Published in final edited form as:

Behav Brain Res. 2011 October 31; 224(2): 344–349. doi:10.1016/j.bbr.2011.06.014.

The role of NTS2 in the development of tolerance to NT69L in mouse models for hypothermia and thermal analgesia

Kristin E. Smith, Mona Boules, Katrina Williams, Abdul H. Fauq, and Elliott Richelson*
Mayo Clinic, 4500 San Pablo Road, Jacksonville, FL, 32224

Abstract

NT69L is a neurotensin (NT)(8–13) analog that binds the two major NT receptors, NTS1 and NTS2, and elicits similar behavioral effects as endogenous NT. Tolerance develops rapidly to some, but not to all of NT69L's effects, and to date, little is known about the mechanisms responsible for this tolerance. The development of tolerance appears to be more prevalent in behavioral effects mediated by NTS1 than by those mediated by NTS2, including hypothermia and thermal analgesia. However, we hypothesize that both NTS1 and NTS2 have important roles in mediating the effects of NT69L. Here we investigate the role of NTS2 on NT69L-mediated hypothermia and thermal analgesia with the use of NTS2 knock-out mice. We show that tolerance develops to NT69L-mediated hypothermia and thermal analgesia following sub-chronic treatment in wild-type mice, and that NTS2 is necessary for the development of that tolerance. Additionally, we suggest potential means by which NTS2 influences these NT69L-mediated behaviors.

Keywords

tolerance; neurotensin; receptor; regulation; NT69L; analgesia

1. Introduction

Neurotensin (NT¹) is an endogenous tridecapeptide that behaves as a neurotransmitter in the brain. It is implicated in the modulation of dopamine (DA) signaling [1–5] and exerts potent effects including hypothermia [6], hypotension [7], and analgesia [8], as well as antipsychotic-like effects [9]. However, NT is easily degraded by peptidases and cannot cross the blood-brain barrier. To circumvent this problem, the Richelson laboratory developed a number of synthetic NT analogs, including NT69L [10, 11], that are more biologically stable, can be administered peripherally, and act as agonists at one or more of the three known NT receptors, NTS1 (high affinity, levocabastine-insensitive), NTS2 (low affinity, levocabastine-sensitive), and NTS3 (sortilin).

© 2011 Elsevier B.V. All rights reserved

*Correspondence: Elliott Richelson Mayo Clinic 4500 San Pablo Road Jacksonville, FL, 32223 richel@mayo.edu Fax: 904-953-7117 Phone: 904-953-8708.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

The development of tolerance to NT-mediated analgesia may be dependent on the effect being tested as well as the primary receptor through which the specific effect is mediated. A major hurdle regarding the clinical use of NT and NT analogs is the tendency for tolerance to develop to many of the clinically relevant effects.

NT69L {[N-methyl-Arg⁸, L-Lys⁹, L-neo-Trp¹¹, tert-Leu¹²] NT(8–13)} has been extensively studied [12] and binds with high affinity to both human NTS1 and human NTS2 with K_d values of 3.1 nM and 2.1 nM, respectively [13]. Additionally, NT69L appears to bind with much lower affinity to NTS3 [14]. Similar to NT, NT69L has hypothermic and antipsychotic-like effects [10, 15] and induces potent analgesia in rodents and in monkeys. In rats, NT69L blocks both thermal and persistent pain as determined by the hot plate and tail immersion assays [10, 16], and by the formalin test [17], respectively. In rhesus monkeys, NT69L blocks capsaicin-induced allodynia in the tail immersion assay [18].

Although NT69L elicits many of the same effects as centrally administered NT, tolerance develops rapidly (after a single dose) to a number of those effects (e.g. hypothermia and conditioned avoidance behavior [19]). Conversely, tolerance develops very slowly, if at all (no tolerance after five daily doses), to others (e.g. blockade of the hyperactivity caused by psychostimulants) [19, 20]. Tolerance may develop as a result of desensitization of the receptors to NT69L as has been described for extended exposure of NTS1 to agonist [21–25]. Following brief agonist exposure, it has been suggested that internalized NT receptors can be recycled to the membrane for quick resensitization [22]; however, after prolonged agonist exposure, receptors are internalized and degraded, requiring *de novo* synthesis for recovery and resulting in a longer resensitization lag time [21]. Importantly, resensitization is not achieved until agonist is removed [22].

It is theorized that tolerance to NT69L builds quickly in those effects mediated by NTS1, but not those mediated by NTS2 [26]. This theory is supported in part by the fact that rats quickly develop tolerance to NT69L-mediated hypothermia [19], which is dependent on NTS1 [27]. Recent studies support a main role for NTS1 in NT69L-mediated analgesia in various rat pain models [17, 27], suggesting that tolerance will develop after repeated NT69L administration. However, we hypothesize that it is not one single receptor that mediates the analgesic effects of NT69L, but the combined effort of two or more NT receptors working in concert. In support of this hypothesis are the data suggesting that NTS1 is modulated by both NTS2 [28, 29] and NTS3 [30]. These associations make it likely that multiple receptors are responsible for the analgesic effects of NT and NT analogs, including NT69L. Here we investigated the role of NTS2 on two specific NT69L-mediated effects, hypothermia and thermal analgesia with the use of NTS2 knock-out (NTS2^{-/-}) mice. Additionally, we use NTS1-selective NT analog, NT72 to examine the importance of NTS2 activation in the development of tolerance to these effects.

2. Materials and Methods

2.1. Animals

Wild-type (WT) and NTS2^{-/-} mice were approximately 45 days old at the time of testing and housed in a temperature controlled room (23 ± 2 °C) with a 12-h light/dark cycle and free access to food and water throughout the study. Animal use was approved by the Mayo Institutional Animal Care and Use Committee and was consistent with the NIH animal use guidelines for the care and use of laboratory animals. NTS2^{-/-} mice were established at Roche (Palo Alto, CA, USA) as previously described [27]. Briefly, the NTS null allele was originally created in Bruce-4 ES cells, which were derived from a C57BL/6 mouse strain. Cells were injected into BALB/c blastocysts to generate chimeras. Male chimeras were mated with female C57BL/6J mice to give rise to the F1 or N1 heterozygotes (+/-). To ensure a pure genetic background, one additional backcross was performed (male F1 het × female C57BL/6J) to generate the N2 heterozygotic mice. Homozygotic knockout (-/-) and wild type (+/+) mice F2 were from N2 heterozygote × heterozygote intercrosses. These knockout and wild type mice were used to establish “in house” knockout and wild type

colonies through heterozygous mating in each strain. The wild type mice used were from the heterozygous progeny.

2.2. Drugs

Levocabastine was purchased from Sigma-Aldrich (St. Louis, MO, USA), reconstituted in DMSO and diluted in 0.9% saline. NT79 was synthesized in the Mayo Proteomics Research Center (Rochester, MN) as previously described [31]. NT69L was synthesized as modified from [10] on an Fmoc-Leu-Wang resin (NovaBiochem) utilizing Fmoc solid-phase protein synthesis on a Liberty Microwave synthesizer (CEM Corp). The amino acids used were *N*-*O*-NBS-*N*-Me-Arg(Pbf), Fmoc-Arg, Fmoc-Lys (Boc), Fmoc-Pro, Fmoc-*neo*Trp(Boc), Fmoc-tert. Leu. Upon completion of the synthesis, the resin was washed with dichloromethane and dried under high vacuum. To remove the *O*-NBS (ortho-nitrobenzoic acid) group of *N*-Me-Arg(Pbf) from the N-terminal of the protected peptide, the dried resin was placed in a 10ml-frit funnel and shaken with 5 mL of DMF containing 5 eq of DBU (diazabicycloundecane) and 10 eq of 2-mercaptoethanol for 30 min at R.T. under nitrogen. The liquids were drained off and the resin was washed with DMF (x5), DCM (x5) and MeOH (x5), and then dried under high vacuum. After suspending and shaking the dried mass of resin with a cocktail consisting of TFA, phenol, thioanisole, water, and ethanedithiol (16.5:1:1:1:0.5) for 2–2.5 h at R.T., the liquids were drained and the resin was washed with TFA. The combined volatiles were evaporated to half their volume. Cold dry methyl *t*-butyl ether was added to precipitate the crude peptide which was washed with more aliquots of the ether. An off-white residue thus obtained was dried in vacuum and the pure NT69L peptide was isolated by RP-HPLC on a prep column (Vydac 2.2 × 25, FR = 8 mL/min, gradient elution with 10% B to 100%B; solvent B = acetonitrile with 0.1% TFA and solvent A = water with 0.1F TFA). The purified and lyophilized peptide (95–100 mg) was obtained as a very light pink powder.

All drugs were administered intraperitoneally and used at the following doses based on previously published data: 2.5 mg/kg levocabastine [16], 5.0 mg/kg NT72 [31], 1.0 mg/kg NT69L [10].

2.3. Core body temperature

WT and NTS2^{-/-} mice were treated with either saline or NT69L. Additionally, a subset of WT mice were pretreated with levocabastine 15 minutes prior to either saline or NT69L. Treatments in all experimental groups were administered daily for 13 consecutive days and body temperature was measured immediately before treatment, and at 30, 90, and 180 min following treatment, on day one (acute treatment) and on days five and 13 (sub-chronic treatment). Mice treated with NT72 were tested for body temperature immediately before treatment and at 30 minutes following treatment on days one and five.

Changes in core body temperature were measured with the use of a thermistor probe inserted 1.5 cm into the rectum of the mouse. Unless otherwise indicated, data were analyzed with the use of one-way ANOVA with Bonferroni posttests (performed using GraphPad Prism version 4.0, San Diego California USA). The following groups were compared: 1) NT69L versus saline-treated mice within the same genotype, and 2) WT mice pre-treated with levocabastine and NT69L versus NTS2^{-/-} mice treated with NT69L (to determine if treatment with levocabastine would phenotypically mimic NTS2^{-/-} mice). Additionally, following acute NT69L treatment, 3) WT mice versus NTS2^{-/-} mice or WT mice pre-treated with levocabastine. These comparisons were made to determine if the absence of NTS2 signaling resulted in significant differences in NT69L-mediated hypothermia following acute treatment. Finally, 4) WT mice treated with NT72 acutely (day 1) versus

sub-chronically (day 5) to determine if mice developed tolerance to the hypothermic effects of NTS1-selective NT72.

2.4. Hot plate

Data from core body temperature measurements followed similar trends between treatment days two and thirteen amongst all treatment groups. Therefore, five consecutive daily treatments were used for the following sub-chronic analgesia experiments. WT and NTS2^{-/-} mice were treated with either saline, NT69L, or NT72. Additionally, a subset of WT mice were pretreated with levocabastine 15 minutes prior to either saline or NT69L. Treatments in all experimental groups were administered once (acute treatment) or daily for five consecutive days (sub-chronic treatment). The analgesic effects of all treatments were determined immediately before final treatment, and then at 30 and 90 minutes following final treatment with the use of the hot plate assay for thermal pain.

Hot plate measurements were taken on a metal surface maintained at a temperature of 52.0±0.2°C. Latency was measured as the time the mouse took either to lick or to shake a hind paw after being placed on the hot plate. To prevent tissue damage, mice that did not respond in 30 s were removed from the plate. Hot plate latency was measured immediately prior to, and 30 and 90 min following final treatment. Mice that did not respond to the hot plate at time zero were excluded from the study. Unless otherwise indicated, data were analyzed using two-way ANOVAs with Bonferroni's multiple comparison tests (performed using GraphPad Prism version 4.0, San Diego California USA). Latency values were also converted to maximum possible effect (MPE) according to the following formula ((post-drug latency - baseline latency) / (cut-off time - baseline latency)), where the cut-off time was 30 sec.

3. Results

3.1 Body Temperature

Acutely, NT69L caused a significant decrease in core body temperature at 30 min post-treatment in both WT ($P < 0.01$) and NTS2^{-/-} ($P < 0.001$) mice compared to that for saline controls (Figure 1). This decrease was also evident at 90 min post-treatment in NTS2^{-/-} mice ($P < 0.001$), but returned to baseline levels for both genotypes 180 min post-treatment. A significantly larger decrease in body temperature was measured in NTS2^{-/-} mice compared to that for WT mice at 30 min post-treatment ($P < 0.05$). The histamine H₁ antagonist, levocabastine, which is also known to be both an antagonist and agonist at NTS2, alone did not change core body temperature (data not shown). However, when levocabastine was given prior to NT69L, significant decreases in body temperature were measured at 30 ($P < 0.001$), 90 ($P < 0.01$), and 180 ($P < 0.01$) min post-treatment as compared to that for WT saline controls. These decreases were significantly larger than in NT69L-treated WT mice not pre-treated with levocabastine at 30 ($P < 0.01$) and 180 ($P < 0.01$) min post-treatment, and in NTS2^{-/-} mice at 180 min post-treatment ($P < 0.01$).

By contrast, subchronic treatment with NT69L caused a decrease in body temperature in NTS2^{-/-}, but not in WT mice compared to that for saline controls. This decrease in NTS2^{-/-} mice was evident at 30 ($P < 0.001$) and 90 ($P < 0.05$) min post-treatment on day five, and at 30 minutes post-treatment on day 13 ($P < 0.001$). Tolerance to the effects of NT69L was detected in WT mice as early as the second day of treatment and persisted throughout the thirteen days tested. Mice pretreated with levocabastine responded to NT69L similarly to NTS2^{-/-} mice, showing a significant decrease in body temperature over the 13 days tested (see Figure 1 for P-values). Notably, pre-treatment with levocabastine (followed by NT69L) resulted in significantly larger decreases in body temperature compared to that

for NTS2^{-/-} mice at 30 ($P < 0.01$), 90 ($P < 0.01$), and 180 ($P < 0.001$) min post-treatment on day 13, but not on day five. WT mice pre-treated with levocabastine were not statistically compared to WT mice following sub-chronic NT69L treatment because WT mice developed tolerance to NT69L after treatment day one. Levocabastine alone did not change core body temperature following sub-chronic treatment (data not shown).

NT72, an NTS1-selective NT analog, significantly reduced body temperature ($P < 0.001$) 30 minutes after acute treatment in WT and NTS2^{-/-} mice. However, following sub-chronic treatment, the drop in body temperature elicited by the analog was considerably reduced in both genotypes, with only WT mice experiencing a significant ($P < 0.05$) effect (Figure 2A).

3.2 Analgesia

Analgesia was directly related to a decrease in core body temperature; mice that displayed a marked decrease in core body temperature ($\leq -2^{\circ}\text{C}$) demonstrated considerably less pain compared to mice that did not, as measured by the hot plate assay. With the exception of sub-chronically treated WT mice (which develop tolerance to the hypothermic effect of NT69L), a $\leq 2^{\circ}\text{C}$ decrease in core body temperature was used to determine if NT69L was successfully administered. In mice that displayed no decrease in body temperature in response to NT69L treatment, NT69L did not have an analgesic effect in any of the pain tests administered. For acute NT69L treatment, 2/10 WT mice (20%), 1/14 WT mice pretreated with levocabastine (7%), and 1/16 NTS2^{-/-} mice (6%) did not demonstrate a decrease in body temperature. For sub-chronic NT69L treatment, 3/13 WT mice pretreated with levocabastine (23%) and 2/24 NTS2^{-/-} mice (8%) did not demonstrate a decrease in body temperature. These mice were excluded from the study.

3.2.1. Hot Plate—Immediately prior to, and at 30 and 90 min after treatment, mice were tested for thermal pain with the use of the hot plate assay (Figure 3). These data were also converted to MPE values (see section 2.4 for calculation). Following acute treatment, NT69L caused a significant increase in hot plate latency at 30 and 90 min post treatment in WT (MPE = 12.4%, $P < 0.05$; 92.1%, $P < 0.001$), WT pretreated with levocabastine (MPE = 100.0%, $P < 0.001$; 93.7%, $P < 0.001$), and in NTS2^{-/-} (MPE = 100.0%, $P < 0.001$; 90.6%, $P < 0.001$) mice, respectively. The increase in hot plate latency was significantly larger in WT mice pre-treated with levocabastine ($P < 0.001$) and in NTS2^{-/-} mice ($P < 0.01$) compared to that for WT mice at 30 min post-treatment, and in WT mice pre-treated with levocabastine compared to that for WT mice at 90 min ($P < 0.001$) post-treatment as determined by t-tests (SigmaStat version 3.5, Ashburn, VA, USA). Following sub-chronic treatment, NT69L caused a significant increase in hot plate latency at 30 and 90 min post treatment in WT mice pretreated with levocabastine (MPE = 78.1%, $P < 0.05$; 50.8%, $P < 0.05$), and in NTS2^{-/-} (MPE = 77.5%, $P < 0.001$; 56.5%, $P < 0.001$) mice, respectively. No increase in hot plate latency was observed for WT mice sub-chronically treated with NT69L. Similar to NT69L, NTS1-selective NT72 caused a significant increase in hot plate latency following acute treatment ($P < 0.001$; 30 minutes post treatment), but not sub-chronic treatment in WT and NTS2^{-/-} mice (Figure 2B). For all mice, acute and sub-chronic treatment with saline (or levocabastine followed by saline) elicited no significant increase in hot plate latency (data not shown).

4. Discussion

NT69L is a NT(8–13) analog that elicits many of the same behavioral effects as does endogenous NT including hypothermia and analgesia. Tolerance develops rapidly to some, but not to all of NT69L's effects, and to date, little is known about the mechanisms responsible for this tolerance. The development of tolerance appears to be more prevalent in

behavioral effects mediated by NTS1 than by those mediated by NTS2 [26], and it is possible that the long half-life of NT69L keeps neurons in an extended period of desensitization.

The tolerance developed to many of the behavioral effects of NT69L may rely on the extreme stability of the peptide once it passes the blood-brain barrier. Donato Di Paola et al. demonstrated that a murine neuroblastoma cell line exposed for 12 h to a neurotensin analog, NT2, required a half-time of three hours for recovery of NT binding. The half-life of NT69L is reported to be 500 h in human plasma and 250 h in rat intestine [35, 36]. If NT69L is still present in the CNS of mice 24 h after treatment, resensitization of the receptors would be arrested, resulting in a failure to respond to subsequent injections of NT69L, a situation that would be observed as tolerance at the behavioral level. This is supported by data showing that 5-day treatment with NT69L causes a decrease in NT binding in the striatum of rats [26, 37].

We hypothesize that in addition to NTS1, NTS2 also plays an important role in the development of tolerance to NT69L, particularly to those effects thought to be NTS1-mediated. In the present study we demonstrated that 1) tolerance develops to NT69L-mediated hypothermia and thermal analgesia following sub-chronic treatment in WT mice, and 2) elimination of NTS2 blocks the development of that tolerance. Specifically, we suggest that prolonged exposure to NT69L causes a NTS2-dependent down regulation of NTS1.

These data for NT69L-mediated thermal analgesia are in contrast to previous data reporting a lack of tolerance to NT analog-mediated analgesia. Dubuc et al. [38] found that tolerance develops in rats to NT analog-mediated hypothermia, but not to analgesia as determined by the plantar test. Similarly, β -lactotensin, a compound with affinity for both NTS1 and NTS2, induces analgesia in mice without the development of tolerance as determined by the tail pinch assay [39]. Finally, Hughes et al. [40] described an NT analog with affinity for both NTS1 and NTS2 that induces analgesia in rats without the development of tolerance as determined by the hot plate assay. This may indicate a mechanistic difference between analgesia produced by NT69L and that produced by other NT analogs.

It is generally accepted that the hypothermic effects of NT and its analogs are mediated by NTS1 [41, 42]. Recently, Mechanic et al. showed that NTS1 plays a major role in NT69L-mediated thermal analgesia (as well as hypothermia) [27]. Here we have shown that WT mice rapidly develop tolerance to both hypothermia and thermal analgesia in as little as one day following repeated treatment with NT69L. These data support the theory that repeated activation of NTS1 leads to tolerance. Also, these data confirm previous reports of tolerance to NT [43]- and NT69L [19]-mediated hypothermia in rats. By contrast, mice deficient in NTS2 failed to develop tolerance to NT69L-mediated hypothermia or thermal analgesia. This strongly suggests that NTS2 plays a role in the development of tolerance to these NTS1-mediated effects of NT69L, possibly through the regulation of NTS1 trafficking.

Notably, there are biological differences between WT and NTS2^{-/-} mice that go beyond the absence of NTS2. NTS2^{-/-} mice exhibit a 15–20 fold increase in NTS1 mRNA compared to that for WT mice [44]. This is likely a mechanism to compensate for the loss of NTS2, although it could indicate the regulation of NTS1 expression by NTS2. To rule out the effect of elevated NTS1 on the development of tolerance to NT69L, mice were pre-treated with levocabastine prior to treatment with NT69L. Levocabastine has been shown to be both an agonist [45] and an antagonist [46] in NT-mediated analgesia depending on the type of pain being assessed. When using the hot plate assay for thermal pain, levocabastine acts as an antagonist [45, 46], and would compete with NT69L for binding to NTS2. WT mice pre-

treated with levocabastine reacted similarly to that seen with NTS2^{-/-} mice, failing to develop tolerance to NT69L-mediated hypothermia or thermal analgesia after repeated treatment. These data support the theory that NTS2 is directly involved in the development of tolerance to NTS1-mediated effects of NT69L.

Behavioral data also support a direct role for NTS2 in NT69L-mediated hypothermia and thermal analgesia. NTS2^{-/-} mice and WT mice pre-treated with levocabastine exhibited significantly larger decreases in core body temperature, as well as longer hot plate latencies compared to those for WT mice following acute NT69L treatment. These data differ from those showing no effect of levocabastine on centrally administered NT-induced hypothermia [45, 46], and suggests that NTS2 is more involved in NT69L-mediated, than NT-mediated, signaling. The present data suggest that when both receptors are present, NTS2 has an inhibitory effect on NTS1. Possible mechanisms for regulation include direct inhibition via physical interaction, or regulation of NTS1 trafficking to the membrane. Indeed, the NT receptors are known to regulate each other *in vitro* [28–30, 47]. When both receptors are expressed, NTS2 forms heterodimers with NTS1, and results in the accumulation of NTS1 in the juxtannuclear compartment, most likely impeding recruitment to the membrane [28, 29].

To determine if the role of NTS2 on the development of tolerance is an active or passive one, we performed similar experiments with the NTS1-selective analog, NT72. If activation of NTS2 is required for tolerance to develop, mice should not develop a tolerance to NT72-mediated effects. Unexpectedly, WT and NTS2^{-/-} mice quickly developed tolerance to both the hypothermic and analgesic effects of NT72. This suggests that NTS2 is playing a role in tolerance developed to NT69L (dual activation of NTS1 and NTS2)-, but not in NT72 (activation of NTS1 only)-mediated effects. Importantly, little is known regarding the role of NTS3, which has previously been shown to interact with NTS1. A thorough analysis of the roles of NT receptors in tolerance would require the use of NTS3^{-/-} mice.

Interestingly, pre-treatment of WT mice with levocabastine resulted in significantly larger decreases in body temperature following NT69L treatment compared to NTS2^{-/-} mice at various time points, most notably on day thirteen. Differences between these two experimental groups are not surprising due to the inherent actions of levocabastine at its receptors. In addition to blocking NTS2 from NT69L binding, levocabastine is also an antagonist at the histamine H₁ receptor. Indeed, there is evidence that NT-induced hypothermia is mediated by histamines [48, 49], and another H₁ receptor antagonist, mepyramine, inhibits NT-induced hypothermia in a dose-dependent manner [48]. However, levocabastine itself does not influence NT-induced hypothermia when given i.c.v, i.p., or injected directly into the PAG [45, 46]. The observation that levocabastine enhances NT69L-mediated hypothermia may be specific to this, and other systemically available NT analogs, and is likely due to the extraneous binding of levocabastine.

Notably, NT69L-induced hypothermia was directly related to NT69L-induced analgesia. Those mice that did not drop body temperature ($\geq 2^{\circ}\text{C}$) in response to NT69L did not exhibit significant thermal analgesia when measured with the use of the hot plate. This suggests that the hypothermic and analgesic effects of NT69L are linked and that the analgesic effect of NT69L in a model of thermal pain may be due to hypothermia-induced analgesia [32–34], or that measures of thermal pain may be skewed by a lower body temperature at time of testing. Indeed, NT69L-induced thermal analgesia has been previously reported to go hand-in-hand with hypothermia in rodents [10, 27]. The first report of NT69L-induced analgesia in rats found significant decreases in body temperature, even at doses lower than that which caused analgesia as measured with use of the hot plate [10]. This suggests that NT69L-induced analgesia is dependent on hypothermia and not

necessarily vice versa. Similarly, Mechanic et al., reported that only those doses of NT69L capable of inducing hypothermia in mice can also induce analgesia with use of the tail immersion assay, and showed that both effects were eliminated when NTS1 was knocked out [27]. This effect may be specific to rodents, as doses of NT69L capable of blocking capsaicin-induced allodynia in the tail immersion assay did not elicit significant hypothermia in rhesus monkeys [18].

5. Conclusions

The data presented here support an inhibitory role for NTS2 in NTS1-mediated behaviors, and suggest that, following NT69L exposure in the absence of NTS2, NTS1 is more available for binding at the membrane. This inhibitory role may be dependent on NTS2 activation by drug, as NTS2 does not appear to play a role in the development of tolerance to NTS1-specific NT72. Following NT69L exposure in the absence of NTS2, it is possible that NTS1 is either 1) not efficiently down-regulated, or 2) more quickly recycled or resynthesized and trafficked to the membrane compared to that for mice with both receptors. This suggests that NTS2 has an important role in keeping NTS1 internalized once the receptor is bound to ligand and down-regulated. An unimpeded supply of membrane NTS1 would explain the heightened NT69L effects seen in NTS2^{-/-} mice, as well as the lack of tolerance. More work is needed to elucidate out the mechanism(s) by which NTS2 regulates NTS1, as well as the mechanisms responsible for the development of tolerance to NTS1-selective analogs such as NT72.

Acknowledgments

This work was supported by the Siragusa Foundation Career Development Award (M. Boules), as well as the Mayo Foundation for Medical Education and Research.

References

- [1]. Pinnock RD. Neurotensin depolarizes substantia nigra dopamine neurones. *Brain Res.* 1985; 338:151–4. [PubMed: 4027584]
- [2]. Seutin V, Massotte L, Dresse A. Electrophysiological effects of neurotensin on dopaminergic neurones of the ventral tegmental area of the rat in vitro. *Neuropharmacology.* 1989; 28:949–54. [PubMed: 2572997]
- [3]. Mercuri NB, Stratta F, Calabresi P, Bernardi G. Neurotensin induces an inward current in rat mesencephalic dopaminergic neurons. *Neurosci Lett.* 1993; 153:192–6. [PubMed: 7687049]
- [4]. Binder EB, Kinkad B, Owens MJ, Nemeroff CB. Neurotensin and dopamine interactions. *Pharmacol Rev.* 2001; 53:453–86. [PubMed: 11734615]
- [5]. Jomphe C, Lemelin P-L, Okano H, Kobayashi K, Trudeau L-E. Bidirectional regulation of dopamine D2 and neurotensin NTS1 receptors in dopamine neurons. *European Journal of Neuroscience.* 2006; 24:2789–800. [PubMed: 17116165]
- [6]. Bissette G, Nemeroff CB, Loosen PT, Prange AJ, Lipton MA. Hypothermia and intolerance to cold induced by intracisternal administration of the hypothalamic peptide neurotensin. *Nature.* 1976; 262:607–9. [PubMed: 8728]
- [7]. Rioux F, Quirion R, St-Pierre S, Regoli D, Jolicœur FB, Belanger F, et al. The hypotensive effect of centrally administered neurotensin in rats. *Eur J Pharmacol.* 1981; 69:241–7. [PubMed: 7215426]
- [8]. Clineschmidt BV, McGuffin JC. Neurotensin administered intracisternally inhibits responsiveness of mice to noxious stimuli. *Eur J Pharmacol.* 1977; 46:395–6. [PubMed: 201475]
- [9]. Jolicœur FB, Gagne MA, Rivest R, Drumheller A, St-Pierre S. Atypical neuroleptic-like behavioral effects of neurotensin. *Brain Res Bull.* 1993; 32:487–91. [PubMed: 8106129]

- [10]. Tyler-McMahon BM, Stewart JA, Farinas F, McCormick DJ, Richelson E. Highly potent neurotensin analog that causes hypothermia and antinociception. *European Journal of Pharmacology*. 2000; 390:107–11. [PubMed: 10708713]
- [11]. Cusack B, Boules M, Tyler BM, Fauq A, McCormick DJ, Richelson E. Effects of a novel neurotensin peptide analog given extracranially on CNS behaviors mediated by apomorphine and haloperidol. *Brain Res*. 2000; 856:48–54. [PubMed: 10677610]
- [12]. Boules M, Fredrickson P, Richelson E. Current topics: brain penetrating neurotensin analog. *Life Sci*. 2003; 73:2785–92. [PubMed: 14511764]
- [13]. Boules M, Liang Y, Briody S, Miura T, Fauq I, Oliveros A, et al. NT79: A novel neurotensin analog with selective behavioral effects. *Brain Res*. 2010; 1308:35–46. [PubMed: 19874804]
- [14]. Munck Petersen, C.; Nissen, P.; Sondergaard Madsen, P.; Meldgaard Høgh Quistgaard, E.; Keller Groftehauge, M.; Skou Thirup, S., et al. Design and use of sortilin specific molecular imaging ligands. G01N 33/68 (2006.01) G06F 19/00 (2006.01) ed2009
- [15]. Secchi RL, Sung E, Hedley LR, Button D, Schreiber R. The neurotensin agonist NT69L improves sensorimotor gating deficits in rats induced by a glutamatergic antagonist, but not by dopaminergic agonists. *Behav Brain Res*. 2009; 202:192–7. [PubMed: 19463701]
- [16]. Boules M, Shaw A, Liang Y, Barbut D, Richelson E. NT69L, a novel analgesic, shows synergy with morphine. *Brain Res*. 2009; 1294:22–8. [PubMed: 19651107]
- [17]. Roussy G, Dansereau M-A, Doré-Savard L, Belleville K, Beaudet N, Richelson E, et al. Spinal NTS1 receptors regulate nociceptive signaling in a rat formalin tonic pain model. *Journal of Neurochemistry*. 2008; 105:1100–14. [PubMed: 18182046]
- [18]. Fantegrossi WE, Ko MCH, Woods JH. E R. Pharmacological effects of a novel neurotensin receptor agonist, NT69L, in rhesus monkeys. *Pharm Biochem & Behavior*. 2005; 80:341–9.
- [19]. Boules M, McMahon B, Wang R, Warrington L, Stewart J, Yerbury S, et al. Selective tolerance to the hypothermic and anticonvulsant effects of a neurotensin analog that crosses the blood-brain barrier. *Brain Res*. 2003; 987:39–48. [PubMed: 14499944]
- [20]. Hertel P, Olsen CK, Arnt J. Repeated administration of the neurotensin analogue NT69L induces tolerance to its suppressant effect on conditioned avoidance behaviour. *Eur J Pharmacol*. 2002; 439:107–11. [PubMed: 11937099]
- [21]. Donato di Paola E, Cusack B, Yamada M, Richelson E. Desensitization and down-regulation of neurotensin receptors in murine neuroblastoma clone N1E-115 by [D-Lys8] neurotensin(8–13). *J Pharmacol Exp Ther*. 1993; 264:1–5. [PubMed: 8380855]
- [22]. Gilbert JA, Strobel TR, Richelson E. Desensitization of neurotensin receptor-mediated cyclic GMP formation in neuroblastoma clone N1E-115. *Biochem Pharmacol*. 1988; 37:2833–8. [PubMed: 2840079]
- [23]. Hermans E, Gailly P, Octave JN, Maloteaux JM. Rapid desensitization of agonist-induced calcium mobilization in transfected PC12 cells expressing the rat neurotensin receptor. *Biochem Biophys Res Commun*. 1994; 198:400–7. [PubMed: 8292048]
- [24]. Yamada M, Richelson E. Further characterization of neurotensin receptor desensitization and down-regulation in clone N1E-115 neuroblastoma cells. *Biochem Pharmacol*. 1993; 45:2149–54. [PubMed: 8390262]
- [25]. Hermans E, Maloteaux JM. Desensitization of neurotensin-induced phosphoinositide hydrolysis in transfected CHO cells. *Biochem Pharmacol*. 1996; 51:1749–52. [PubMed: 8687490]
- [26]. Wang Rui MB, Tiner William, Richelson Elliott. Effects of repeated injections of the neurotensin analog NT69L on dopamine release and uptake in rat striatum in vitro. *Brain Res*. 2004; 1025:21–8. [PubMed: 15464740]
- [27]. Mechanic JA, Sutton JE, Berson AE, Wu X, Kwan J, Schreiber R, et al. Involvement of the neurotensin receptor 1 in the behavioral effects of two neurotensin agonists, NT-2 and NT69L: lack of hypothermic, antinociceptive and antipsychotic actions in receptor knockout mice. *Eur Neuropsychopharmacol*. 2009; 19:466–75. [PubMed: 19223157]
- [28]. Perron A, Sharif N, Sarret P, Stroh T, Beaudet A. NTS2 modulates the intracellular distribution and trafficking of NTS1 via heterodimerization. *Biochem Biophys Res Commun*. 2007; 353:582–90. [PubMed: 17188644]

- [29]. Hwang JR, Baek MW, Sim J, Choi HS, Han JM, Kim YL, et al. Intermolecular cross-talk between NTR1 and NTR2 neurotensin receptor promotes intracellular sequestration and functional inhibition of NTR1 receptors. *Biochem Biophys Res Commun*. 2010; 391:1007–13. [PubMed: 19968961]
- [30]. Martin S, Navarro V, Vincent JP, Mazella J. Neurotensin receptor-1 and -3 complex modulates the cellular signaling of neurotensin in the HT29 cell line. *Gastroenterology*. 2002; 123:1135–43. [PubMed: 12360476]
- [31]. Rossi GC, Matulonis JE, Richelson E, Barbut D, Pasternak GW. Systemically and topically active antinociceptive neurotensin compounds. *J Pharmacol Exp Ther*. 2010; 334:1075–9. [PubMed: 20576795]
- [32]. Suckow MA, Terril LA, Grigdesby CF, March PA. Evaluation of Hypothermia-Induced Analgesia and Influence of Opioid Antagonists in Leopard Frogs (*Rana pipiens*). *Pharmacology Biochemistry and Behavior*. 1999; 63:39–43.
- [33]. Sadowski B, Konarzewski M. Analgesia in Selectively Bred Mice Exposed to Cold in Helium/Oxygen Atmosphere. *Physiology & Behavior*. 1999; 66:145–51. [PubMed: 10222487]
- [34]. Lapo IB, Konarzewski M, Sadowski B. Analgesia induced by swim stress: interaction between analgesic and thermoregulatory mechanisms. *Pflügers Archiv European Journal of Physiology*. 2003; 446:463–9.
- [35]. Tyler-McMahon BM, Boules M, Richelson E. Neurotensin: peptide for the next millennium. *Regul Pept*. 2000; 93:125–36. [PubMed: 11033059]
- [36]. Richelson, E.; Cusack, B.; Pang, Y.; McCormick, D.; Fauq, A.; Tyler, BM., et al. Neo-Tryptophan. Mayo Foundation for Medical Education and Research; USA: 2006. p. 1-30.
- [37]. Wang R, Boules M, Gollatz E, Williams K, Tiner W, Richelson E. Effects of 5 daily injections of the neurotensin-mimetic NT69L on the expression of neurotensin receptors in rat brain. *Molecular Brain Research*. 2005; 138:24–34. [PubMed: 15878217]
- [38]. Dubuc I, Pain C, Suaudeau C, Costentin J. Tolerance to the hypothermic but not to the analgesic effect of [D-Trp1]neurotensin during the semichronic intracerebroventricular infusion of the peptide in rats. *Peptides*. 1994; 15:303–7. [PubMed: 8008636]
- [39]. Yamauchi R, Sonoda S, Jinsmaa Y, Yoshikawa M. Antinociception induced by beta-lactotensin, a neurotensin agonist peptide derived from beta-lactoglobulin, is mediated by NT2 and D1 receptors. *Life Sci*. 2003; 73:1917–23. [PubMed: 12899917]
- [40]. Hughes FM Jr, Shaner BE, May LA, Zotian L, Brower JO, Woods RJ, et al. Identification and functional characterization of a stable, centrally active derivative of the neurotensin (8–13) fragment as a potential first-in-class analgesic. *J Med Chem*. 2010; 53:4623–32. [PubMed: 20481538]
- [41]. Pettibone DJ, Hess JF, Hey PJ, Jacobson MA, Leviten M, Lis EV, et al. The effects of deleting the mouse neurotensin receptor NTR1 on central and peripheral responses to neurotensin. *J Pharmacol Exp Ther*. 2002; 300:305–13. [PubMed: 11752130]
- [42]. Remaury A, Vita N, Gendreau S, Jung M, Arnone M, Poncelet M, et al. Targeted inactivation of the neurotensin type 1 receptor reveals its role in body temperature control and feeding behavior but not in analgesia. *Brain Res*. 2002; 953:63–72. [PubMed: 12384239]
- [43]. Popp E, Schneider A, Vogel P, Teschendorf P, Bottiger BW. Time course of the hypothermic response to continuously administered neurotensin. *Neuropeptides*. 2007; 41:349–54. [PubMed: 17655926]
- [44]. Liang Y, Boules M, Li Z, Williams K, Miura T, Oliveros A, et al. Hyperactivity of the dopaminergic system in NTS1 and NTS2 null mice. *Neuropharmacology*. 2010; 58:1199–205. [PubMed: 20211191]
- [45]. Dubuc I, Remande S, Costentin J. The partial agonist properties of levocabastine in neurotensin-induced analgesia. *Eur J Pharmacol*. 1999; 381:9–12. [PubMed: 10528128]
- [46]. Tyler BM, Groshan K, Cusack B, Richelson E. In vivo studies with low doses of levocabastine and diphenhydramine, but not pyrilamine, antagonize neurotensin-mediated antinociception. *Brain Res*. 1998; 787:78–84. [PubMed: 9518559]
- [47]. Souza F, Forgez P. Molecular and cellular regulation of neurotensin receptor under acute and chronic agonist stimulation. *Peptides*. 2006; 27:2493–501. [PubMed: 16889873]

- [48]. Kandasamy SB. Neuropeptides, radiation, and thermoregulation. *Ann N Y Acad Sci.* 1997; 813:344–52. [PubMed: 9100905]
- [49]. Kandasamy SB, Hunt WA, Harris AH. Role of neurotensin in radiation-induced hypothermia in rats. *Radiat Res.* 1991; 126:218–22. [PubMed: 2023992]

Research Highlights

- Tolerance develops to NT69L-mediated hypothermia and thermal analgesia in WT mice
- Tolerance to NT69L does not develop in the absence of NTS2
- NTS2 may have a regulatory role in NTS1 expression and trafficking

Figure 1.

Core body temperature measurements. Core body temperature was measured in NT69L- or saline-treated WT, NTS2^{-/-} mice, and WT mice pre-treated with levocabastine [WT (L)] at 0, 30, 90 and 180 min post-treatment. Measurements taken on days 1, 5 and 13 are shown. Asterisks represent statistical significance between mice treated with NT69L and the saline control of the respective genotype. Crosses represent statistical significance between NT69L-treated NTS2^{-/-} mice and WT mice pre-treated with levocabastine. Xs represent statistical significance between NT69L-treated WT mice and WT mice pre-treated with levocabastine. Dollar signs represent statistical significance between NT69L-treated WT and NTS2^{-/-} mice. ***, †††: $P \leq 0.001$; **, ††, xx: $P \leq 0.01$; *, \$: $P \leq 0.05$.

Figure 2.

Core body temperature (A) and hot plate latency (B) data for treatment of WT and NTS2^{-/-} with NT72 for one or five days. A) Core body temperature is graphed as the drop in temperature from immediately prior to, to 30 minutes after treatment. Asterisks represent statistical significance between measurements taken immediately prior to, and those taken 30 minutes after treatment with NT72. ***: $P \leq 0.001$; *: $P \leq 0.05$.

Figure 3.

Hot plate latencies. WT mice, NTS2^{-/-} mice, or WT mice pre-treated with levocabastine [WT(L)] were treated with saline or NT69L for one or five days. The hot plate assay was used to measure thermal pain immediately prior to, and at 30 and 90 min post treatment. Asterisks represent statistical significance between post-treatment time points as compared to pre-treatment baseline. ***: $P \leq 0.001$; *: $P \leq 0.05$.