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An NTS2 Analog Enhances the Analgesic Effects of Morphine in an Animal Model of Persistent Pain and Does not Exhibit Tolerance

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Abstract: The analgesic efficacy of neurotensin agonists depends on their activation of two receptor subtypes, NTS1 and/or NTS2. In this study we determined the role of NTS2 in an animal model of persistent pain (intraplantar injection of formalin) with the use of the NTS2-selective analog, NT79 and NTS2-knockout mice ($NTS2^{-/-}$). Wild type (WT) and $NTS2^{-/-}$ mice were pretreated with NT79 and tested for formalin-induced lifting and biting. Additionally, the effect of repeated administration of NT79 and morphine alone and in combination was determined in WT mice. Intraplantar injection of formalin produced the typical biphasic nociceptive response of this persistent pain model. Formalin evoked lower pain intensity in $NTS2^{-/-}$ mice as compared to that for WT mice. Pretreatment with NT79 attenuated formalin-induced nociception throughout phase II in the WT mice, and in early phase II in the $NTS2^{-/-}$ mice. Lifting and biting responses were attenuated, indicating spinal and supra-spinal modulation of persistent nociception. More importantly, repeated injection of NT79 enhanced, while that of morphine reduced their antinociceptive effects, respectively. Subchronic co-administration of NT79 and morphine enhanced the analgesic effect over either drug alone. These data support the role of NTS2 in modulating formalin-induced pain. Additionally, these data provide a rationale for the potential therapeutic role of NTS2-selective analogs in chronic pain management alone or in combination with morphine and without the development of tolerance.

Keywords: Formalin test, morphine, neurotensin, neurotensin receptors, pain, tolerance.

1. INTRODUCTION

Chronic pain, whether the result of nerve trauma or persistent inflammation, is a debilitating condition that exerts a high social cost in terms of productivity, economic impact, and quality of life [1]. Opioids such as morphine are widely used for treatment of pain, but are associated with potentially serious side effects and the risk of addiction. Also, with long-term use of opioids antinociceptive tolerance develops. Thus, alternative, non-opioid, non-addicting pharmacological treatments for persistent pain are needed, to be administered alone or in combination with opioids, such as morphine. Combination, synergistic therapies of non-opioid and opioid drugs, such as we have shown between the non-selective NT receptor agonist NT69L and morphine [2] and the NTS2-selective analog NT79 and morphine [3] could mitigate the side effects of morphine, such as constipation, physical dependence, addiction, and delay the development of tolerance to the opioid drug.

1.1. NT and Pain

Neurotensin (NT) is a 13-amino acid neuropeptide that produces antinociception in several animal models of pain [4, 5]. Central injection of NT produces naloxone-independent spinal and supra-spinal analgesic effects [6-9]. Molecular cloning and pharmacological data have demonstrated the existence of at least three NT receptor subtypes: the high-affinity levocabastine-insensitive NT receptor subtype 1 (NST1) [10, 11], the low-affinity levocabastine-sensitive NT receptor subtype 2 (NTS2) [12, 13], and sortilin/NTS3 [14, 15]. However, only NTS1 and NTS2 have been implicated in the analgesic properties of NT [4, 5, 16].

Studies by our group and others show that NT and NT analogs are effective in treating thermal, visceral (acetic acid-induced writhing), persistent inflammatory (formalin-induced) pain, and neuropathic pain [2, 17-20]. However, evidence suggests that the analgesic efficacy of NT analogs varies with their selectivity for NTS1 and NTS2, the pain model, and probably the animal species used.

1.2. NT Receptors and Pain

Reports on mice lacking the NTS1 gene reveal that NT and NT analogs fail to induce antinociception in the hot plate (HP) test [21]. Consistent with the knockout mice studies, our group showed that the inhibition of NTS1 synthesis with the use of antisense peptide nucleic acids (PNAs) targeting NTS1 also results in loss of the analgesic properties of NT in the hot plate test [22]. On the other hand, others [23] working with NTS1- and NTS2 knockout (NTS1-/- and NTS2-/-) mice suggested that NTS2 plays an important role in thermal nociception compared to NTS1 under physiological conditions. Similar conclusions come from studies using the HP test in mice and the NTS2-selective ligand levocabastine, which block the effects of a NT agonist [24]. Others also established that NTS2 are extensively associated with spinal nociceptive pathways and implicate NTS2 in the analgesic effect of NT [16, 23-27].

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Recently, our group has developed a novel NTS2-selective analog named NT79. This NT(8-13) analog while ineffective in reducing thermal pain, blocks acetic acid-induced writhing [20], without development of tolerance to its analgesic effects (Boules *et al.*, unpublished data). Interestingly, NT79 does not cause hypothermia, suggesting that this effect is needed to block thermal pain. NT79 also reduces formalin-induced pain and does so in synergy with morphine [3]. While many studies have implicated NTS2 in mediating analgesia in various animal pain models [16, 23, 28], few provided evidence for the involvement of NTS2 in reducing persistent pain with the use of knockout mice. Lafrance *et al.*, 2010 [29] demonstrated that mice lacking NTS2 exhibit significantly lower stress-induced analgesia following cold-water swim stress as compared to that for their wild type littermates. Roussy *et al.*, [30] tested the pain behavioral responses to formalin following systemic administration of morphine with the use of $NTS1^{-/-}$ mice.

1.3. NT, Morphine, and Pain

Most studies show that antinociception mediated by either NT agonists or opioids are independent of one another [7, 31]. However, there are reports suggesting that the two systems have some interactions. Receptors for NT and for opioids co-localize in brain and in spinal cord in areas that are important for pain perception. The midbrain periaqueductal grey (PAG), which is a major region for the site of action of opioids and other agents, has nerve terminals containing opioid peptides and NT, as well as high densities of opioid and NT receptors [32, 33]. Antinociception produced by μ-opioid receptor activation in the amygdala was reported as partly dependent on activation of both the μ-opioid and NT receptors in the ventral PAG [34]. In addition, it has been suggested that morphine administration in the PAG activates endogenous NT in the nucleus raphe magnus and that NT might serve a modulatory role in morphine's antinociceptive response [35]. Furthermore, there is evidence that in the hot plate test, the NT system regulates the jump response to either morphine or a NT receptor agonist [24]. Interestingly, mice that were made tolerant to morphine showed reduced analgesic effects of NT [36].

Chronic treatment with opioids is associated with the development of tolerance to its analgesic effects, thus requiring dosage increases over time, to attain a consistent level of analgesia. Researchers have tried combinations of drugs to mitigate these problems. For example, the N-methyl-D-aspartate (NMDA) receptor antagonist ketamine was combined with opioids not only to enhance the efficacy of low doses, but also to attenuate the development of tolerance [37, 38]. However, serious motor impairment is observed at doses of ketamine that are antinociceptive in the rat, and in humans, ketamine can be psychotomimetic.

The present study was done to test: 1) the effect of subchronic administration of the NTS2-selective analog, NT79, in an animal model of persistent pain (intraplantar injection of formalin) with the use of NTS2 knockout mice ($NTS2^{-/-}$); 2) the effect of subchronic administration of morphine on formalin-induced pain; and 3) the effects of administration of both NT79 and morphine together on the development of tolerance to either drug. Such data would support the use of NTS2-selective compounds, alone or in combination with morphine as a new class of analgesic drugs for the treatment of persistent pain. Additionally, as the results show, the combined use of NT79 and morphine could potentially reduce the development of analgesic tolerance to morphine. The use of lower doses of morphine to achieve the same analgesic response and delaying the development of tolerance to morphine would be clinically invaluable to patients suffering from chronic pain.

2. MATERIALS AND METHODS

2.1. Animals

Adult male wild type (WT), and $NTS2^{-/-}$ mice (30-34g) were used in all experiments. $NTS2^{-/-}$ mice were generated as described by our group [39]. Animals were kept 4 per cage in a temperature-controlled room with 12 h light/dark cycle. Food and water were provided *ad libitum*, and all experimental procedures were approved by Mayo Clinic Institutional Animal Use and Care Committee.

2.2. Chemicals, Drugs

NT79 was synthesized as described previously [20]. Formalin was purchased from (Fisher Scientific, Kalamazoo, MI, USA).

2.3. Behavioral Testing

The formalin test was conducted as previously described for mice [40]. On the day of the experiment, mice were placed in clear plastic testing chambers for 60 min for habituation. Mice were then injected with either saline or NT79 (5 mg/kg, i.p.) and 30 min later with 20 μl of 5% formalin subcutaneously (s.c.) into the plantar surface of the right hind paw. The mice were then placed in clear plastic chambers for observation for 60 min. The data are presented as the time in seconds per 5-min interval the animals spent lifting/biting in a 1 h observation period as previously described [41, 42]. For the subchronic studies, groups of WT mice were tested for formalin-induced pain (day 1) and after five daily injections of NT79 (5 mg/kg, i.p.), morphine (1 mg/kg, s.c.) or the combination of both NT79 and morphine (day 5). The doses of NT79 and morphine used were based on our previous studies and were the minimum doses that will cause a significant analgesic effect in the formalin test [3]. Control animals were injected with saline and tested for formalin-induced pain on day 1 and day 5. On day 5 the mice were injected in the plantar surface of the left hind paw.

2.4. Statistical Analysis

Statistical analysis was performed with Sigma Stat software (SPSS, Inc., Chicago, Illinois, USA). A two-way ANOVA with repeated measures was performed within each genotype with variation of treatment across time (Fig. **1**). Average data were analyzed with one-way or two-way ANOVA followed by Holm-Sidak test for multiple comparisons. A *P*-value less than 0.05 is considered significant.

3. RESULTS

3.1. Effect of acute NT79 on formalin-induced pain

Subcutaneous injection of formalin into the plantar surface of the hind paw of the WT and $NTS2^{-/-}$ mice evoked

the biphasic (phase I and phase II) responses characteristic for this test (Fig. **1**).

A two-way ANOVA with repeated measures within each genotype showed a significant effect of treatment for WT mice ($F_{1,119}$=305.96 P<0.001) on formalin-induced lifting and biting throughout phase II but only initially (t=20 to 30 min) for the NTS2$^{-/-}$ mice (P=0.10) in the same phase. Time significantly affected the pain response for WT mice, ($F_{11,119}$=271.381 P<0.001), and NTS2$^{-/-}$ mice ($F_{11,119}$=37.04 P<0.001), and there was significant interaction of time x treatment for WT mice ($F_{11,119}$=96.295, P<0.001), and NTS2$^{-/-}$ mice ($F_{11,119}$=4.115 P<0.001), Fig. (**1**).

NT79 attenuated the pain response during phase II in the WT mice. A two-way ANOVA showed that genotype ($F_{1,19}$=15.749 P<0.001) and treatment ($F_{1,19}$=200.85 P<0.001) significantly affected formalin-induced pain. This was supported by significant genotype x treatment interaction ($F_{1,19}$=134.318 P<0.001). NT79 had a very mild analgesic effect in the NTS2$^{-/-}$mice, Inset Fig. (**1**).

With respect to individual pain behaviors, NT79 significantly blocked lifting (P<0.001) and biting (P<0.001) responses in the WT mice, but was without any effect in NTS2$^{-/-}$ mice (Fig. **2**).

3.2. Effect of Sub-Chronic Administration of NT79 Alone and in Combination with Morphine on Formalin-Induced Pain in WT Mice

Fig. (**3**) shows the effects of subchronic injections of NT79, morphine, and the combination of both drugs on formalin-induced pain. Injection of saline on day 1 and day 5 did not show significant difference (P=0.516) and thus these data were combined and presented as the saline group. One-way ANOVA showed a significant ($F_{6,47}$ = 56.011, P<0.001) effect of treatment (NT79, morphine, or the combination of NT79 and morphine) on reducing formalin-induced pain both on day 1 ($F_{3,29}$=12.447, P<0.001) and on day 5 ($F_{3,27}$=13.64, P<0.001).

More importantly, five daily injections of NT79 did not result in tolerance to its analgesic effects as there was no significant difference in response between animals injected with NT79 on day 1 or on day 5 (P=0.771). Repeated injections of morphine significantly (P=0.044) reduced its analgesic properties in the formalin test as compared to that for the saline control. The combination of NT79 and morphine had an enhanced analgesic effect as compared to saline (P<0.001) with no difference between day 1 and day 5 (P=0.807). The reduction in formalin-induced pain behaviors was stronger when NT79 and morphine were combined as compared to morphine alone (P=0.002) and to NT79 alone (P=0.025).

4. DISCUSSION

The present study was carried out to test the effects of subchronic administration of the NTS2-selective analog, NT79, on its analgesic properties in the formalin test, which is frequently used as a nociceptive assay for modeling persistent pain in laboratory animals [43]. The effect of repeated administration of NT79, morphine, and a combination of both drugs on antinociceptive tolerance was also assessed.

4.1. NTS2 and Formalin-Induced Pain

The formalin test measures the response to continuous pain generated by injured/inflamed tissue [43]; [44] and thus is considered to be more relevant to clinical pain states bridging the gap between acute and chronic pain [45]. Formalin injection evoked the two peaks of pain-related behaviors characteristic of this test [46]. Phase I is due to the effects of formalin on sensory receptors, and phase II is due to inflammation and central sensitization [28]. The duration of the two behaviors was similar to those of the rat [3]. Interestingly, the intensity of the pain response was lower in the NTS2$^{-/-}$ mice as compared to that of the WT mice. NTS2 exhibits constitutive activity [47], thus the lack of NTS2 might account for the reduced sensitivity to pain due to reduced constitutive signaling in NTS2$^{-/-}$ mice. This notion is

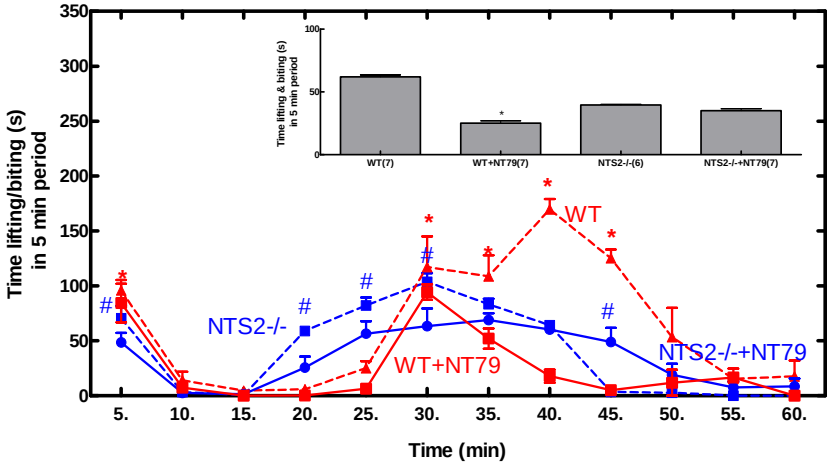

Fig. (1). Time course for the effects of NT79 on formalin-induced lifting and biting in WT and NTS2$^{-/-}$ mice. WT and NTS2$^{-/-}$ mice were divided into four groups. Animals were injected with NT79 (5 mg/kg i.p.) or saline and the formalin test was performed as described in the Methods section. The data are depicted as the average time (mean ± SEM) the animal spent lifting and/or biting in a 5 min period. Inset shows the average time (s) (mean +/- SEM) the animals spent lifting and/or biting per 5 min across 1 h period. *Significantly different from NT79-pretreated WT mice. #Significantly different from NT79-pretreated NTS2$^{-/-}$ mice. WT=wild type; NTS2$^{-/-}$=NTS2 knockout mice; (n) = number of animals in each group.

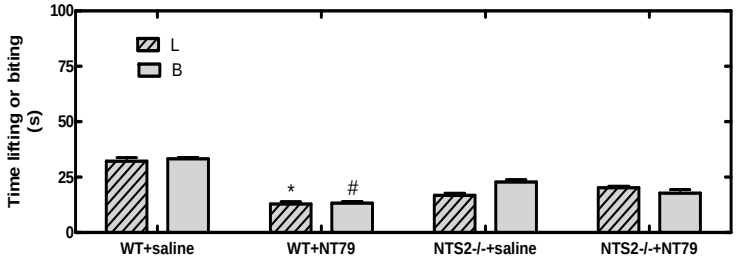

Fig. (2). Effects of NT79 on individual nociceptive behaviors in formalin tonic pain model in WT and NTS2$^{-/-}$ mice. Animals were maintained, treated, and tested as described in the legend to Fig. (**1**). Results come from data obtained with animals used in Fig. (**1**) and are presented as the average time (s) (mean +/- SEM) the animals spent lifting or biting per 5 min across 1 h period *Significantly different from lifting within the same genotype without NT79 pretreatment. #Significantly different from biting within the same genotype without NT79 pretreatment. P<0.05 is considered significant.

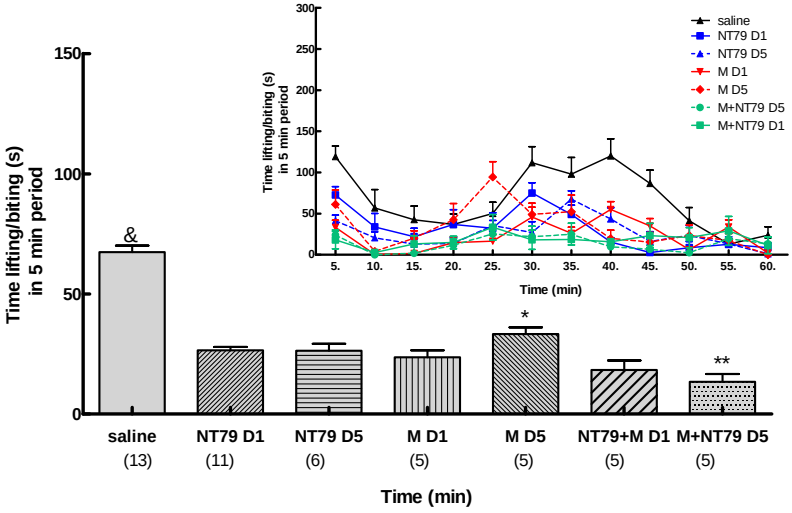

Fig. (3). Effects of acute (D1) and 5 daily treatments (D5) of NT79 (5 mg/kg), morphine (1 mg/kg), and their combination on formalin-induced lifting and biting in WT mice. Groups of animals were injected daily with NT79, morphine, or the combination of the two drugs and tested for formalin-induced lifting and biting on day 1 and day 5 as described in the Method section. $^{\&}$Significantly different from all treatments; *significantly different from M, D1; **significantly different from M, D5 and NT79, D5. M=morphine; D1=day 1; D5=day 5; (n) = number of animals in each group. Inset shows the time course for the data depicted in the bar graph.

further supported by the increase in pain intensity evoked by formalin in NTS1$^{-/-}$ mice (data not shown). NTS1$^{-/-}$ mice have higher expression of NTS2 [39], which might be associated with increased constitutive signaling.

NT receptors have been reported to play an important role in mediating the analgesic effects of the endogenous peptide as evidenced by the release of NT in the spinal cord [48] and by the up-regulation of NT-like immunoreactivity in fibers and terminals in superficial laminae of the dorsal horn ipsilateral to formalin injection [49]. Our data demonstrate, with the use of an NTS2-selective analog, NT79, and NTS2 knockout mice the importance of NTS2 in mediating formalin-induced nociceptive behaviors similar to its role in thermal nociception [16, 23], inflammatory pain [16], and tonic pain [28]. However, the role NTS2 plays in nociceptive behaviors is still controversial. Although Roussy *et al.* reported that NTS1 mediates the analgesic effects of NT in tonic spinal pain paradigms [50], the same group showed that intrathecal administration of NTS2 agonists (levocabastine and JMV-431) was effective in inhibiting the aversive behaviors induced by formalin. These results implicate NTS2 in mediating the analgesic effects of these compounds [28]. Maeno *et al.* reported that NTS2$^{-/-}$ mice display increased jump latency in the hot plate test [23], while others reported that NTS2$^{-/-}$ mice submitted to both acute and tonic pain stimuli show a greater sensitivity to pain in comparison to that for wild-type littermates [29]. Additionally, our data support reports indicating that the antinociceptive properties of NT agonists in the formalin test depend on the animal species tested [51].

4.2. Effect of Acute NT79 on Formalin-Induced Pain

As with our previous study in Sprague-Dawley rats [3], NT79 significantly attenuated the formalin-induced lifting and biting responses during phase II in the WT mice. As expected, based on its NTS2-selective properties, NT79 did not significantly attenuate formalin-induced lifting and biting responses in the NTS2$^{-/-}$ mice except for the 10 min of phase two (Fig. **1**). The limited analgesic activity in the NTS2$^{-/-}$ mice might be due to the low activity of NT79 at NTS1. These data strongly indicate that the antinociceptive effects of NT79 in phase II of the formalin test are mediated through NTS2. The analgesic effects of NT79 during phase II of the formalin test are shared by NT and NT receptor-active

compounds, such as NT69L, JMV-431, and levocabastine. NT and NT69L, which bind equally to both NTS1 and NTS2, significantly reduce pain-evoked responses during phase II of the formalin test. Accordingly, pretreatment with the NTS2-active compounds, JMV-431 and levocabastine, is effective in inhibiting the aversive behaviors induced by formalin in late phase II [28]. The difference between the effects of the JMV-431 and levocabastine and the effects of NT79 in the present study could be due to the partial affinity of NT79 for NTS1 [20], as well as the difference in the route of administration (intrathecal versus systemic, respectively).

Furthermore, assessment of the stereotypic pain behaviors of lifting and biting revealed that, unlike its effects in the rats, where NT79 reverses the formalin-induced lifting, but not the biting response, NT79 reversed all nociceptive endpoint behaviors in the mice. This effect is similar to the non-selective NTS1/NTS2 analogs. Thus, contrary the findings in rats, where NT79 causes spinal analgesia only, administration of NT79 in the mice affects both spinal and supra-spinal pain pathways. These results further support the notions that there is species variation in response to NT analogs and that variations in response are also dependent on the analog used and its NT receptor subtype selectivity.

4.3. Effect of Sub-Chronic Administration of NT79 on Formalin-Induced Pain

Perhaps the most important finding in this study was the lack of tolerance to the antinociceptive effects of NT79 after repeated administration of this peptide. We found similar results for its antinociceptive effects in the acetic acid-induced writhing test, where NT79 had significant antinociceptive effects after eight daily injections (Boules *et al.*, unpublished data). It is interesting that tolerance does not appear to develop or develops very slowly to NT effects thought to be mediated by NTS2. These results support previous studies by our group and others showing that the development of tolerance is more prevalent in behavioral effects mediated by NTS1, as compared to those mediated by NTS2 [52-55]. Conversely, subchronic administration of morphine significantly reduced its analgesic effect as has been reported by others for formalin-induced pain [56, 57]. Interestingly, the analgesic effects of the combined administration of NT79 and morphine were enhanced over either drug alone after five daily injections (Fig. **3**). This result potentially has great clinical significance for the treatment of persistent pain in patients, who very frequently are treated long-term with opioids.

5. CONCLUSION

In conclusion, the use of NTS2 knockout mice provided evidence that NTS2 plays a major role in the regulation of spinal and supra-spinal nociception in mice. Also, NTS2-selective compounds may be a new class of novel analgesics for the treatment of persistent pain without the development of tolerance. Finally, the co-administration of an NTS2-selective compound in combination with an opioid could provide a paradigm shift in the treatment of chronic pain.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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PATIENT'S CONSENT

Declared none.

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