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Effects of Neurokinin-1 Receptor Agonism and Antagonism in the Rostral Ventromedial Medulla of Rats with Acute or Persistent Inflammatory Nociception

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Abstract

The rostral ventromedial medulla (RVM), a central relay in the bulbospinal pathways that modulate nociception, contains high concentrations of Substance P (Sub P) and neurokinin-1 (NK1) receptors. However, the function of Sub P in the RVM is poorly understood. This study characterized the actions of Sub P in the RVM in the absence of injury and then used two NK1 receptor antagonists, L-733,060 and L-703,606, to probe the role of endogenously released Sub P in the development and maintenance of persistent inflammatory nociception of immune or neurogenic origin. In uninjured rats, microinjection of Sub P in the RVM produced a transient thermal antinociception that was attenuated by pretreatment with L-733,060 or L-703,606. It did not alter threshold to withdrawal from tactile stimulation with von Frey filaments. Microinjection of the antagonists alone did not alter paw withdrawal latency or threshold suggesting that Sub P is not tonically released in the RVM in the absence of injury. However, microinjection of either antagonist in the RVM was sufficient to reverse heat hyperalgesia 4 h, 4 days or 2 weeks after intraplantar injection of complete Freund's adjuvant (CFA). Antagonism of NK1 receptors in the RVM did not prevent or reverse tactile hypersensitivity induced by CFA, but did attenuate that produced by capsaicin. NK1 receptor antagonism did not prevent the development of thermal hyperalgesia, tactile hypersensitivity or spontaneous pain behaviors induced by mustard oil. The results suggest that Sub P has bimodal actions in the RVM and that following inflammatory injury, it can play a critical role as a pronociceptive agent in the development and maintenance of hyperalgesia and tactile hypersensitivity. However, its actions are highly dependent on the stimulus modality and the type of injury, and this may be an additional basis for the poor efficacy of NK1 receptor antagonists in clinical trials.

Keywords

hyperalgesia; substance P; neurokinin; complete Freund's adjuvant; capsaicin; mustard oil

In the focus on Substance P (Sub P) as a mediator of nociception in the periphery and spinal cord (reviewed by Baranauskas and Nistri, 1998; Sandkuhler et al., 2000; Schaible et al.,

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2002; Keeble and Brain, 2004), its ability to produce antinociception when administered supraspinally has often been overlooked. For example, intracerebroventricular injection of Sub P increases the threshold for vocalization and vocalization afterdischarge to noxious electrical stimulation of the tail (Naranjo et al., 1982). Microinjection of Sub P in the periaqueductal gray (PAG) or the A7 catecholamine nucleus increases withdrawal latencies to heat stimulation of the tail or the hindpaw (Malick and Goldstein, 1978; Mohrland and Gebhart, 1979; Yeomans and Proudfit, 1992; Rosén et al., 2004), and increases threshold to noxious mechanical stimulation of the hindpaw after injection in the PAG (Rosén et al., 2004). Finally, microinjection of the NK1 receptor agonist GF73632 in the ventral tegmental area or the nucleus accumbens suppresses flinching in the formalin test (Altier and Stewart, 1997). Studies with neurokinin-1 (NK1) receptor antagonists have further established the antinociceptive action of endogenously released Sub P. Microinjection of RP-8950 in the ventral tegmental area antagonizes foot shock stress-induced antinociception (Altier and Stewart, 1999), while microinjection of L-703,606 in the rostral ventromedial medulla (RVM) or the PAG reverses the antinociception produced by microinjection of carbachol in the lateral hypothalamus (Holden and Pizzi, 2008; Holden et al., 2009).

Although these data provide substantive evidence for an antinociceptive role of Sub P and NK1 receptors at supraspinal sites in conditions of acute nociception, the function of this neurotransmitter in conditions of persistent pain is not nearly as well understood. Persistent inflammatory nociception alters the physiology and pharmacology of neurons in the rostral ventromedial medulla (RVM), a central relay in the bulbospinal pain inhibitory and pain facilitatory pathways that modulate the synaptic transmission of sensory information in the spinal cord. Persistent inflammatory injury leads to time-dependent changes in the actions of both inhibitory and excitatory amino acid (Gilbert and Franklin, 2001; Guan et al., 2002; Miki et al., 2002; Guan et al., 2004), as well as opioid neurotransmitters in the RVM (Hurley and Hammond, 2000, 2001; Schepers et al., 2008). The RVM contains high concentrations of Sub P and NK1 receptors (Ljungdahl et al., 1978; Nakaya et al., 1994). Yet, very little is known about their role in the modulation of acute nociception by the RVM or whether these actions change under conditions of persistent nociception. Simone and colleagues have suggested that Sub P may function to facilitate nociception under conditions of acute inflammatory injury induced by capsaicin (Pacharinsak et al., 2008), and indeed determined that pain facilitatory neurons ("ON" cells) express NK1 receptors (Budai et al., 2007). To further pursue this proposal, the present study first characterized the actions of Sub P in the RVM in the absence of inflammatory injury. It then used NK1 receptor antagonists to probe the role of endogenously released Sub P in the RVM in the modulation of thermal or mechanical threshold during acute, subacute or persistent inflammation induced by intraplantar (ipl) injection of complete Freund's adjuvant (CFA) or transient neurogenic inflammation induced by capsaicin or mustard oil (MO). The results suggest that conditions of persistent inflammatory nociception can cause a phenotypic switch in the function of Sub P in the RVM. However, the effects are highly dependent on the stimulus modality and the type of injury. These results highlight how the development of centrally penetrant analgesics requires a better understanding of the consequences of persistent pain for the function of targeted receptors and neurotransmitters at supraspinal sites.

METHODS AND MATERIALS

These experiments were approved by the University of Iowa Animal Care and Use Committee, and conducted in accordance with the *Guide for Care and Use of Laboratory Animals* as promulgated by the National Institutes of Health and the guidelines of the International Association for the Study of Pain (Zimmermann, 1983). Every effort was made to minimize the number of rats used and their suffering.

Animal Preparation

Adult male Sprague-Dawley rats (Charles River, Raleigh, NC) weighing 275–325 g were used in these studies. Rats were anesthetized with a mixture of ketamine (70 mg/kg i.p.) and xylazine (13 mg/kg i.p.), placed in a stereotaxic instrument and implanted with an intracerebral guide cannula (26-gauge; Plastics One, Roanoke, VA) using aseptic and sterile tip methods. The guide cannula terminated 3 mm dorsal to the RVM. Cannulae were affixed to the skull with stainless steel screws and dental acrylic, and a stainless steel stylet was inserted in each guide cannula to maintain patency. After surgery, animals were housed individually and maintained on a 12-h light/dark cycle with free access to food and water. Cannulae were implanted seven days before behavioral testing.

Models of Inflammatory Injury

Complete Freund's adjuvant (CFA) was used to model an immune mediated acute, subacute and prolonged inflammatory injury. The rats were lightly anesthetized with isoflurane and the thickness of the hindpaw in the dorsoventral axis was measured with digital calipers. The left hind paw was then injected with 150 μ l of CFA (150 μ g of *Mycobacterium butyricum*, 85% Marcol 52, and 15% Aracel A mannide monoemulsifier; Calbiochem, San Diego, CA) or saline, pH 7.4. Rats were returned to their cages for four h, four days, or two weeks. These experiments were conducted using cohorts of rats in which rats from each shipment (18–24 at a time) were allocated among the six different treatment conditions (i.e., CFA or saline injection; four h, four day or two week survival time). Multiple replicates of each cohort were performed.

Transient inflammatory injury of neurogenic origin was modeled by ipl injection of the transient receptor potential V1 (TRPV1) channel agonist capsaicin (5 μ g in 10 μ l; 0.05% w/v) or the TRPA1 channel agonist mustard oil (MO) (500 μ g in 50 μ l; 1% v/v). The rats were swaddled in a towel for injection of capsaicin or MO in the left hind paw.

Tactile Hypersensitivity

Rats were acclimated to the testing room for 30 min. To assess tactile hypersensitivity, rats were placed on an elevated mesh surface and allowed to acclimate for a further 15 min. Monofilaments of increasing force (Smith & Nephew, Germantown, WI, USA) were applied to the webbing between the 3rd and 4th digits of the hindpaw. Paw withdrawal threshold (PWT) was determined using the Up-and-Down method of Dixon as described by Chaplan et al. (1994). The maximum filament used was 28.8 g because this force passively lifted the hindpaw of rats of this weight. Rats that did not respond to this filament were assigned this value.

Heat Hyperalgesia

Rats were acclimated to the testing environment for 30 min and then placed in individual Plexiglas chambers situated on a 25°C glass surface for another 15-min period of acclimation. Nociceptive sensitivity was measured by focusing a high-intensity beam of light on the plantar surface of the hindpaw. The time required for the rat to withdraw its hindpaw from the thermal stimulus was termed the paw withdrawal latency (PWL). The lamp was adjusted to elicit baseline PWL of 8 to 12 s. If a withdrawal response did not occur within 20 s, the test was terminated to prevent tissue injury and the rat was assigned this latency.

Microinjection Procedure and Drugs

All agents were microinjected in a volume of 0.50 μ l via a 33-gauge stainless steel injector needle that extended 3 mm beyond the guide cannula tip. Drug delivery was monitored by following the movement of an air bubble in the tubing that connected the injector to the syringe pump. The needle was left in place for 60 s after the injection to minimize diffusion of drug

up the injection tract. Each rat was used once. The investigator was blinded to the test drug. The oxalate salt of L-703,606 was placed into solution in 1% β -hydroxycyclodextrin at pH 7.0, which served as its vehicle. The hydrochloride salt of L-733,060 readily dissolved in saline at pH 7.0, which served as its vehicle. Solutions of Sub P were adjusted to pH 6.8 because it is not stable at a neutral pH; saline at pH 6.8 served as its respective control. All drugs were obtained from Sigma-Aldrich (St. Louis, MO.)

Histology

After testing, animals were euthanized by CO₂ inhalation. Brains were removed and fixed by immersion in 10% formalin containing 30% sucrose. Transverse sections were cut on a cryostat microtome and stained with cresyl violet. Injection sites were identified by a person blinded to the behavioral outcome. Sites were plotted on an atlas of the rat brain and then verified by a second individual also blinded to behavioral outcome. Microinjection sites that were located in the nucleus reticularis gigantocellularis pars α , usually 1–2 per treatment condition were included with those in the nucleus raphe magnus and collectively referred to as the RVM.

Statistical Analysis

Data were expressed as the mean and S.E.M. Two-way repeated measures analysis of variance, in which the repeated factor was time and the other factor was treatment condition, was used to compare PWL among the different groups. Post-hoc comparisons among mean values for the individual treatment groups were made using Newman-Keul's test. As PWT was non-parametric, a Mann–Whitney U test was used to compare the median values of the groups at each time point tested. A $P \leq 0.05$ was considered significant.

RESULTS

Effects of Sub P in the brainstem in the absence of inflammation: heat

The first set of experiments examined the effect of Sub P on responses to noxious heat stimuli in naïve rats. Microinjection of 100 pmol Sub P at sites within the RVM (circles, Fig. 1A) produced a transient increase in PWL that was followed 30 min later by a decrease in PWL compared to saline (Fig. 1B). Lower doses of Sub P did not increase PWL, but did decrease PWL at later times (Fig. 1B, C). Microinjection of 100 pmol Sub P at sites dorsal or dorsal lateral to the RVM (squares, Fig. 1A) also increased PWL to a lesser extent, but did not decrease PWL at any time (Fig. 1D). Microinjection of 100 pmol Sub P in the vestibular nuclei, rostral and dorsal to the RVM, or in the pyramids did not alter PWL (pentagons, Fig. 1A).

The second set of experiments established effective doses of two different NK1 receptor antagonists. These experiments were conducted in rats that had received an ipl injection of saline 4 days earlier (hereafter referred to as saline-treated rats) to more closely parallel other studies in which CFA was injected. Microinjection of either 20 nmol L-703,606 or 10 pmol L-733,060 in the RVM antagonized the increase in PWL produced by microinjection of 100 pmol Sub P at the same site (Fig. 2). Both antagonists produced a significant inhibition, although the optimal pretreatment time differed. L-733,060 was most effective when administered 15 min before Sub P (Fig. 2A), and was ineffective when administered 30 min before the agonist (data not shown). In contrast, L-703,606 was most effective when administered 30 min before Sub P (Fig. 2B), and was ineffective when administered 15 min before the agonist (data not shown). Microinjection of 10 pmol L-733,060 by itself in the RVM did not alter PWL (Fig. 2C). Although 20 nmol L-703,606 produced a statistically significant increase in PWL compared to saline, the effect was not considered biologically significant as the values did not differ from baseline (Fig. 2D).

Effects of Sub P in the brainstem in the absence of inflammation: tactile

The effect of Sub P on responses to tactile stimulation with von Frey filaments was also examined in saline-treated rats. Microinjection of 100 pmol Sub P in the RVM of rats that had received an injection of saline at the same site 15 min earlier was followed by a progressive decrease in PWT of both hindpaws over the next hour (Fig. 3A,B). Interestingly, microinjection of L-733,060 in the RVM 15 min before microinjection of Sub P at the same site delayed, but did not prevent the progressive decrease in PWT in both hindpaws (Fig. 3A,B). While these results initially suggested a pronociceptive effect of Sub P, in the course of testing the effects of L-733,060 alone it was determined that microinjection of saline alone in the RVM was also followed by a progressive decrease in PWT over time, as was microinjection of 10 pmol L-733,060 alone (Fig. 3C,D and insets Fig. A,B). The effects of L-733,060 alone and saline did not differ. Furthermore, the blinded observer reported that, regardless of the agent administered, the rats often became more attentive and more responsive to repeated applications of von Frey filaments to both hindpaws over a period of 60 min. Thus, the apparent pronociceptive effect of Sub P could not be clearly dissociated from a possible increase in the animal's vigilance due to repetitive stimulation. The ability of L-733,060 to obtund the decrease in PWT observed after microinjection of saline in the RVM was not tested. The effect of L-703,606 on PWT was not determined.

Antagonism of NK1 receptors in the RVM reverses heat hyperalgesia induced by CFA

Having established effective doses of L-733,060 and L-703,606, the next set of experiments examined the role of endogenous Sub P and NK1 receptors in acute, subacute and persistent heat hyperalgesia produced by ipl injection of CFA. L-733,060 was used for the majority of subsequent studies because it was readily soluble in saline, nearly a thousand-fold more potent, and produced a more complete antagonism of the effects of Sub P.

Antagonism of NK1 receptors by microinjection of L-733,060 or L-703,606 in the RVM reversed heat hyperalgesia induced by CFA (Fig. 4). Four h after injection of CFA, microinjection of 10 pmol L-733,060 in the RVM partially reversed the heat hyperalgesia on the ipsilateral inflamed hind paw (Fig. 4A). Microinjection of 10 pmol L-733,060 in the RVM completely reversed the heat hyperalgesia in rats four days and two weeks after injection of CFA (Fig. 4B,C). Antagonism by L-733,060 was maximal 15 min after its microinjection, diminished by 30 min and nearly absent by 45 min. Microinjection of L-733,060 in the NGC also reversed heat hyperalgesia when examined in rats treated 4 days earlier with CFA (Fig. 4B). A lower dose of L-733,060 (2 pmol) in the RVM was also able to obtund heat hyperalgesia when tested in rats treated four h earlier with CFA. The reversal was less (to 6.6 ± 0.7 sec; $N = 9$) and only evident at 15 min, so this dose was not tested further. Comparable results were obtained with 20 nmol of L-703,606 when microinjected in the RVM of rats four days after CFA (Fig. 4D). However, the reversal of the heat hyperalgesia was more prolonged and persisted through 60 min.

Paw withdrawal latencies of the contralateral, uninflamed hind paw were not affected in a consistent manner. Microinjection of L-733,060 in the RVM four h (Fig. 5A), but not four days or two weeks (Fig. 5B,C) after CFA treatment produced a modest increase in PWL (Fig. 5A). Microinjection of L-703,606 in the RVM also increased PWL four days after CFA treatment (Fig. 5D).

Antagonism of NK1 receptors in the RVM modestly enhances tactile hypersensitivity induced by CFA

Microinjection of 10 pmol L-733,060 in the RVM did not reverse the established tactile hypersensitivity induced by ipl injection of CFA either 4 h or 4 days earlier; effects at two weeks were not tested. Rather, it caused a further decrease in PWT compared to saline (Fig.

6A,B). To determine whether the lack of antagonism reflected an inability to reverse an established tactile hypersensitivity, L-733,060 or saline was injected in the RVM 15 min before ipl injection of CFA (Fig. 6C). However, pretreatment with L-733,060 did not prevent or obtund the development of tactile hypersensitivity on the ipsilateral hindpaw. Microinjection of L-733,060 also enhanced tactile hypersensitivity on the contralateral hindpaw of CFA-treated rats (data not shown).

Antagonism of NK1 receptors in the RVM prevents the tactile hypersensitivity induced by capsaicin, a TRPV1 agonist

The inability of NK1 receptor antagonism to reverse or prevent tactile hypersensitivity induced by CFA was unexpected given a prior report that it prevented tactile hypersensitivity induced by capsaicin (Pacharinsak et al., 2008). Figure 7 illustrates that, as previously reported, tactile hypersensitivity induced by ipl injection of capsaicin was significantly reduced in rats in which 10 pmol L-733,060 was microinjected in the RVM compared to the effect of saline. The antagonism was maximal 5 min after the injection of capsaicin (20 min after L-733,060), and then diminished (Fig. 7A). Paw withdrawal threshold was unchanged in the contralateral hindpaw of capsaicin-treated rats in either the saline- or L-733,060 treatment group (data not shown).

NK1 receptor antagonism in the RVM does not prevent thermal hyperalgesia, tactile hypersensitivity or spontaneous pain behaviors induced by MO, a TRPA1 agonist

Microinjection of 10 pmol L-733,060 in the RVM 15 min before ipl injection of MO did not diminish either the magnitude or the duration of the heat hyperalgesia ($P > 0.4$) or tactile hypersensitivity ($P > 0.1$) (Fig. 8A,B). Mustard oil did not alter the PWL in the contralateral hindpaw in either saline- or L-733,060 treated rats (data not shown). Repeated application of von Frey filaments to the contralateral hindpaw of MO-treated rats caused a progressive decrease in PWT from approximately 28 g to 10 g over the 60 min testing period. The decline was equivalent in both saline- and L-733,060 treated rats (data not shown). Mustard oil also evoked a brief period of licking and lifting of the ipsilateral hindpaw in the 15 min following its injection (Fig. 8C). Pretreatment with L-733,060 in the RVM did not reduce these behaviors (Fig. 8C; $P > 0.2$ for lifting, licking or total). Spontaneous nociceptive behaviors were not observed in the contralateral hindpaw of MO-treated rats.

DISCUSSION

Actions of Sub P in the absence of injury

This study extends the supraspinal sites at which Sub P acts to produce thermal antinociception to include the RVM and the NGC immediately dorsal or dorsolateral to the RVM. The antinociceptive effect was mediated by NK1 receptors because the increase in PWL was attenuated by pretreatment with two structurally dissimilar NK1 receptor antagonists. Spantide, a peptidergic NK1 receptor antagonist, similarly reverses the antinociceptive effect of Sub P in the PAG (Rosén et al., 2004). The antinociceptive effect of Sub P was very short-lived, consistent with the brief half-life of this peptide (Eison et al., 1982; Michael-Titus et al., 2002). The antinociceptive effect was followed approximately 30 min later by a modest heat hyperalgesia as compared to saline. However, the effect was not dose-dependent, PWLs were not significantly less than baseline values in these rats, and the hyperalgesia was not replicated in another experiment when Sub P was microinjected in rats in which saline had been injected in the RVM 15 min earlier. The present study further established that Sub P is not tonically released in the RVM in the absence of inflammation because neither NK1 receptor antagonist decreased PWL when microinjected in the RVM, confirming a recent report (Holden and Pizzi, 2008). Collectively, these data indicate that in the absence of injury, Sub P acts in the RVM to

produce thermal antinociception, but is not tonically released in the RVM in absence of inflammation.

The ability of Sub P to produce mechanical antinociception was not tested, although its effects on tactile threshold were examined. Paw withdrawal threshold progressively decreased in the 60 min after microinjection of Sub P in the RVM. A similar progressive decrease in PWT occurred after microinjection of pH-matched saline in the RVM suggesting that the effect was secondary to changes in the vigilance of the animals. Nonetheless, pretreatment with L-733,060 delayed or obtunded the decrease in PWT observed after microinjection of Sub P. These data suggest that Sub P has a pronociceptive action in the RVM with respect to mechanical stimuli; however, changes in vigilance could also contribute. Because PWT decreased in a comparable manner after microinjection of L-733,060 by itself or saline, it is unlikely that prolonged repetitive testing of both hindpaws with von Frey filaments causes a significant release of Sub P in the RVM in the absence of injury.

Actions of endogenous Sub P in under conditions of inflammatory injury

Having established effective doses of L733,060 and L703,606, this study then investigated the role of endogenously released Sub P in the RVM under conditions of persistent inflammatory nociception. Three different models of inflammatory injury, each of which induces heat hyperalgesia and tactile hypersensitivity, were used. Intraplantar injection of CFA was used to model an immune-based inflammatory injury of acute (4 h), subacute (4 days), and prolonged (2 weeks) duration. Capsaicin and MO were used to model neurogenic inflammation of brief duration, and to respectively activate TRPV1- and TRPA1-expressing primary afferent neurons. All three approaches examined primary hyperalgesia or hypersensitivity, i.e. responsiveness at the site of injury. The role of medullary Sub P in the development and maintenance of secondary heat hyperalgesia and tactile hypersensitivity remains to be tested.

In the CFA model, antagonism of NK1 receptors in the RVM or NGC was sufficient to reverse established heat hyperalgesia 4 h, 4 days and 2 weeks after inflammatory injury. These data suggest that Sub P is released in the RVM after inflammatory injury and has a critical role in both the initiation and maintenance of heat hyperalgesia. The finding that antagonism of NK1 receptors in the RVM did not alleviate tactile hypersensitivity in the CFA model was unexpected. It was previously reported (Pacharinsak et al., 2008), and replicated in this study, that pretreatment with L-733,606 in the RVM prevented tactile hypersensitivity produced by ipl injection of capsaicin. However, L-733,060 neither reversed nor prevented tactile hypersensitivity induced by CFA. Rather, it slightly enhanced established tactile hypersensitivity suggesting that endogenously released Sub P may have anti-allodynic or antinociceptive actions in the RVM for tactile stimuli under conditions of inflammatory injury. This finding is not the first instance in which Sub P and NK1 receptors have been shown to be differentially involved in the processing of heat and mechanical stimuli. Lamina I and V neurons in mice that lack Sub P exhibit deficits in their response to noxious heat, but not to noxious mechanical stimulation. In addition, in mice lacking Sub P, MO-induced sensitization to heat stimuli was greatly reduced in these neurons, whereas sensitization to mechanical stimuli was retained (Mazario and Basbaum, 2007). Interestingly, Simone and colleagues recently reported that ablation of NK1 expressing RVM neurons by microinjection of saporin-Sub P more effectively reduced capsaicin- and CFA-induced thermal hyperalgesia than mechanical hypersensitivity (Brink et al., 2009).

Mustard oil shares mechanisms of nociception with capsaicin, including neurogenic release of Sub P (Andrade et al., 2008; Ruparel et al., 2008). Further, it produced heat hyperalgesia and tactile hypersensitivity equivalent to that produced by capsaicin. It was therefore expected that NK1 receptor antagonism in the RVM would prevent the heat hyperalgesia and tactile hypersensitivity produced by MO. This was not the case. A possible explanation can be

proposed based on differences in the spontaneous pain behaviors produced by capsaicin and MO. The total duration of licking and lifting after ipl injection of capsaicin in five rats was 241.8 ± 67.7 and 48.6 ± 16.9 sec, respectively. These values did not differ from those recorded after MO. However, licking and lifting behaviors elicited by capsaicin persisted throughout the 15 min observation period (median: 136, 37.5 and 50.9 s for lifting and 48, 1.6 and 0 s for licking in each successive 5 min block) whereas 90% of these behaviors occurred in the first 5 min after MO. Thus, L-733,060 may not prevent MO-induced heat hyperalgesia or tactile hypersensitivity because the nociceptive behaviors evoked by MO are brief and of insufficient duration to evoke a release of Sub P in the RVM. Alternatively, the tactile hypersensitivity and heat hyperalgesia may be mediated by pain modulatory neurons in the RVM that are not subject to regulation by Sub P.

The present results suggest the actions of Sub P in the RVM are modality specific, and that it can function as both an antinociceptive and pronociceptive agent. The mechanisms by which Sub P exerts antinociceptive and pronociceptive effects in the RVM are not entirely clear. Whole-cell recordings from RVM neurons in uninjured rats *in vitro* indicate that Sub P produces inward currents in ~70% of non-serotonergic spinally projecting neurons (Zhang and Hammond, 2009). Immunohistochemical studies indicate that a large portion of spinally projecting neurons express the NK1 receptor (Pinto et al., 2008). Direct excitation of bulbospinal pain inhibitory neurons could be one means by which Sub P can exert an antinociceptive effect in the RVM, the duration of which is limited by the short half-life of the peptide and receptor desensitization. However, whole-cell recordings also determined that, in CFA-treated rats, glutamatergic AMPA-mediated inputs to certain types of non-serotonergic spinally projecting RVM neurons were markedly enhanced and that this enhancement could be prevented by an NK1 receptor antagonist (Zhang and Hammond, 2009). Extracellular recordings from physiologically-characterized *ON* cells in the RVM indicate that the response of these neurons to iontophoretic application of NMDA is enhanced in capsaicin-treated rats and that the enhancement is reversed by an NK1 receptor antagonist (Budai et al., 2007). These data indicate that Sub P may exert a pronociceptive effect under conditions of inflammatory nociception through its ability to potentiate glutamatergic inputs to spinally projecting pain facilitatory neurons in the RVM. This role of Sub P may become predominant after certain, but not all types of inflammatory injury. Finally, not all RVM neurons exhibit a facilitated response to glutamate under conditions of persistent inflammatory nociception, nor do all RVM neurons respond to Sub P (Zhang and Hammond, 2009). Their respective roles remain unresolved at present.

The prediction that NK1 receptor antagonists would be effective analgesics was supported by a large body of preclinical data (reviewed by Hill and Oliver, 2006). Although CP-99,994 was efficacious in a model of acute third molar extraction (Dionne et al., 1998), subsequent trials of several NK1 receptor antagonists could demonstrate no efficacy against long-standing pain of osteoarthritis, diabetic neuropathy or migraine (Rupniak and Kramer, 1999; Goldstein et al., 2000; Goldstein et al., 2001a; Goldstein et al., 2001b; Sindrup et al., 2006). Of the many explanations proffered for these unexpected findings (Rupniak and Kramer, 1999; Hill, 2000; Urban and Fox, 2000; Laird, 2001), perhaps most significant is the fact that these clinical trials did not ascertain whether the agents were centrally penetrant *in humans*. The sole exception is a preliminary report that a dose of aprepitant known to provide 90% occupancy of human NK1 receptors (Hargreaves, 2002) was ineffective in postherpetic neuralgia (Block et al., 1998). Thus, while these clinical studies indicate that antagonism of NK1 receptors and Sub P in the periphery is not sufficient to produce analgesia, they do not necessarily exclude a role of NK1 receptors in the CNS. This study and another recent report (Pacharinsak et al., 2008) suggest that Sub P has a pronociceptive action in the RVM under conditions of persistent inflammatory nociception. Orally bioavailable, centrally penetrant NK1 receptor antagonists have now been developed as anti-emetic agents (Diemunsch et al., 2009). Thus, it may be time

to revisit the analgesic efficacy of centrally penetrant NK1 receptor antagonists that could simultaneously antagonize the pronociceptive actions of Sub P in the spinal cord and brainstem. However, given the evidence of modality specificity in the actions of Sub P in conditions of persistent pain, the incorporation of quantitative sensory testing using mechanical and thermal stimuli in these trials, would be warranted as well as highly informative. Finally, the dependence on the type of injury may also explain why NK1 receptor antagonists were not globally effective in clinical trials.

Abbreviations

CFA	Complete Freund's adjuvant
Ipl	intraplantar
MO	Mustard oil
NGC	Nucleus reticularis gigantocellularis
PWL	Paw withdrawal latency
PWT	Paw withdrawal threshold
RVM	Rostral ventromedial medulla
Sub P	Substance P

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Figure 1.

(A) Distribution of sites at which 100 pmol Sub P was microinjected in the brainstem. Numbers to the left of the sections are the distance from the interaural line. Circles indicate sites in the rostral ventromedial medulla (RVM), squares represent sites in the nucleus reticularis gigantocellularis (NGC) dorsal or dorsolateral to the RVM, and pentagons represent out sites. Solid symbols indicate sites at which Sub P increased paw withdrawal latency (PWL) to > 16.1 sec, or 2 S.D. greater than the mean PWL measured 5 min after microinjection of saline. (B) Time course of the effects of Sub P after microinjection in the RVM. B1 and B2 are baseline PWL determined before and 4 days after intraplantar injection of saline in the hindpaw, respectively. Numbers of animals ranged from 6–12. † $P < 0.05$, ‡ $P < 0.01$ compared to saline at the corresponding time point. (C) Dose-response relationship determined 5 min after microinjection of Sub P in the RVM for each hindpaw. Symbols are the mean $\pm$ S.E. of

determinations made in 6–12 rats. (D) Time course of the effects of 100 pmol Sub P (solid symbols) or saline (open symbols) microinjected in the RVM (circles) or NGC (squares). Numbers of animals ranged from 5–15. Where S.E.M. is not visible, it is encompassed by the symbol

Figure 2.

Determination of effective doses of NK1 receptor antagonists in the rostral ventromedial medulla. Microinjection of (A) 10 pmol L-733,060 or (B) 20 nmol L-703,606 attenuates the increase in paw withdrawal latency (PWL) produced by microinjection of 100 pmol Sub P at the same site. The optimal pretreatment time was 15 min for L-733,060 and 30 min for L-703,606. Microinjection of (C) L-733,060 or (D) L-703,606 alone did not substantively alter PWL. B1 and B2 are the PWL determined before and again 4 days after ipl injection of saline in the hindpaw. Numbers in parentheses are the number of rats. Symbols are mean $\pm$ S.E.M. * $P < 0.05$, ** $P < 0.01$ compared to B2, † $P < 0.05$, ‡ $P < 0.01$ compared to vehicle at corresponding time.

Figure 3.

Effects of antagonism of NK1 receptors in the rostral ventromedial medulla (RVM) on paw withdrawal threshold (PWT). Panels A and B illustrate that microinjection of 10 pmol L-733,060 in the RVM, delays the progressive decrease in PWT of both ipsilateral and contralateral hindpaws that occurs after microinjection of Sub P at the same site. Insets illustrate that a progressive decrease of similar magnitude and time course occurs after microinjection of just saline in the RVM. Panels C and D illustrate that a progressive decrease in PWT occurs after microinjection of 10 pmol L-733,606 or saline (duplicated in insets) in the RVM and that with one exception, these treatments do not differ. † $P < 0.05$, ‡ $P < 0.01$ compared to saline at corresponding time. B1 and B2 are the PWT determined before and again 4 days after ipl injection of saline in the hindpaw. Symbols are mean $\pm$ S.E.M. Numbers of rats are indicated in parentheses.

Figure 4.

Antagonism of NK1 receptors in the rostral ventromedial medulla (RVM) or in the nucleus reticularis gigantocellularis (NGC) reverses thermal hyperalgesia induced by ipl injection of complete Freund's adjuvant (CFA) in the hind paw (A) 4 h, (B, D) 4 days or (C) two weeks earlier. B1: Paw withdrawal latency determined before injection of CFA in the left hindpaw. B2: Paw withdrawal latency determined at the designated time after injection of CFA and before microinjection of 10 pmol L-733,060, 20 pmol L-703,606, or their respective vehicles in the RVM (arrow). Panels depict withdrawal latency of the ipsilateral, inflamed hindpaw. * $P < 0.05$, ** $P < 0.01$ compared to B2, † $P < 0.05$ ‡ $P < 0.01$ compared to vehicle at corresponding time. Symbols are mean $\pm$ S.E. M. Parentheses indicate number of rats.

Figure 5.

Effects of NK1 receptor antagonism in the rostral ventromedial medulla (RVM) or in the nucleus reticularis gigantocellularis (NGC) on PWL of the contralateral hindpaw of rats that received an ipl injection of complete Freund's adjuvant (CFA) in the hindpaw (A) 4 days, (B, D) 4 days or (C) two weeks earlier. B1: Paw withdrawal latency determined before injection of CFA in the left hindpaw. B2: Paw withdrawal latency determined at the designated time after injection of CFA and before microinjection of 10 pmol L-733,060, 20 pmol L-703,606 or their respective vehicles in the RVM (arrow). Panels depict withdrawal latency of the contralateral, uninflamed hindpaw. * $P < 0.05$ ** $P < 0.01$ compared to B2, † $P < 0.05$ ‡ $P < 0.01$ compared to vehicle at corresponding time. Symbols are the mean $\pm$ S.E.M. Parentheses indicate number of rats.

Figure 6.

Antagonism of NK1 receptors in the rostral ventromedial medulla further enhances tactile hypersensitivity in the ipsilateral hindpaw and induces tactile hypersensitivity in the contralateral hindpaw of rats that received an ipl injection of complete Freund's adjuvant (CFA) four h (A) or four days (B) earlier. Panel C depicts inability of pretreatment with L-733,060 to obtund the development of tactile hypersensitivity after CFA. B1: Paw withdrawal threshold determined before injection of CFA in the left hindpaw. B2: Paw withdrawal threshold determined at the designated time after injection of CFA and before microinjection of 10 pmol L-733,060 or saline in the RVM (arrow). Panels depict withdrawal threshold of the ipsilateral,

inflamed hindpaw † $P < 0.05$ ‡ $P < 0.01$ compared to vehicle at corresponding time. Symbols are mean $\pm$ S.E.M. Parentheses indicate number of rats.

Figure 7.

Antagonism of NK1 receptors in the rostral ventromedial medulla (RVM) prevents the induction of tactile hypersensitivity in rats that received an ipl injection of 0.05% capsaicin in the hindpaw. B1: Baseline paw withdrawal threshold. Paw withdrawal threshold was re-determined 15 min after microinjection (left arrow) of 10 pmol L-733,060 or saline in the RVM and immediately before ipl injection of capsaicin. Abscissa is time relative to injection of CAP (right arrow). † $P < 0.05$ compared to vehicle at corresponding time. Symbols are mean $\pm$ S.E.M. Number of rats is indicated in parentheses.

Figure 8.

Antagonism of NK1 receptors in the rostral ventromedial medulla (RVM) does not prevent the induction of (A) thermal hyperalgesia, (B) tactile hypersensitivity or (C) spontaneous nociceptive behaviors in rats that received an ipl injection of 1% mustard oil (MO) in the hindpaw. BL: Baseline paw withdrawal latency or threshold. Either 10 pmol L-733,060 (solid squares) or saline (open squares) was microinjected in the RVM 15 min before intraplantar injection of MO. Abscissa is time relative to injection of MO (arrow). Panels depict withdrawal latency, threshold, or licks and lifts of the ipsilateral hindpaw. Symbols are mean $\pm$ S.E.M. Numbers in columns are the N for each treatment group.