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ALTERED MORPHINE-INDUCED ANALGESIA IN NEUROTENSIN TYPE 1 RECEPTOR NULL MICE

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Abstract—Both neurotensin (NT) and opioid agonists have been shown to induce antinociception in rodents after central administration. Besides, previous studies have revealed the existence of functional interactions between NT and opioid systems in the regulation of pain processing. We recently demonstrated that NTS1 receptors play a key role in the mediation of the analgesic effects of NT in long-lasting pain. In the present study, we therefore investigated whether NTS1 gene deletion affected the antinociceptive action of mu opioid drugs. To this end, pain behavioral responses to formalin were determined following systemic administration of morphine in both male and female NTS1 knockout mice. Acute injection of morphine (2 or 5 mg/kg) produced strong antinociceptive effects in both male and female wild-type littermates, with no significant sex differences. On the other hand, morphine analgesia was considerably reduced in NTS1-deficient mice of both sexes compared to their respective controls, indicating that the NTS1 receptor actively participates in mu opioid alleviating pain. By examining specifically the flinching, licking and biting nociceptive behaviors, we also showed that the functional crosstalk between NTS1 and mu opioid receptors influences the supraspinally-mediated behaviors. Interestingly, sexual dimorphic action of morphine-induced pain inhibition was found in NTS1 null mice in the formalin test, suggesting that the endogenous NT system interacts differently with the opioid network in male and female mice. Altogether, these results demonstrated that NTS1 receptor activation operates downstream to the opioidergic transmission and that NTS1-selective agonists combined with morphine may act synergistically to reduce persistent pain.

Key words: tonic pain, formalin, opioid, supraspinal, crosstalk.

Both neurotensin (NT) and opioid agonists have been shown to induce antinociception in rodents after intracerebral administration. It is now well established that the spinal nociceptive transmission is modulated by inhibitory and facilitatory systems that originate from supraspinal sites in the CNS (Porreca et al., 2002; Gebhart, 2004; Vanegas, 2004; Heinricher et al., 2009). In particular, the neuronal network connecting the midbrain periaqueductal gray (PAG) and the rostral ventromedial medulla (RVM) actively contributes to the analgesic actions of mu opioid agonists, including morphine as well as to those mediated by NT agonists (Dobner, 2006; Loyd and Murphy, 2009). In support, opioid- and NT-peptide immunoreactive nerve terminals, as well as high densities of opioid- and NT-receptors are reported in most descending antinociceptive circuits (Basbaum and Fields, 1984; Sarret and Beaudet, 2003). Therefore, from an R&D perspective, it is of particular interest to know whether the coexistence of NT and opioid systems in cerebral structures implicated in pain modulation leads to their functional interactions.

Originally, NT was found to exert a potent antinociceptive effect in a mu opioid-independent manner in a variety of analgesic screening tests, including tail-flick, hot plate and writhing induced by acetic acid (Clineschmidt et al., 1979, 1982). Indeed, the analgesic effects of NT, reported as being more potent than morphine at equimolar doses, were still effective in the presence of two structurally related opioid antagonists, naloxone or naltrexone (Nemeroff et al., 1979; Osbahr et al., 1981; Behbehani and Pert, 1984; Coquerel et al., 1986; al-Rodhan et al., 1991; Sarhan et al., 1997; Boules et al., 2009). In that respect, the neurotensinergic system plays a pivotal role in the non-opioid form of stress-induced analgesia (Seta et al., 2001; Gui et al., 2004; Lafrance et al., 2010). There is, however, existing data indicating the analgesic actions of NT which are dependent on the functional integrity of the brain opioid system (van Wimersma Greidanus et al., 1982; Yaksh et al., 1982; Furuta et al., 1984). Above all, mice rendered tolerant to morphine show an attenuated antinociceptive profile to central NT administration (Luttinger et al., 1983). Based on these studies, it would appear that NT-induced pain suppression is, at least in part, generated by distinct mechanisms from those of the opioid peptides.

Of obvious importance is whether opioid-induced antinociception is also independent of the endogenous NT system. In this regard, morphine and the mu opioid receptor-selective agonist DAMGO both induced a naloxone-reversible increase of the NT release in the medial PAG, suggesting that local NT release from either intrinsic neurons or afferent terminals within the PAG may be involved in opioid-induced analgesia (Stiller et al., 1997). Similarly, the antinociception induced by the mu-opioid receptor activation in the amygdala is partly dependent on the recruitment of NT receptors in the ventral PAG (Tershner and Helmstetter, 2000). However, it has also been demon-

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Abbreviations: ANOVA, analysis of variance; AUC, area under curve; NT, neurotensin; PAG, periaqueductal gray; RVM, rostral ventromedial medulla; SEM, standard errors of mean.

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strated that NT signaling disruption, by the use of either NT antiserum or NT receptor antagonists reinforced the antinociceptive action of morphine, revealing this time an "anti-opioid" effect of NT (Urban and Smith, 1993, 1994; Smith et al., 1995, 1997). These opposing findings may nevertheless be explained by the bidirectional effect of NT: NT inducing pain-facilitatory or -inhibitory dose-related behavioral responses (Urban and Gebhart, 1997; Urban et al., 1999; Neubert et al., 2004). The fact that NT produces dose-dependent antinociceptive and anti-analgesic effects within the descending PAG-RVM pathway also suggests interaction with multiple pain modulatory systems, possibly via distinct NT receptor subtypes. Accordingly, the central role of NT in pain modulation is dependent on the activation of two seven-transmembrane domain G protein-coupled receptors, designed NTS1 and NTS2 (Dobner, 2006; Sarret et al., 2007). Among the studies demonstrating the involvement of NT receptors in nociceptive processing, we recently demonstrated that NTS1 receptors play a key role in mediating the analgesic effects of NT in long-lasting pain (Roussy et al., 2008).

Based on the pivotal role of NT and opioid peptides in regulating pain transmission, the current study was undertaken to determine if alterations in NTS1 signaling would affect the antinociceptive action of systemically administered morphine. For this purpose, we evaluated the analgesia induced by morphine in mice deficient for the high affinity NTS1 receptor (NTS1-KO), by measuring the formalin-evoked nociceptive behaviors in a widely accepted model of persistent inflammatory pain (Tjolsen et al., 1992; Coderre et al., 1993; Sawynok, 2004). Since gender/sex-related differences in pain and analgesia are reported in humans and rodents, both male and female mice were used to study the functional interaction between NT and opioid receptor systems (Kest et al., 2000; Mogil et al., 2000; Craft, 2003; Greenspan et al., 2007; Loyd and Murphy, 2009).

EXPERIMENTAL PROCEDURES

Animals, housing and habituation

NTS1-KO mice were backcrossed with C57BL/6 mice (Charles River, St-Constant, QC, Canada). NTS1-KO and wild-type littermate adult male and female mice (20–30 g) used throughout the experiments were generated by mating heterozygous mice. The absence of expression of the NTS1 gene in NTS1-deficient mice was confirmed by RT-PCR analyzes, as previously described (Maeno et al., 2004). Routine genotyping was conducted by PCR on purified tail DNA using two NTS1-specific primers (5′-GTT AAC ACC TTC ATG TCC TTC CTG-3′, 5′-TAC GTA AGA CGA GGA CTC CAT GGC G-3′) and two neo primers (5′-GGA TCG GCC ATT GAA CAA GAT GG-3′; 5′-CTT CAG CAA TAT CAC GGG TAG CC-3′). The expected sizes for the WT and KO alleles were 200 and 700 bp, respectively. Animals were kept on a 10-h light/14-h dark cycle and allowed *ad libitum* access to food and water. Animals were individually acclimatized to Plexiglas enclosures and handling for 3 consecutive days prior testing. Mice were randomly assigned to control and drug treatments. Furthermore, the behavioral observations were performed by two experimenters blinded to the genotypes in a quiet room, between 9:00 and 12:00 PM to reduce any variation related to circadian rhythm. Experiments were approved by the animal care committee at the Université de Sherbrooke in compliance with the policies and directives of the Canadian Council on Animal Care and guidelines from the International Association for the Study of Pain.

Behavioral studies

Intraperitoneal administration of mu-opioid receptor agonist before formalin injection. Mice were injected i.p. with morphine (2 or 5 mg/kg, Sabex Inc., Boucherville, QC, Canada) diluted in physiological saline (0.9% NaCl) 15 min before formalin administration. Control mice received physiological saline.

Formalin test. Antinociception was assessed using the formalin test as a model of tonic pain. For this purpose, mice were placed for a 60-min habituation period in the experimentation room. Thereafter, mice received a 20 μl intradermal injection of 2% formaldehyde solution (i.e. 5.4% formalin, Fisher Scientific, Montreal, QC, Canada) into the plantar surface of the right hind paw. Following this, mice were placed in clear plastic chambers ($30\times30\times30$ cm^3) positioned over a mirror angled at 45° in order to allow an unobstructed view of the paws. Their behaviors were then observed for the next 60 min. An intraplantar injection of formalin produced a biphasic nociceptive response, typical of this tonic pain model (Tjolsen et al., 1992; Coderre et al., 1993). The two distinct phases of spontaneous pain behaviors that occur in rodents are proposed to reflect a direct effect of formalin on sensory receptors (phase I) and a longer lasting pain due to inflammation and central sensitization (phase II). These two phases are separated by a period of quiescence, namely the interphase characterized by an active inhibition of the formalin-induced nociceptive behaviors (Sawynok, 2004).

Nocifensive behaviors were assessed using a weighted score method as described previously (Dubuisson and Dennis, 1977; Coderre et al., 1993). Following an injection of formalin into the right hind paw, the experimenter measured the time spent in each of four behavioral categories: 0, the injected paw is comparable to the contralateral paw; 1, the injected paw has little or no weight placed on it; 2, the injected paw is elevated and is not in contact with any surface; 3, the injected paw is licked, bitten, or flinched. The behaviors believed to represent higher levels of pain intensity were given higher weighted scores. The weighted average pain intensity score ranging from 0 to 3 was then calculated by multiplying the time spent in each category by the category weight, summing these products and dividing by the total time in a given time interval. The pain score was thus calculated from the following formula $(1T1+2T2+3T3)/180$ where T1, T2 and T3 are the durations (in sec) spent in behavioral categories 1, 2 or 3 respectively during each 180 s block. Phase I and II values were calculated between 0–9 min and 21–60 min, respectively. To statistically compare the magnitude of the morphine analgesia between wild-type and NTS1-KO mice, dose-response curves were generated for phase II by calculating the area under the curve (AUC) for each dose and for each genotype. The AUC of "pain score–time" above the weighted pain score of 1 were calculated by the trapezoidal rule using Prism 4.0. Alternatively, formalin-induced pain-related behaviors were quantified by monitoring the cumulative time spent in flinching/licking/biting during the inflammatory phase (phase II) (Tjolsen et al., 1992). This representation of the data allowed to determine whether the differences in pain responses between wild-type and NTS1-KO mice occurred at the supraspinal level.

Statistical analysis. Data are presented as means±standard errors of the mean (SEM). All calculations and statistical analysis were performed using Prism 4.0 and Instat 3.05 (Graph Pad Software, San Diego, CA, USA). Nociceptive scores over the 3 min time blocks were analyzed using a two-way analysis of variance for repeated measures, with comparisons between experimental groups