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The Role of Neurotensin Receptors on Visceral Pain and Activity Levels in Mice.

Christopher J. Walker

University of North Florida

The Role of Neurotensin Receptors on Visceral Pain and Activity Levels in Mice.

By

Christopher James Walker

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This Thesis titled The Role of Neurotensin Receptors on Visceral Pain and Activity Levels in Mice is approved:

_____ _____

Dr. Lori Lange Committee Chair

_____ _____

Dr. John Fryer Committee Member

Accepted for the Psychology Department:

_____ _____

Dr. Micheal Toglia Department Chair

Accepted for the College of Arts and Sciences:

_____ _____

Dr. Barbara A. Hetrick
Dean of the College of Arts and Sciences

Accepted for the University:

_____ _____

Dr. John Kantner
Dean of the Graduate School

DEDICATION

To my loving wife Stephanie.
Without her I would have never been able to accomplish so much.
She is my love and my life.

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ABSTRACT

This study examines the effects of neurotensin (NT) receptor sites on the sensation of visceral pain. Previous work by researchers has found, through the use of NT analogs, that visceral pain is closely associated with NT receptor 2 (NTSR2). This study tested 70 genetically modified mice. The mice were either missing NTSR1, NTSR2, or were wild-type (WT) mice that were not missing any NT receptors. The mice were injected intraperitoneally with either saline or acetic acid then observed for a 60 minute period and writhing behavior was recorded. Twenty four hours later activity levels were recorded in the open field assay. We found that contrary to previous research, NTSR2 is not solely responsible in the sensation of visceral pain. We also found that NTSR1 plays a more significant role than NTSR2, contrary to previous research. Additionally, we found that the NT receptors may be affected by age related factors. The findings of this study suggest that NTSR2 does in fact play a role in the sensation of visceral pain but that NTSR1 may modulate the degree of activation of NTSR2. It can also be concluded that age may have a role in the effectiveness of NTSR sites in visceral pain. This information allows for further research to analyze possible age-dependent effects of NT receptor sites that could alter the possible usefulness of NT analogues in the future.

The Role of Neurotensin Receptors on Visceral Pain and Activity Levels in Mice.

Dr. Robert Carraway and Dr. Susan Leeman (1973) first discovered neurotensin from a bovine hypothalamic extract, which also identified substance P. Through the use of chromatography, Carraway and Leeman (1973) found that neurotensin is a tridecapeptide that is composed of Glu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu. A tridecapeptide is defined as peptide consisting of 13 amino acids. Since the discovery of neurotensin (NT), it has been found in not only the central nervous system (CNS) but also in several peripheral areas of the body, such as the gastro-intestinal tract (GI) (Kitabgi, Carraway, and Leeman, 1976). Because NT is found in several tissues, it has been suggested to serve multiple purposes throughout the body. In the CNS is has been found to have hypothermic qualities as well as antinociception or the reduction a sensitivity to pain (Tyler-McManhon, Boules, Richelson, 2000). In regards to NT's effects on the GI tract, NT has been shown to stimulate growth in the small and large bowel as well as inhibit small bowl motility in animal models (Anderson et al, 1977; Evers et al., 1992). Research has shown varying applications of NT, such as use in therapy for neuropsychiatric disorders and controlling growth of cancerous cells in the digestive tract (Boules et al., 2013).

In order to better understand the multitude of potential applications of NT, it is necessary to better understand the processes through which NT signaling is mediated throughout the body. The most important feature of NT is that the 8-13 a.a. fragment of the peptide is all that is necessary for biological activation (Tyler-McMahon, et al., 2000). All four neurotensin receptors (NTSR), NTSR1, NTSR2, NTSR3, and NTSR4, have an affinity for the C-terminal 8-13 a.a. fragment of NT (Tyler-McMahon, et al., 2000; Boules et al, 2013). Differences in affinity and NTSR location accounts for the variety of functions that NT serves throughout the body.

NTSR1 is the most studied of the four different receptor sites (Boules et al, 2013). NTSR1 is co-localized with dopaminergic neurons in the ventral mesencephalon, which may be related to sensitization to drugs (Boules et al, 2013). It is important to note that the four different receptors are categorized as either high or low affinity. High affinity receptors such as NTSR1 are sensitive to Na^+ ions and Guanosine-5'-triphosphate (GTP), which lowers sensitivity of the receptor site to NT (Vincent, Mazella, Kitabgi, 1999). Much of the recent research conducted on the four types of NT receptors are related to the use of either NT analogs that have a higher affinity for certain receptors or through the use of mice that have had, through genetic alteration, one of their receptors removed (Boules et al, 2013). Recent work using NTSR1 knockout mice ($NTSR1^{-/-}$), whose NTSR1 receptors have been removed, has found that these mice show greater hyperactivity and have a greater response to amphetamine-induced hyperactivity (Boules et al., 2010). Additionally, this research shows that $NTSR1^{-/-}$ mice have a greater amphetamine induced dopamine release than control mice (Liang et al., 2010). NTSR1 was also shown to promote endogenous glutamate signaling in the brain (Boules et al, 2013). It is important to note that NTSR1 is found throughout the CNS and is found in both neurons and glial cells (Elde et al., 1990).

In contrast to NTSR1, NTSR2 is relatively localized. NTSR2 is found primarily in the olfactory system, the cerebral and cerebellar cortices, and specific hypothalamic nuclei (Boules et al, 2013). NTSR2 is considered a low affinity receptor and is less sensitive to Na^+ ions and is not affected by GTP at all (Vincent, Mazella, Kitabgi, 1999). NTSR2 is also the receptor that is most closely associated with pain due to its presence in the periqueductal gray matter and the rostral ventrolateral medulla (Boules et al, 2013). When NT is injected into the previously mentioned areas, opioid-independent analgesia is produced (Behbehani, 1992). Recent research

using an NTSR2 selective agonist along with NTSR2 knockout mice ($NTSR2^{-/-}$) has shown a decrease in analgesia, which solidifies NTSR2's role in pain (Remaury et al., 2002; Boules, 2010). It is important to note that although relatively similar, NTSR1 and NTSR2 are structurally different which leads to differences in affinity for the Na^{+} ion (Vincent et al., 1999).

NTSR3 is unrelated to NTSR1 or NTSR2 structurally and is found throughout the brain (Boules et al., 2013). NTSR3 has an association with organelles like the Golgi apparatus and glucose transporter vesicles (Sarret et al., 2003). NTSR3 is thought to be an integral part of cell death due to its ability to bind with unprocessed nerve growth factor (Nykjaer et al., 2004). Additionally, when NT binds with NTSR3 there is a modular effect on NTSR1. The response elicits growth in certain forms of cancer cells, such as colon cancer cells (Martin et al., 2002; Dal Farra et al., 2001). However, NTSR3 is still vastly understudied as compared to the other NT receptors. To better understand NTSR3 and its full effects on the body, further research is required.

The final type of NT receptor is NTSR4. This NT receptor site is the most recently discovered NT receptor and is similar to NTSR3 in structure (Boules et al., 2013). NTSR4 is found throughout the brain, but is primarily located in the hippocampus and cerebellum (Motoi et al., 1999). Currently, it is thought that NTSR4 is related to intracellular trafficking and the termination of NT signals, but little else is known about NTS4 (Jacobsen et al., 2001).

The purpose of explaining the affinities and locations of the NT receptors is to explain the varying applications of NT. Due to either location or affinity, NT may have an effect on cancerous cell growth or be used as an anti-psychotic (Boules et al., 2013). Location can also demonstrate the relation between NT and many other neurotransmitters and hormones. In regards to the hypothalmic-pituitary-adrenal (HPA) hormone system, the amount of NT administered

appears to determine the effect on the system whether it be excitatory or inhibitory (Malendowicz and Nussdorfer, 1994). If a lower dose of NT is administered to the paraventricular nucleus, an area in the hypothalamus associated with hormone excretion, NT appears to have a stimulatory effect where as the opposite occurs when a higher dose is used in the same region (Malendowicz and Nussdorfer, 1994). Moreover, NTSR1 is related to a hormone known as the gonadotropic-releasing hormone and seems to allow for NT to act as a modulator of other neurotransmitter systems (Rakovska et al., 1998).

Arguably, NT's most co-localized neurotransmitter is dopamine (DA). This co-localization of dopamine is not for one specific area that dopamine influences but rather the entire dopomanergic system (Tyler-McMahon et al., 2000). Several studies have looked into NT's influence on DA, including several on central nervous system (CNS) disorders, such as Parkinson's disease and schizophrenia. These disorders have been researched because of the interaction between NT and DA in the nigrostraital and mesocortical pathways, which are pathways in the brain associated with these disorders (Tyler-McMahon et al., 2000). NT is primarily a modulator for DA in two ways: by regulating tyrosine hydroxylase TH gene expression and by decreasing DA binding affinity for D_2 receptors (Burgevin et al, 1992; Fuxe et al., 1992; Li et al., 1995). NT receptors can either increase or decrease DA transmission depending on whether the receptors are on the pre- or post- synaptic terminal (Boules et al., 2013). NT counters the effects of DA meaning that if the NT receptors are on the pre-synaptic terminal, the NT receptor facilitates DA transmission by hindering reuptake, and thus making the transmission more potent (Boules et al., 2013). The opposite is true if the NT receptors are on the post-synaptic terminal because NT receptors on the post-synaptic side hinder propagation of the DA signal.

NT also has an effect on several other neurotransmitters such as serotonin and glutamate (Boules et al., 2013). NT is present in serotonergic neurons and causes an increase in firing rates and also increases the release of glutamate in other areas, such as the frontal cortex and the striatum (Ferraro et al, 2011, 2012; Jolas and Aghajanian, 1996; Li et al., 2001). Because NT is related to so many neurotransmitters, NT has been linked to many possible applications. The primary applications that are covered include NT being used as an anti-psychotic for schizophrenia, NT being used for possible psychostimulant, NT's use in Parkinson's disease, and lastly NT's relation to pain and its synergistic qualities with opioids.

The first application to be discussed is the use a NT as a possible anti-psychotic for schizophrenia. The disorder effects roughly 1 percent of the global population and causes both positive (delusions and hallucinations) as well as negative (social withdrawal and cognitive blunting) symptoms (Boules et al., 2013). One hypothesis about the origin of symptoms of schizophrenia posits that these symptoms originate in the dopaminergic system (Boules et al., 2013). NT receptors are found on DA neurons in the ventral tegmental area of the brain, among several other regions that are closely linked with the dopamine system in the brain (Tyler-McMahon, 2000). Studies have found that individuals with schizophrenia have less NT receptors and have decreased levels of NT in their cerebrospinal fluid (Sharma et al., 1997; Wolf et al., 1995). NT receptors have been shown to have an excitatory effect on DA release, increase the firing rate, and increase the rate of DA synthesis (Jomphe et al., 2006). The close association between NT and its apparent ability to moderate DA synthesis and transmission has led to the hypothesis that NT could be used as a possible treatment for schizophrenia.

There are primarily four avenues of research that suggest that NT can be used as a form of treatment for schizophrenia. First, effective antipsychotics have shown alterations in the NT

systems of rats (Boules et al., 2013). Second, certain anti-psychotics have a tendency to increase NT mRNA after treatment (Boules et al., 2013). Third, both typical and atypical antipsychotics effect the mesolimbic and nigro-striatal NT systems in contrasting ways (Boules et al., 2013). Lastly, when NT is administered directly to the CNS, it evokes similar behaviors when atypical antipsychotics are administered centrally (Boules et al., 2013). These rationales each contribute the notion that NT is involved in the process of schizophrenia.

NT has also been associated with certain psychostimulant effects because of its properties as a modulator of DA neurotransmission. When NT is directly injected into the ventral tegmental area there is hyperactivity and DA is released in the nucleus accumbens (NA) area of the brain, which produces a reaction similar to the effects of psychostimulants (Kalivas and Duffy, 1990; Kalivas, 1994). NT has also been shown to reduce the effects of a psychostimulant, such as nicotine or cocaine when NT is administered to the NA (Boules et al., 2013). Specifically, a NT agonist known as NT69L has been shown to block the acute locomotor effects associated with cocaine and d-amphetamine (Boules et al., 2001). This agonist has also been shown to block sensitization to nicotine, which suggests that the use of this agonist could help with certain forms of addiction treatment (Fredrickson et al., 2003). It is important to note that agonists of NT, such as NT69L, do not show the same addictive qualities of other antipsychotics even though the effects are similar (Fantegrossi et al., 2005). This quality makes these agonists good candidates for treatment because they do not have the potential to become addictive (Fantegrossi et al., 2005).

NT has been shown to have effects on Parkinsonian patients. As previously mentioned, NT is elevated in the plasma of patients with Parkinson's disease compared to unaffected individuals or patients that are not treated with levodopa (Schimpff et al., 2001). Autopsies

preformed on patients with Parkinson's disease have shown that individuals with the disease have fewer dopaminergic neurons as well as fewer NTSR1 receptors (Yamada and Richelson, 1995). The higher concentrations of NT in tissue, CSF, and plasma is a possible reactionary method the body uses as an attempt to compensate for loss of motor function or alternative pathways being used as the original pathways are destroyed (Caceda et al., 2006). It is important to note that when injected NT results in muscular rigidity and tremors related to the dose of NT when injected bilaterally in the medial forebrain bundle (Jolicoeur et al., 1991). This research suggests there is an association between NT and Parkinson's. However, to what degree and by what means is relatively unknown.

Arguably the most prolifically studied quality is NT's ability as an analgesic as well as its synergistic qualities with opiates. This paper's focus is as on the analgesic qualities of NT. In order to properly understand the analgesic qualities of NT, it is necessary to explain the physiology behind pain.

The Institute of Medicine has recently stated that roughly one hundred million adults are afflicted by chronic pain annually in America. This costs between 560 - 635 billion dollars annually in disability, lost wages, and decreased productivity (Institute of Medicine (US) Committee on Advancing Pain Research, Care, and Education, 2011). Chronic pain is just one subset of a larger portion of research involving pain and pain management. Pain is a negative feeling that is associated with a specific point in the body (Vanderah, 2007). This negative feeling is associated with damage or potential damage to the body, which is referred to as "noxious"(Vanderah, 2007). This noxious stimulus is picked up by receptors referred to as nociceptors (Vanderah, 2007). These nociceptor nerves have cell bodies in the dorsal root ganglia and terminate in the spinal cord (Vanderah, 2007).

NT may be involved in this process, by way of the descending modulator system, in which opioid receptors in the periaqueductal gray region of the midbrain modulate neural firing (Vanderah, 2007). The descending pain modulator system uses neurons that release serotonin or norepinephrine (Vanderah, 2007). Serotonin and norepinephrine interact with opioid-containing interneurons in the spinal dorsal horn that release opioid peptides (Vanderah, 2006). These peptides either directly inhibit the release of pain transmitters from afferent nociceptive signals or inhibit second order pain transmission cells (Vanderah, 2006). Second order pain transmission cells are cells that transmit the noxious signal from the peripheral system to the spinal cord and then the thalamus (Vanderah, 2006). By releasing the opioid peptides, the signal never reaches the thalamus to be processed as pain. The presence of opioid receptors further solidifies NT's synergistic qualities with opioid-based analgesics.

The most common method of antinociception related to opioid peptides is morphine. The problem with such methods is that the use of opioids traditionally comes with the development of tolerance to such treatment (Boules et al., 2014). With this tolerance comes increased dosages of opioids; a point is then reached where the negative side effects such as hypothermia begin to manifest (Boules et al., 2014). Currently, NT has also shown that it has an effect in multiple areas of the descending pain modularity system. Research has shown that NT has an analgesic effect if administered centrally (Boules et al., 2013). It also has a long-term analgesic affect if injected into the rostroventral medulla (Boules et al., 2013). NT, when used concurrently with traditional opioids, allows for lower doses of opioids needed to reach intended analgesia (Boules et al., 2014).

It is important to differentiate the roles that NT receptors have with pain because there are differences between the category of pain and the NT receptor that the pain is associated with

(Boules et al., 2013). This information has been determined primarily from using NT analogs that have higher affinities for specific NT receptors and through the use of genetically altered mice with certain NT receptors removed (Smith et al., 2012). Research using several NTSR1 agonists has found that NTSR1 may be involved in the modulation of thermal pain as well as long-term persistent pain (Boules, et al., 2013). Through similar means it has been concluded that NTSR2 receptors modulates visceral pain, the paradigm that was chosen for this study (Boules et al., 2013).

It is important to note that current research has only used NT analogs to assess the association of NT receptors and certain forms of pain. The purpose of this study was to test mice that have been genetically altered to have their NT receptors deleted, ($NTSR1^{-/-}$ or $NTSR2^{-/-}$ mice). This is significant because past research has been limited by the effectiveness of NT analogs. By studying the effects of NT receptors on visceral pain, the data gathered are directly associated with either the presence or absence of NT receptors and are not contingent upon the effectiveness of a drug or possible off target or side effects. Overall, this study was desinged to provide a more comprehensive understanding of NT receptors and their association with pain.

Focus of Current Study

As previously mentioned, the purpose of the study was to investigate the interaction between NT receptors and visceral pain. The majority of research that has been conducted involves the use of NT analogs due to NT's unstable nature. Because NT is a neuropeptide, if it is injected peripherally in the body, it will break down from peptidases before it can reach any NT receptors in the CNS (Smith et al., 2012). These analogs are more stable derivatives of the 8-13 a.a. of NT, which is the only part of NT that is necessary for biological activation (Vincent et al., 1999). These NT analogs, due to the chemical composition, have for the most part higher affinity

for either NTSR1 or NTSR2. By testing responses in mice that have been injected with one of these analogs it is possible to investigate the effects of either NTSR1 or NTSR2, depending on the affinity of the compound.

There are many analogs being studied but, for the focus of this study, the primary NT analogs involved are PD149163, NT69L, and NT79. PD149163 is a NTSR1 selective agonist that has been shown to reduce inflammatory-based pain brought on by formalin injection. The procedure for a common formalin test is to inject formalin into the front paw to measure nociception, or the ability to sense pain, in rodents. This injection causes moderate continuous pain in rodents and is measured by paw lifts. It can be inferred that NTSR1 is involved in the induction of inflammatory pain because pain is reduced in reaction to an NTSR1 agonist (Roussy et al., 2008).

NT69L is a nonselective NT agonist that has also been shown to demonstrate analgesia in formalin tests, thermal pain tests, and visceral pain tests (Roussy et al., 2008; Smith et al., 2012; Tyler et al., 1998). However, NT69L is non-selective and activates NTSR1 and NTSR2, and as a result, the specific NT receptor cannot be associated with specific forms of pain. NT79 is a NTSR2 selective agonist that does not reduce thermal pain; however, it has been shown to diminish acetic acid-induced writhing and formalin-induced pain (Boules et al., 2010; Boules et al., 2011). Additionally, NT79 does not produce the negative effects associated with other analogues, such as PD149163 and NT69L. These negative side effects include hypothermia and hypotension, which suggest that these negative side effects are associated with the activation of NTSR1 but not the activation of NTS2 (Boules et al., 2013). Because of the negative side effects associated with NTSR1, the focus of this study is on the ability NT79 to lessen acetic acid induced writhing and its connection with NTS2.

It is also important to note that in previous studies the mice were 45 days old; therefore, age related differences were not taken into account when it came to NT receptors effects on visceral pain (Smith et al., 2012). This study uses two cohorts of mice: one group being approximately 18 months or older (referred to as the “old” group) and the second group being approximately 5 to 6 months in age (referred to as the “young” group) in order to provide a more comprehensive understanding of NT receptors and their relationship to visceral pain.

Hypotheses

There are three hypotheses of the study. One hypothesis is that mice that have an acetic acid injection will exhibit a higher index of visceral pain measured by writhing behavior (Smith et al., 2012). Another is that mice lacking NTSR2 will experience less visceral pain as measured by less writhing behavior than unaltered mice referred to as wild type (WT) or compared to NTSR1 knockout mice (Boules et al., 2011). Mice that exhibit more writhing will also exhibit lower involuntary activity levels i.e. movement in a one-hour period (Cattaruzza et al., 2013).

Method

The study is a two by two design in which the independent variables are the presence of NT receptor sites and the use of either saline or acetic acid in the IP injections. The dependent variables are the visceral pain as measured by writhes and activity levels. Within group variability was analyzed and considered negligible. The first portion of the study we examined visceral pain on mice that have been genetically altered so that certain receptors are absent. By testing pain on mice who have had their receptors knocked-out, it will allow for a better understanding of what types of pain are associated with different receptors. Determining which types of pain are associated with the different receptors could provide a more accurate treatment of pain by using analogs that have higher affinities for different receptors. "Visceral Pain" was

determined by measurement of writhes in a five-minute segment over a one-hour period. Writhes were defined as the extension of at least one front and back paw paired with abdominal stretching. A writhe was measured as either full point or no point at all. A scaled format was not used for this study.

Procedures

Animals

Male mice were divided into two groups, one being the young group which contained mice approximately 5 to 6 months in age, and the old group which contained mice approximately 18 months in age. Each of the two age groups contained 35 mice that were categorized into three genetic varieties of mice: wild type (WT), NTSR1 knockout ($NTSR1^{-/-}$), and NTSR2 knockout mice ($NTS2^{-/-}$). The animals were held in climate controlled housing ($23\pm2^{o}C$) and were allowed free access to food and water and had a 12 hour light / dark cycle. The animals tested were approved and held to the standards of the Mayo Institutional Animal Care and Use Committee as well as National Institute of Heath laboratory animal use guidelines. The $NTS1^{-/-}$ and $NTS2^{-/-}$ mice were established genetically at Roche (Palo Alto, CA, USA) and/or at The Mayo Clinic (Rochester, MN, USA).

Acetic acid-induced writhing

All injections were made intraperitoneally (ip) and volumes injected were based off of standards set according to weight and age set by the Institutional Animal Care and Use Committee. Intraperitoneally is an injection into the peritoneum, the cavity in the abdomen between the lower abdominal organs and the abdominal wall. .1 milliliters of either saline or acetic acid was given to the 18 month old mice and 0.08 milliliters were given to the 5 to 6 month old mice due to the young mice weighting less than their older counterparts. The mice

were marked on their tails in order to ensure accuracy in later measures. The mice were then placed in an enclosure, similar in design to their housing, and were observed for a one hour period. Writhing was defined as abdominal stretching accompanied with extension of either one or both hind legs and was quantified in an all or nothing scale. Either an action counted as a writhe or it was not counted as a writhe (not a graded scale). Writhes were recorded in five-minute increments to allow for accuracy as well as a time course if necessary. Once the one-hour period had elapsed, the mice were promptly returned to their home cage.

Activity measures

Twenty-four hours after the IP injection of either saline or acetic acid the activity levels of the mice were recorded for a one-hour period. Four mice were run simultaneously in order to ensure that activity measures were recorded as close to twenty-four hours after the initial induction of stress. Activity levels were recorded by way of a Plexiglas Opto-Varimex Minor motility chamber (Columbus Instruments, Columbus, OH). Activity was recorded by a computer in centimeters per hour. Activity was registered by use of an infrared grid system in which movement was registered by way of the lasers being broken. Fecal samples were gathered from the chamber for later analysis. Once the samples were gathered and the activity recorded, the mice were then perfused, in which blood was drained from their tissue and replaced with saline, for later studies.

Data

Figure 1 displays the writhing data for the young mice cohort and Figure 2 displays the writhing data for the older mice cohort. In each of the figures an (*) indicates a p value < 0.05, (**) indicates a p value < 0.01, (***) indicates a p value < 0.001, and (****) indicates a p value < 0.0001. The data were analyzed by ANOVA with a post-hoc Fisher's t-test. The populations

for all groups in the older cohort contained six mice for each group, except for $NTS2^{-/-}$ acetic acid group which contained eight. In regards to the populations for the younger cohort, due to limited access to rodents, the populations were different for each of the groups and are as follows: Saline WT n=6, Saline $NTSR1^{-/-}$ n=5, Saline $NTSR2^{-/-}$ n=6, AA WT n=7, AA $NTSR1^{-/-}$ n=5, AA $NTSR2^{-/-}$ n=6. Because of this unequal distribution of the young cohort among the groups along with a one month delay between groups, across cohort analysis was not possible.

For the younger cohort, there was more variability among the controls as seen in Figure 1 but not to the degree that it confounds the data. In Figure 1 it was evident that in the younger cohort there seems to be an alternate effect on the amount of writhing because the $NTSR2^{-/-}$ group had the most amount of writhes. There was a significant difference between $NTSR2^{-/-}$ and the WT group (p value < 0.01). There was also a significant difference between the $NTSR1^{-/-}$ and the $NTSR2^{-/-}$ groups (p value < 0.001). It is important to note that there is no significant difference between $NTSR1^{-/-}$ and the WT group.

For the older cohort there was a significant difference between the wild type group (WT) and the $NTSR1^{-/-}$ group (p value < 0.0001) as well as the $NTS2^{-/-}$ group (p value <0.01). When looking at the controls for each of the subsets for each of the group, there are no significant differences. This means that the writhing differences in the acetic acid groups are due to the differences in the NT receptor and not pre-existing group differences. Of the acetic acid group, the least amount of writing was observed in the $NTSR1^{-/-}$ group, followed by the $NTSR2^{-/-}$ group. As expected, the group with the most writhing was the WT group.

Activity levels were gathered 24 hours after the initial injections. Although young WT mice had a significant amount of visceral pain (Figure 1), no lasting effects on activity were observed nor were any differences observed in $NTSR1^{-/-}$ or $NTSR2^{-/-}$ mice (Figure 3). However,

old WT mice that had experienced visceral pain also had a lasting effect on activity (Figure 4). Additionally, there were significant differences between the saline and the acetic acid groups for the old and young cohorts. The saline groups were consistent in writhes, which indicates the validity of saline as a control. In regards to activity levels, there was no significant difference in activity between the acetic acid and the saline groups in the young cohort as seen in Figure 3. There was some internal differences in activity level of the older cohort but no significant differences between the acetic acid and saline groups as seen in Figure 4.

Discussion

In both young and old mice, there were significant differences in writhing between the acetic acid and the saline groups. This finding is to be expected and is similar with all previous research found on visceral pain induced by acetic acid (Smith et al., 2011). This finding confirms the hypothesis of the study, which states that mice that have been injected with acetic acid would exhibit more writhing behavior.

The hypothesis was confirmed that the acetic acid induced more writhing in both age groups than the saline injection. The confirmation of this hypothesis verifies that the IP injections were made correctly and that the quantification of the writhes was also done properly. The second hypothesis was not fully confirmed. We found that when either NTSR1 or NTSR2 were not present, there were significantly less writhes. This confirms previous research that presumes that NTSR1 and NTSR2 are associated with visceral pain. The data also shows that NTSR1's absence has a greater affect on writhes than NTSR2's absence. This is contrary to hypothesis two. This corresponds with previous research that shows there is modular effect between NTSR1 and NTSR2 when it comes to pain. It would appear as though previous work by Smith and collogues (2012) is correct in that NTSR1 may in fact regulate signaling of

NTSR2. These findings suggest that when analyzing the analgesic properties of NT receptor sites, it would appear that it is necessary to consider the interactive properties of these receptors. Previous research has suggested that focusing on the potential analgesic properties of NTSR2 only may be more beneficial because NTSR1 is associated with hypothermia and hypotension (Boules et al., 2013). However with our research it would appear as though focusing solely on NTSR2 may not allow for the consideration of the potential interaction of NTSR1 and NTSR2. That being the case, NT analogs such as NT69L may need to be reevaluated due to its affinity for both NTSR1 and NTSR2. Further research could examine the possibility of NT analogues that react with NTSR1 in more specific areas that are not associated with hypothermia or hypotension but still modulate NTSR2.

In regards to hypothesis two, the hypothesis is only partially confirmed for the older mice. There is a significant difference between the NTSR2-/- mice treated with acetic acid and the WT group because NTSR2-/- mice writhed less than the WT group. Also, the NTSR1-/- mice, the one in which NTSR1 is absent, writhed significantly less than the WT group and *also* writhed less than the NTSR2-/- mice. This may be explained by what previous researchers have found that both NTSR1 and NTSR2 play a role in analgesia. This concept is supported by research found by Smith et al that suggest NTSR1 having a mediating role of NTSR2's analgesic properties (Smith et al., 2012). Therefore, in this case it may be that NTSR1 plays a larger role in visceral pain (Boules et al., 2013). To summarize, in old mice, the mice lacking either NTSR1 or NTSR2 had significantly less writhes than the WT group (with NTSR1 and NTSR2 present). Additionally, NTSR2-/- mice writhed more than NTSR1-/- mice which denotes that the hypothesis was not fully confirmed. In regards to application, NT analogs that have previously been found to activate both NTSR1 and NTSR2 may have been considered less viable due to the

negative reactions associated with NTSR1 may have merit for their usefulness as analgesics as suggested in previous research by Boules et al. (2013).

The third hypothesis was not confirmed; in actuality, the opposite was supported by our data. The activity levels were highest in WT treated with acetic acid, which also had the most recorded writhes of any of the groups tested in the older population. There was no significant difference in activity levels in the younger population. There are two possible explanations for these results. The research by Cattaruzza et al., (2013) looked at the affects of visceral pain and activity levels found that voluntary wheel running decreased which was categorized as "activity". This differs from our study because we measured activity in centimeters of *all* movement in a one hour period when placed in a novel open field environment. Another explanation may be that in Cattaruzza's (2013) study the visceral pain inflicted was through pancreatic inflammation and was chronic. In our study, the viscerally induced pain was acute, lasting for roughly one hour. Both of these reasons could be explanations for the differences in results. It may be that acute pain does not carry over a twenty-four hour period to the same extent of chronic visceral pain.

In regards to activity levels, the data are inconclusive when referring to pain having an effect on activity levels in that higher pain levels were not associated with any changes in activity levels. There does appear to be an age related effect on activity level and pain in that the younger population appears to have higher activity level in general. These data differs from previous research but the explanation may be that "activity" is measured in many different ways and in some cases activity is more voluntary rather than direct movement which was measured in this study.

There was a degree of difference between the young and the old cohort. Unfortunately, due to constrains in the populations of the mice and that there was a 30 day gap in time between the old and the young cohort cross cohort analysis is not possible. There does seem to be an indication of an age affect taking place. As seen in Figure 1 as compared to Figure 2, the absence of NTSR2 seems to have a different effect on the young mice that on the old mice. The potential for this effect will be discussed further in the future research section.

Limitations

There are several limitations to this study including population limitations and possible compensatory effects of genetic alternation in mice. The first and major limitation is the overall population size. Due to situations within the institution there was a limited population of WT, NTSR1, and NTSR2 mice available for the young and old cohorts. There was intra-group reliability within the controls, so the limited population did not hinder the data to such a degree as to make the data unusable.

There are several problems related to using genetically modified strains of mice for studies. The problem relates primarily to the use of KO mice, which are mice that have had their genes altered. In our study, receptor sites have been removed from the mice through genetic alteration. As a result of the breeding process for these mice, it is possible for "the flanking gene problem" to occur (Wolfer, Crusio, Lipp, 2012). This occurs when the alleles next to the altered gene(s) are also affected and could pose a potential confound to the study (Wolfer, Crusio, Lipp, 2012). Another problem that can occur is that KO mice can have altered developmental compensation of alternative genetic pathways. For example, knockout mice for Agouti Related Protein (AgRP-KO mice) are relatively normal but acute removal of AgRP in adult mice using Cre/Lox technology results in immediately lethality (Luquet et al, 2005).

Implications

One of the most exciting findings from our study is that there may in fact be an age-dependent effect for NT receptors, a result that has not been previously described. There were differences between the acetic acid groups for both age cohorts, but the effect is greater in the older mice. This could be for any number of reasons, from the development of NT receptors to how pain is processed in older populations. Additionally, the writhing for the NTSR1$^{-/-}$ mice for the older cohort and WT young cohort were similar. Moreover, the findings of this study show that NTSR1 does appear to have an effect on visceral pain in this paradigm but it may also be modulated by NTSR2. Age may also be a factor in the degree to which NTSR1 and NTSR2 are involved in the transmission of visceral pain.

Further Research

Several aspects of this study could benefit from further research. First, the age factor could be further researched by examining how the age of the mice affects the influence of NTSR1 and NTSR2 on visceral pain. Potential studies could include longitudinal studies in which mice are observed as they age or observing two age cohorts within a shorter time span to account for temporal variations. Second, the influence of more recently described NT receptors such as NTS3 and NTS4 and their involvement in visceral pain could be further studied. Third, by using the same genetically altered mice employed by this study, we could analyze NT receptor effects on other forms of pain, such as thermal or pressure based pain. Lastly, further researchers could look into whether NT receptors have any relation to activity level in rodent models and whether age is a dependent factor.

Figure 1 Young Cohort Writhing

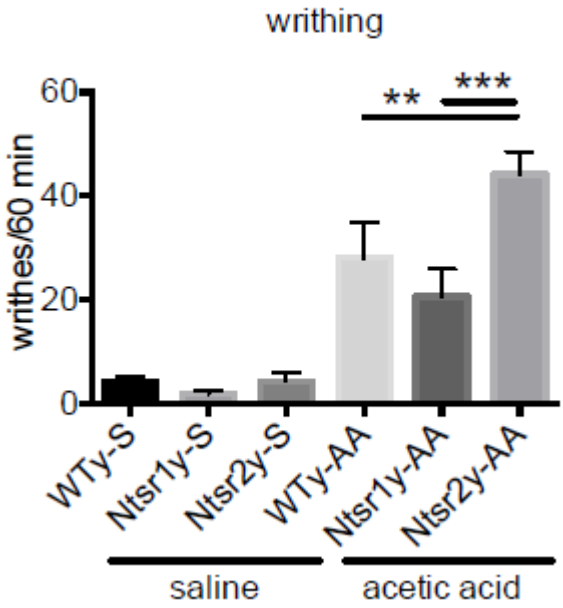


Figure 2 Old Cohort Writhing

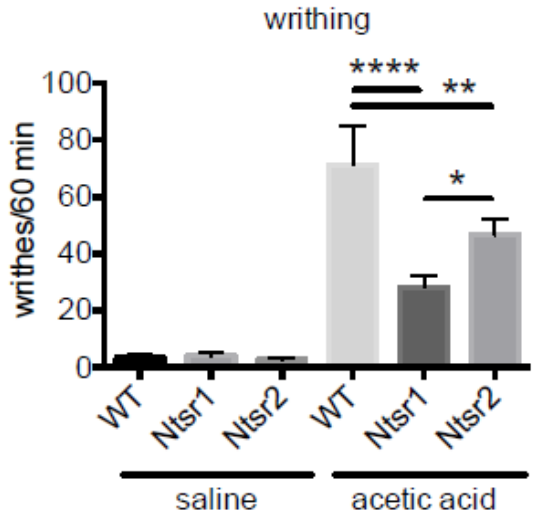


Figure 3 Young Cohort Activity

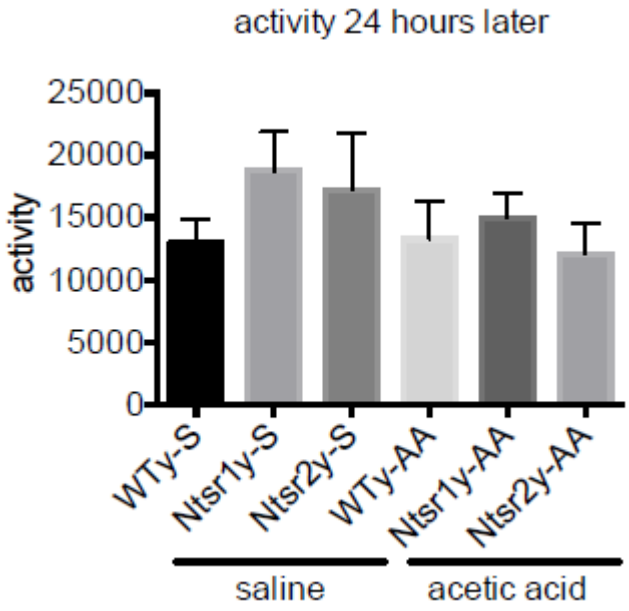
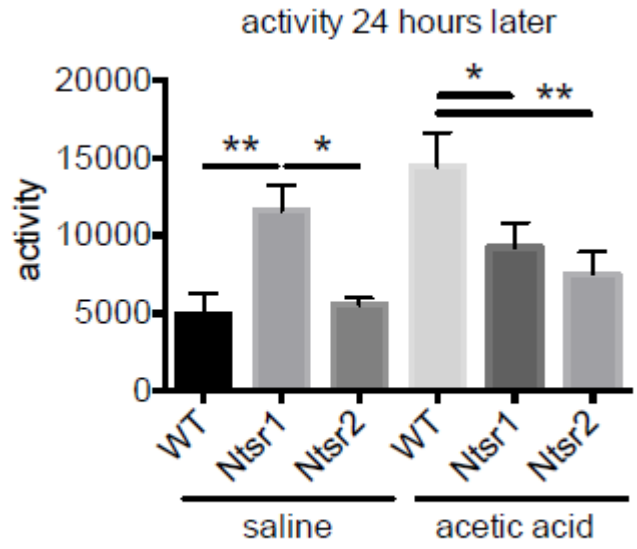


Figure 4 Old Cohort Activity



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