodies in the medial
vestibular, dorsal cochlear nuclei, and the parvocellular reticular formation. Intense
signals were detected on cell bodies and dendrites within the nucleus raphe pallidus, the
inferior olivary nucleus, and the paragigantocellular nucleus. Caudally, NTS₁ receptors
were detected on cell bodies and dendrites within the dorsal motor nucleus of the vagus,
and on axon terminals within the nucleus of the solitary tract. Cell bodies and dendrites
within the external cuneate and lateral reticular nucleus, as well as motor neurons in the
hypoglossal nerve nucleus also displayed immunoreactivity (Boudin et al., 1996).
Effects of neurotensin on dopaminergic neurotransmission

Many studies have been performed to examine the influence application of agonists at the NTS\textsubscript{1} receptor have on dopaminergic neurotransmission. These have included application of agonists into the ventral tegmental area, the origin of the mesocortical and mesolimbic dopamine streams, the substantia nigra pars compacta, the origin of the nigrostriatal dopamine tract, as well as the terminal fields of these dopamine projection pathways.

Application of either the full peptide form of neurotensin or two analogs, the 8-13 fragment of the neurotensin peptide, or [D-Tyr-11]neurotensin directly into the ventral tegmental area produces differential effects on dopamine efflux in the terminal regions of the mesocortical/mesolimbic dopamine streams (Sotty et al., 2000). Injection of all three compounds at concentrations of \(10^{-3}\) M produced elevations in extracellular dopamine levels in the prefrontal cortex as measured by differential normal pulse voltammetry or differential pulse amperometry, though the elevations in dopamine produced by [D-Tyr-11]neurotensin lasted longer (Sotty et al., 2000). Injections of \(10^{-5}\) M concentrations of neurotensin and (8-13) neurotensin produced results similar to those produced by \(10^{-3}\) M concentrations of these two compounds, while \(10^{-5}\) M concentrations of [D-Tyr-11]neurotensin did not significantly alter dopamine efflux into the prefrontal cortex. Similar patterns of activity were found in the nucleus accumbens, where all three compounds produced elevations in extracellular dopamine levels at concentrations of 10\(^{-5}\) M.
$10^{-5}$ M, with [D-Tyr-11]neurotensin producing more prolonged increases in dopamine levels (Sotty et al., 2000).

Systemic administration of NT69L, an analog of the 8-13 amino acid sequence of the neurotensin peptide also influences dopaminergic neurotransmission in the prefrontal cortex and nucleus accumbens as measured by microdialysis (Prus et al., 2007). At doses of 1.0 and 3.0 mg/kg, NT69L produced significant increases in dopamine levels in the prefrontal cortex, while only the 1.0 mg/kg dose of NT69L produced significant increases in dopamine levels within the nucleus accumbens (Prus et al., 2007).

Stimulation of NTS$_1$ receptors in the prefrontal cortex may have modulatory effects on feedback pathways transmitting from the prefrontal cortex back to midbrain dopamine producing nuclei. Microinjection of the full peptide form of neurotensin or the 8-13 fragment of the neurotensin peptide into the rat prefrontal cortex produces increased firing rates in ventral tegmental dopamine-producing neurons, while microinjection of the 1-8 fragment of the neurotensin peptide does not (Fatigati et al., 2000). Injection of neurotensin into the rat prefrontal cortex also facilitates dopaminergic release in the prefrontal cortex, an effect that can be antagonized by application of the NTS$_1$ receptor antagonist SR 48692 (Petkova-Kirova et al., 2008).

Neurotensin also plays a modulatory role on dopaminergic functioning in the substantia nigra pars compacta. Microinjections of neurotensin into the substantia nigra pars compacta produces elevated levels of dopamine efflux into the rodent basal ganglia, an effect which has been demonstrated to last for as long as 20 hours (Napier et al., 1985).
Because injection of neurotensin into the origins and terminal fields of the midbrain dopamine streams can produce elevated dopamine levels, the facilitatory effects of neurotensin on dopamine release may be due to mixed presynaptic/somatodendritic stimulatory effects depending on the locus of action. These effects also seem to be dependent on NTS$_1$ receptor activation, as application of selective NTS$_1$ receptor antagonists prevents these effects. However, the full neurotensin peptide is a large, rapidly degraded, brain impenetrable peptide, which makes it unsuitable for central nervous system activity when administered systemically. Fortunately, analogs of the biologically active 8-13 fragment of the neurotensin peptide are systemically administrable, and have demonstrated efficacy in the facilitation of dopamine release.

*Effects of neurotensin on serotonergic neurotransmission*

Although it has not received quite as much attention as the dopaminergic system, the literature suggests that neurotensin also plays a facilitatory role in serotonergic neurotransmission. *In vitro*, application of the full neurotensin peptide, and the 8-13 fragment of the neurotensin peptide increases the firing rate of dorsal raphe serotonergic neurons in a concentration dependent manner, while application of the selective NTS$_1$ receptor antagonist SR 48692 blocks these effects (Jolas & Aghajanian, 1996). Application of the 1-8 fragment of the neurotensin in the same set of experiments did not induce increases in firing rates; more evidence supporting the efficacy of analogs of the neurotensin peptide.
In the raphe magnus, application of neurotensin to *in vitro* tissue preparations also induced depolarization of serotonergic neurons and increased the generation of action potentials (Li et al., 2001). Further experiments were carried out to determine the mechanisms by which neurotensin exerted these effects. Application of anti-\(G_{q/11}\) antiserum prevented the excitatory effects neurotensin had on raphe magnus serotonin neurons. Application of the IP3 inhibitor heparin, and BAPTA, a Ca\(^{+2}\) chelator also impaired the excitatory effects exhibited by neurotensin (Li et al., 2001). These experiments provide further support for the \(G_{q/11}\), IP3, Ca\(^{+2}\) signal cascade in mediating the excitatory effects of neurotensin.

Application of both the full neurotensin peptide and the 8-13 fragment induce increases in prefrontal cortical serotonin release *in vitro* (Heaulme, M., 1998), effects that are antagonized by application of the NTS\(_1\) receptor antagonist SR 48692. *In vivo*, microinjection of neurotensin into the prefrontal cortex of rats also induces sustained elevations in serotonin release, an effect that is also inhibited by application of SR 46892 (Petkova-Kirova et al., 2008).

Though much of the research on the neurochemical modulations induced by neurotensin has focused on dopamine, the serotonergic system is also affected. Further study into the modulatory role the neurotensin system plays on serotonergic neurotransmission could further validate the use of neurotensin analogs in the treatment of disorders involving imbalances in serotonergic neurotransmission, including depression.
Effects of neurotensin on noradrenergic neurotransmission

If research into the role neurotensin plays in serotonergic neurotransmission is lacking, the role neurotensin plays in noradrenergic neurotransmission is even scarcer. However, the minimal evidence gathered into the modulatory role neurotensin plays on noradrenaline in the central nervous system indicates there may be interactions at play worth further study. In one such study, rats trained to self-administer nicotine were forced to undergo a withdrawal period. During this withdrawal period, animals that previously had received saline/nicotine treatment displayed significantly higher concentrations of noradrenaline within the striatum than animals that received neurotensin/nicotine treatment (Boules et al., 2011). These results may indicate that neurotensin administration could provide protective benefits against increased noradrenergic neurotransmission in response to the stress induced by nicotine withdrawal.

Evidence from animal models of depression

Neurotensin analog drugs have been most thoroughly researched as therapeutic agents to treat schizophrenia. The NTS$_1$ receptor agonist PD149163 has displayed antipsychotic effects in variety of animal models of schizophrenia such prepulse inhibition (Feifel et al., 2011), and conditioned avoidance tasks (Holly et al., 2011), where it displays antipsychotic-like effects without producing catalepsy.

In addition to its role in the treatment of schizophrenia, the neurotensin system may also represent a novel target for the treatment of depression. Animals with a genetic
deletion of the NTS₁ receptor display several anxiety- and depression-like behaviors. Deletion of the NTS₁ receptor results in alterations in sleep architecture and abnormal recovery from periods of sleep deprivation (Fitzpatrick et al., 2012), which may correlate with sleep disturbances seen in human patients with depression. In the tail suspension test, an animal behavioral model of depression, NTS₁ receptor knockout mice display greater levels of immobility than their wild type counterparts. In another experiment using the same animals, NTS₁ receptor knockout mice displayed greater levels of anxiety-like behavior in an open field test, spending more time in the corners of the apparatus and less time in the center, indicative of an enhancement of thigmotaxia. The NTS₁ agonist PD149163 may also possess anxiolytic effects, as acute administration of PD149163 reduces conditioned foot shock-induced ultrasonic vocalizations (Prus et al., 2014).

**Neurotensin in the forced swim test**

The measurement of depression-related behaviors in animals is a difficult task, as animals are unable to directly report feelings of sadness or despair. However, several screening models do exist in which drugs that produce antidepressant effects in human beings exert a consistent effect on animal behavior. One such test is the forced swim test.

The forced swim test is a commonly used screening model for novel antidepressant medications in which animals are placed in a cylinder partially filled with water from which there is no escape. After some time, the animals cease swimming and instead adopt a passive, immobile posture characterized by the animal only emitting
movements necessary to stay afloat. Drugs that have antidepressant efficacy in humans reliably decrease the amount of time that animals spend in this immobile posture, while increasing escape, exploratory, or general swimming behaviors. However, drugs that produce stimulant like effects such as caffeine, nicotine, or amphetamine produce a false positive in the forced swim test as they decrease immobility behaviors while not possessing antidepressant efficacy (Castagne et al., 2009). For this reason, a locomotor assessment is generally performed in conjunction with the forced swim test to ensure that any reductions in immobility observed are not due to a general stimulatory effect on locomotor behavior. If a drug produces a reduction in immobility in the forced swim test without increasing locomotor behavior in the locomotor assessment, then the test substance may produce antidepressant effects in humans.

Very few studies to date have explored the role of the neurotensin system on animal behavior in the forced swim test. One study reported an increase in immobility behavior in mice with a genetic deletion of the NTS₁ receptor (Li et al., 2010), while another did not find significant differences between wild type mice and NTS₁ receptor knockout mice (Fitzpatrick et al., 2012). Although the levels of immobility between the two groups in the second study did not reach statistical significance, the authors did report a trend in their data toward greater levels of immobility in NTS₁ receptor deficient mice. Microinjection of the full peptide form of neurotensin directly into the ventral tegmental area resulted in antidepressant-like effects in rats during the forced swim test (Cervo et al., 1992), indicating that in normal animals, exogenous stimulation of NTS₁ receptors may exert antidepressant effects. What is unknown is whether systemically
administrable neurotensin analog drugs like PD149163 will also produce antidepressant effects in the forced swim test.
RATIONALE

Due to the large number of patients that do not respond to antidepressant treatment and the long latency to the onset of therapeutic effects in existing options, novel mechanisms for the treatment of depression are needed. According to the monoaminergic theory of depression, imbalances in one or more of the brains monoamine neurotransmitter systems may lead to the genesis of depressive symptoms. Most of the currently available antidepressant medications aim to increase the synaptic availability of monoamines by a variety of mechanisms such as preventing their reuptake or metabolism. Neurotensin is an endogenous neuropeptide that has modulatory influences on monoaminergic neurotransmission in areas of the brain involved in depression. The neurotensin system has been implicated in a wide variety of psychiatric disorders including schizophrenia, anxiety, and depression. Agonists for the NTS₁ receptor have displayed therapeutic potential in a variety of animal models of psychiatric disorders but what is unclear is whether NTS₁ agonists exhibit antidepressant properties in animal models of depression.

The current study aimed to evaluate the effects of the NTS₁ agonist PD149163 in the forced swim test, a rodent screening model for putative antidepressant medications. The effects of PD149163 and the tricyclic antidepressant imipramine were compared to saline control animals to determine if these compounds may possess antidepressant properties in human patients. To ensure that any alterations in swimming behavior
observed in the forced swim test were not due to alterations in motor behavior alone a locomotor assessment was also performed.
METHODS

Experiment 1

Subjects

Forty-eight male C57/B16 mice (Charles River Laboratories, Portage, MI) were used in this study. All animals were approximately 2 months of age and weighed between 23-28 grams at the beginning of these procedures. Mice were group housed in a temperature-controlled room (22±3°C) with a 12-hour light/dark cycle and were provided free access to food and water in their home cages. The housing and experimental procedures were approved by the Northern Michigan University Institutional Animal Care and Use Committee (#227).

Apparatus

The forced swim test apparatus was a 2L glass beaker (19 cm height, 14 cm diameter) filled with 12 cm of water warmed to a temperature of 23 °C. Twelve cm of water was of sufficient height so that at no point during testing could any part of the animals body touch the bottom of the beaker. Three animals were run simultaneously each in their own individual beaker with cardboard dividers positioned on the left and right sides of each beaker so the animal could not see the adjacent testing apparatus, or the surrounding experimental room. 8x11” sheets of
white computer paper were taped to the cardboard dividers and the background wall to provide contrast to the animals black color.

**Drugs**

Imipramine hydrochloride was obtained from Sigma-Aldrich (St. Louis, MO), and PD149163 was obtained from RTI International (Research Triangle Park, NC). Imipramine and PD149163 were dissolved in 0.9% saline and administered at doses of 1.0 mg/kg or 10.0 mg/kg, and 0.1 mg/kg or 1.0 mg/kg respectively. Imipramine, PD149163 and saline were administered 30 minutes prior to experimental procedures via intraperitoneal injection at a volume of 10 mL/kg. All of the drugs were in salt form.

**Behavioral procedures**

The experimental procedures began one month after the animals arrived from the breeder. Day one of the procedures consisted of acclimation to the testing environment for one hour, followed by the forced swim test procedure. One week after the forced swim test, the locomotor assessment occurred.
Forced Swim Test

The forced swim test procedures were similar to those described by Castagne, Moser, and Porsolt (2009). On the first day of testing, mice were allowed to acclimate to the environment of the experimental room for 1 hour in their home cages. Testing sessions lasted a total of 360 seconds and upon completion of the session animals were placed in a cage lined with paper towels positioned next to a space heater to dry and warm the animals. To minimize distress, animals were not manually dried by the experimenters, but instead were allowed to remain in the paper towel lined cages until sufficiently dry before being returned to their home cages. According to standard scoring methods for this procedure (Castagne et al., 2009), immobility time is typically only scored in the final 240 seconds of the trial sessions as animals usually display very little immobility in the first 120 seconds (Castagne et al., 2009). For the purposes of this study, animal behavior was recorded for the entire 360 seconds of the testing session, and behavior from the first two minutes of the testing session was scored and analyzed separately from behavior in the final four minutes of the testing session. Mice were randomly assigned to a drug treatment group and received a single injection of imipramine (1.0 mg/kg or 10.0 mg/kg), PD149163 (0.1 mg/kg or 1.0 mg/kg) or saline 30 minutes prior to the testing session. Immobility was defined as a lack of all movement except those movements necessary to keep the animals head above the water. Testing sessions were recorded on video and the total duration of immobility and the latency to the first episode of immobility were scored by an observer blind to the experimental conditions. A reduction in time
spent immobile and increased latencies to the first episode of immobility were used as indices of antidepressant efficacy.

*Locomotor assessment*

To rule out non-specific motor effects as a causal factor for any observed differences in immobility behavior between groups a locomotor assessment was performed one week following the forced swim test procedure. The locomotor assessment was carried out in the same room as the forced swim test and all animals received the same test substance and dose that were given before the forced swim test. Animals received the test substance 30 minutes prior to being placed in the locomotor apparatus. Testing sessions lasted for 20 minutes, and the total distance traveled was calculated using Noldus Ethovision XT 7.0 software (Noldus Information Technology, Leesburg, VA).

*Apparatus*

The locomotor apparatus was a wooden box with four separate identical compartments measuring 45.72 cm long x 45.72 cm wide and 27 cm tall. The floor and side walls were painted white in color to provide contrast to the animals black color. Animals were tested three at a time each in their own individual compartment of the apparatus.
Statistical analysis

The dependent variables measured for the forced swim test were total time spent immobile in the first two minutes, and the final four minutes of the testing session, and the latency to the first episode of immobility. The dependent variable measured for the locomotor analysis was total distance traveled. A one-way between groups analysis of variance was used to analyze the effects of each drug (doses and saline) on each measure. Any statistically significant differences were further analyzed using a Dunnet’s post-hoc analysis to determine which treatment groups differed from saline control groups. Also, an independent samples t-tests was performed to determine if the measures taken from the control animals for imipramine differed from the control animals for PD149163. All analyses were conducted using GraphPad Prism for Windows version 6.0 (GraphPad Software, La Jolla, CA).
RESULTS

Forced Swim Test

Time spent immobile: final 4 minutes

Figure 1 represents the mean for the total time spent immobile in the final four minutes of the testing session for imipramine- and saline-treated mice. Administration of imipramine significantly changed the total time spent immobile $F(2,21)=7.014, p<0.01$. A significant decrease in the total time spent immobile was found at the 10.0 mg/kg dose of imipramine in comparison to saline.

Figure 2 represents the mean for the total time spent immobile in the final four minutes of the testing session for PD149163- and saline-treated mice. Administration of PD149163 significantly altered the total time spent immobile $F(2,21)=7.505, p<0.01$, which was due to a significant decrease in the total time spent immobile at both the 0.1mg/kg and the 1.0 mg/kg dose compared to saline.

Figure 3 represents the mean for the total time spent immobile in the last four minutes of the testing session for the imipramine-paired control animals versus the PD14916-paired control animals. The average time spent immobile between the 2 groups was not significantly different $t(14)=1.689, p>0.05$. 
**Time spent immobile: first two minutes**

Figure 4 represents the mean for the total time spent immobile in the first 2 minutes of the testing session for imipramine- and saline-treated mice. Administration of imipramine significantly affected the total time spent immobile $F(2,21)=13.18, \ p<0.001$, due to a significant reduction in the time spent immobile at both the 1.0 mg/kg and the 10.0 mg/kg doses in comparison to saline.

Figure 5 represents the mean for the total time spent immobile in the first 2 minutes of the testing session for PD149163- and saline-treated mice. Administration of PD149163 significantly changed the total time spent immobile $F(2,21)=5.108, \ p<0.05$, due to a significant decreased the total time spent immobile at the 0.1 dose compared to saline.

Figure 6 represents the mean for the total time spent immobile in the first 2 minutes of the testing session for the imipramine-paired versus PD149163-paired control animals. Immobility times for the two groups were not significantly different $t(14)=0.265, \ p>0.05$.

**Latency to the first episode of immobility**

Figure 7 represents the mean latency to the first episode of immobility for the imipramine- and saline-treated mice. Administration of imipramine significantly altered the latency to the first episode of immobility $F(2,21)=6.939, \ p<0.01$, due to a significant increase in the latency at the 10.0 mg/kg dose versus saline.
Figure 8 represents the mean latency to the first episode of immobility for the PD149163- and saline-treated mice. PD149163 treated animals did not significantly differ from their saline control counterparts F(2,21)=2.887, p>0.05.

Figure 9 represents the mean latency to the first episode of immobility for imipramine-paired and PD149163-paired control animals. The mean latencies for the two groups were not significantly different t(14)=0.863, p>0.05.

**Locomotor Assessment**

**Total distance traveled**

Figure 10 represents the mean for the total distance traveled during the open field test for imipramine- and saline-treated mice. Administration of imipramine significantly affected the total distance traveled F(2,21)=14.830, p<0.001, which was due to a significant decrease in the total distance traveled at the 10.0 mg/kg dose of imipramine compared to saline.

Figure 11 represents the mean for the total distance traveled during the open field test for PD149163- and saline-treated mice. Administration of PD149163 significantly altered the total distance traveled F(2,21)=4.733, p<0.05, due to a significant reduction in the distance traveled at the 1.0 mg/kg dose in comparison to saline.

Figure 12 represents the mean for the total distance traveled for imipramine-paired and PD149163-paired control animals. The total distance traveled for the two groups did not significantly differ t(14)=0.5033, p>0.05.
All figures represent the mean plus the standard error of the mean.

Figure 1: Imipramine: Immobility in the Final 4 Minutes

Figure 1 represents the mean for the time spent immobile in the final four minutes of the testing session for imipramine- and saline- treated mice. Treatment with 1.0 mg/kg of imipramine had no significant effect when compared to saline control animals (p>0.05). Treatment with 10.0 mg/kg of imipramine significantly reduced the time spent immobile (p<0.01) compared to saline-treated animals.
Figure 2: PD149163: Immobility in the Final 4 Minutes

Figure 2 presents the mean for the total time spent immobile in the final 4 minutes of the testing session for PD149163- and saline- treated mice. Treatment with 0.1 mg/kg of PD149163 produced a significant reduction in the time spent immobile compared to saline controls (p<0.01). Treatment with 1.0 mg/kg of PD149163 also significantly decreased the time spent immobile in comparison to saline control animals (p<0.05).
Figure 3 represents the mean for the total time spent immobile in the last four minutes of the testing session for the imipramine-paired control animals versus the PD14916-paired control animals. The mean time spent immobile for the two control groups did not significantly differ (p>0.05).
Figure 4 represents the mean for the total time spent immobile in the first 2 minutes of the testing session for imipramine- and saline- treated mice. Treatment with 1.0 mg/kg of imipramine produced a significant decrease in the time spent immobile compared to saline treatment (p<0.05). Treatment with 10.0 mg/kg of imipramine also significantly decreased the time spent immobile in comparison to saline administration (p<0.0001).
Figure 5 represents the mean for the total time spent immobile in the first 2 minutes of the testing session for PD149163- and saline- treated mice. Administration of 0.1 mg/kg of PD149163 produced a significant decrease in the time spent immobile in comparison to saline administration (p<0.01). Treatment with 1.0 mg/kg of PD149163 did not significantly alter the time spent immobile (p>0.05).
Figure 6: Comparison of Controls: Immobility in the First 2 Minutes

Figure 6 represents the mean for the total time spent immobile in the first 2 minutes of the testing session for the imipramine-paired versus PD149163-paired control animals. The mean time spent immobile for the imipramine and PD149163 paired control animals did not significantly differ (p>0.05).
Figure 7 represents the mean latency to the first episode of immobility for the imipramine- and saline-treated mice. Administration of 1.0 mg/kg of imipramine did not significantly alter the latency to the first episode of immobility (p>0.05). Treatment with 10.0 mg/kg produced a significant increase in the latency to the first episode of immobility (p<0.01).
Figure 8: PD149163: Latency to First Episode of Immobility

Figure 8 represents the mean latency to the first episode of immobility for the PD149163- and saline-treated mice. Application of 0.1 mg/kg or 1.0 mg/kg PD149163 failed to produce a significant change in the latency to the first episode of immobility versus saline administration (p>0.05).
Figure 9 represents the mean latency to the first episode of immobility for imipramine-paired and PD149163-paired control animals. The mean latencies to the first episode of immobility for the PD149163- and imipramine-paired control groups did not significantly differ (p>0.05).
Figure 10: Imipramine: Total Distance Traveled

Figure 10 represents the mean for the total distance traveled during the open field test for imipramine- and saline-treated mice. Administration of 1.0 mg/kg of imipramine did not significantly alter the total distance traveled versus saline administration (p>0.05). Treatment with 10.0 mg/kg of imipramine produced a significant decrease in the total distance traveled versus saline administration (p<0.01).
Figure 11 represents the mean for the total distance traveled during the open field test for PD149163- and saline-treated mice. Treatment with 0.1 mg/kg of PD149163 did not significantly affect the total distance traveled in comparison to saline-treated animals. Administration of 1.0 mg/kg of PD149163 produced a significant reduction in the total distance traveled versus saline control animals (p<0.05).
Figure 12: Comparison of Controls: Total Distance Traveled

Figure 12 represents the mean for the total distance traveled for imipramine-paired and PD149163-paired control animals.
DISCUSSION

In the final four minutes of the testing session, the time period analyzed in a typical forced swim test procedure, imipramine produced a significant decrease in the time spent immobile at the 10.0mg/kg dose, while failing to produce a significant decrease at the 1.0 mg/kg dose. These results are consistent with previous experiments where doses of 1.0 mg/kg failed to produce reductions in immobility while the 10.0 mg/kg dose produced consistent reductions, even after the animals were given the test substances daily for 14 consecutive days (Kitamura et al., 2004). PD149163 produced significant decreases in immobility in the final four minutes of the testing session at both the 0.1 mg/kg and 1.0 mg/kg doses. The results of this experiment provide the first report of the effects of PD149163 in the forced swim test, although previous research has demonstrated the ability of intracranial administration of the full neurotensin peptide to reduce immobility in rats (Cervo et al., 1992). The mean time spent immobile in the final four minutes of the testing session for the PD149163- and imipramine-paired saline control groups did not significantly differ, indicating that any observed differences between the treatment groups and their saline counterparts were not due to abnormalities in the controls their results were compared to.

According to the original forced swim test procedures, the first two minutes are typically excluded from analysis as animals generally display little immobility during this time period (Castagne et al., 2009). However, the present study sought to examine if differences in these first two minutes exist. Both doses of imipramine produced
significant reductions in the time spent immobile in the first two minutes, while only the 10.0 mg/kg did in the final four minutes of the session. Only the 0.1 mg/kg dose of PD149163 produced a significant decrease in immobility in the first two minutes, while both doses significantly reduced immobility in the final four minutes. These results indicate that additional inquiry may be needed into the delay to the onset of these substances, and how the therapeutic response differs with varying pretreatment times. In the first two minutes no differences were observed between the imipramine- and PD149163-paired saline controls, indicating again that any observed differences were not due to abnormalities in control group performance.

While the latency to the first episode of immobility is not a result frequently reported in a forced swim test, the present study sought to examine whether either of the test substances would cause a delay in the animals adoption of the immobile posture. If the duration of immobility in the forced swim test can be considered an index of behavioral despair, and a reduction in this behavior is an indication of therapeutic efficacy, then a protracted period of escape-related or swimming behaviors before the animal adopts the passive posture may also be a possible manifestation of antidepressant effects. Imipramine significantly increased the latency to the onset of immobility at the 10.0 mg/kg dose, while the 1.0 mg/kg dose of imipramine and both doses of PD149163 failed to produce a significant effect. However, the differences between the results from the first two minutes and the final four minutes of the testing session indicate the possibility that altering pretreatment time courses may yield different results. The latencies to the onset of the first immobility displayed by the imipramine- and PD149163-paired control mice did not differ, indicating again that any observed
differences between the treatment groups and their controls were not due to abnormalities
in the control groups.

The reasons for excluding the first two minutes of the testing session from
analysis are not adequately explained in any version of the protocol encountered during
the course of this experiment. In Porsolt’s forced swim test protocol he simply states that
animals do not spend very much time immobile in the first two minutes, and the more
stable levels of immobility in the final four minutes of the testing session provide a more
reliable baseline for making comparisons between treatment groups (Castagne et al.,
2010). One possible explanation comes from observing the species differences between
the forced swim test for rats and the forced swim test for mice. In the rat protocol as
described by Porsolt, animals are subjected to two periods of swimming and three
administrations of the test substances. The first day is used as a habituation period
wherein the animals are placed in the apparatus for 15 minutes and no behavioral
recording occurs. Following the habituation period the animals receive an injection of the
test substance. The next day, 4 hours before the testing session, the animals receive
another administration of the test substance. Finally, 30 minutes before the test, the
animals receive a third and final administration of the test substance. The testing session
lasts for a total of 5 minutes and all of the behavior is recorded. The habituation period is
performed in rats because unlike mice they perform other behaviors like diving that
significantly alter baseline immobility times. Therefore, subjecting them to the
habituation period allows for higher levels of immobility on the testing day. Excluding
the first two minutes of the testing session for mice could possibly function as a
habituation period in compensation for the lack of an actual pre-test habituation period.
On the other hand, the habituation period performed in the rat protocol has generated some criticism. This initial exposure could actually reflect a learning period, allowing the rat to learn the most efficient way to survive in this inescapable environment. In that sense, immobility is an adaptive behavioral mechanism and may not be considered an index of despair. Alternatively, an increase in immobility on the second day in the rat protocol could exemplify learned helplessness, wherein the animal has actually learned that the environment is inescapable and ceases efforts to escape (Petit-Demouliere et al., 2005). When mice are exposed to a pre-test habituation like the one performed in the typical rat protocol, the experienced animals exhibit higher levels of immobility than naïve animals when compared during a 5 minute testing session (Alcaro et al., 2002). This pre-exposure could also either be a case of adaptation, or stress induced learned helplessness. Either way, skipping the pre-test habituation session in the mouse protocol removes this possible source of influence, and may therefore provide for more stable behavior in mice. Whatever the case, it is impossible to infer any absolute evidence of human affective behavioral states from observing animal behavior. What we do know is that drugs with antidepressant effects in humans reduce the amount of time rats and mice spend immobile in this task, despite the species differences in protocols.

In the locomotor assessment, none of the drug treatments produced an increase in general locomotor behavior. This is an important fact to note, as this particular screening model is prone to producing false positives. These results indicate that the reductions in immobility observed in the forced swim test were not due to a stimulant-like effect, and may actually represent potential antidepressant effects of the test substances. The 10.0 mg/kg dose of imipramine and the 1.0 mg/kg dose of PD149163 actually produced a
decrease in the total distance traveled during the locomotor assessment. While the ability of imipramine to reduce locomotor behavior has been noted (Gutierrez-Garcia et al., 2009), one possible explanation for the observed reduction in locomotor behavior is the action of imipramine, or its metabolite desipramine, as an antagonist at the histamine H₁ receptor (Owens et al., 1997; Sugar et al., 1984). The sedative effects of tricyclic antidepressant medications are well documented and thus they are frequently prescribed as sleep aids (Plattner et al., 2011).

Previous research has shown that mice lacking the NTS₁ receptor exhibit higher levels of locomotor activity than their wild type counterparts (Li et al., 2010). This hyperactivity has been attributed to disregulation of dopamine transmission in the striatum as a result of the deletion of NTS₁ receptor. In wild type mice, administration of neurotensin or neurotensin analog drugs such as PD149163 effectively block increases in locomotor behavior in response to stimulant treatment (Kalivas et al., 1984; Feifel et al., 2008), an effect that is thought to be due to possible interactions between the NTS₁ and D₂ receptors (Tanganelli et al., 1993; Fuxe et al., 1992; Li et al., 1995). Although it is unclear precisely how neurotensin modulates locomotor behavior under normal circumstances, the possibility for influences exist through its influence on mesolimbic dopaminergic functioning, and input from the nucleus accumbens to cortical glutamatergic neurons and their input to feedback loops returning to midbrain dopamine producing nuclei (Antonelli et al., 2002).

This study has provided the fist indication that the neurotensin analog drug PD149163 exhibits antidepressant-like effects in the forced swim test. Additional
research into the time course, and the effects of this compound at varying doses will provide more insight into the potential to use PD149163 as a novel antidepressant drug. These findings, coupled with the other evidence gathered thus far into the therapeutic potential of the neurotensin system indicate that neurotensin analog drugs like PD149163 may have the potential to alleviate the symptoms of depression in human patients.
REFERENCES


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Schwartz, T., (2013), A neuroscientific update on monoamine oxidase and its inhibitors. *CNS Spectrums*, 00, pp. 4-12.


SIGNATURE PAGE
IACUC #: 227  PROPOSAL TITLE (From cover page): PD149163 and Motivation Effects

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:  
Principal Investigator  06/02/2014

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:  
Department Head  06/09/2014

Date

XII. REVIEWED AND APPROVED BY IACUC REVIEWERS

Signature:  
Institutional Animal Care and Use Committee Chair  06/09/2014

Date

Signature:  
Institutional Animal Care and Use Officer  06/09/2014

Date

Following action on this application, copies of approval or denial letters will be sent to the applicant, Department Head, and appropriate College Dean who will also receive a copy of this application.

IACUC Application · Revised September 16, 2010
## APPENDIX B

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<td>2I12</td>
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