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The role of CNS NMDA receptors and nitric oxide in visceral hyperalgesia

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Abstract

The studies summarized here document the role of NMDA receptors and nitric oxide in the lumbosacral spinal cord and rostral ventromedial medulla in the maintenance of visceral hyperalgesia. Experiments were conducted in rats in which drugs were administered into either the lumbosacral intrathecal space or directly into the rostral ventromedial medulla. The visceral stimulus was noxious colorectal distension, administered before and 3 h after intracolonic instillation of either saline or 25% zymosan. The visceromotor response to colonic distension was quantified and found to be significantly enhanced in rats in which the colon had previously been treated with zymosan. Enhanced responses to distension were attenuated dose-dependently by intrathecal administration of the NMDA receptor channel blocker MK-801 and by inhibition of the neuronal isoform of nitric oxide synthase (nNOS). In corresponding studies wherein drugs were administered directly into the rostral ventromedial medulla, NMDA receptor antagonism and NOS inhibition dose-dependently attenuated exaggerated responses to colonic distension. Taken together, these data suggest that zymosan-produced visceral hyperalgesia is influenced both at the level of the spinal cord and rostral ventromedial medulla, and that descending facilitatory influences from the rostral ventromedial medulla are important to the maintenance of visceral hyperalgesia.

Keywords: Inflammation; Colorectal distension; NMDA receptor; *N*-nitro-L-arginine methyl ester (L-NAME); Rostral ventromedial medulla

1. Introduction

A growing body of evidence suggests that the pain and discomfort characterizing functional bowel disorders represents the equivalent of hyperalgesia (e.g., Mayer and Gebhart, 1994). It has been shown that chemical irritation of the viscera leads to an alteration of normal reflexes (Burton and Gebhart, 1995; Langlois et al., 1994; Rice and McMahon, 1995) and an increased activity (sensitization) of the afferent innervation of those structures (Häbler et al., 1993; Sengupta and Gebhart, 1994; Coutinho et al., 2000). This in turn leads to excitation of spinal neurons accompanied by a change in their receptive field properties (Gebhart and Ness, 1991; Gebhart, 1993). It is well known that excitatory amino acid receptors are involved in activity-dependent changes in the excitability of central neurons and synaptic plasticity. These receptors in the spinal cord have been shown to play a key role in central mechanisms associated with hyperalgesia following peripheral injury, principally in models of cutaneous pain (see Dubner and Ruda, 1992 for overview).

Among the cellular events associated with NMDA receptor activation is influx of calcium, which triggers a cascade of intracellular events, including activation of nitric oxide synthase (NOS) (see Meller and Gebhart, 1993 for review). In neurons in the central nervous system (CNS), a constitutive isoform of NOS (neuronal or nNOS, also referred to as type I NOS) catalyzes the conversion of L-arginine and molecular oxygen to $NO^{\cdot}$ and L-citruline. As reviewed by Meller and Gebhart (1993), there is considerable evidence suggesting that activation of NOS in the spinal cord as a consequence of NMDA receptor activation plays a key role in mediating hyperalgesia in models of cutaneous pain.

In addition to these changes initiated at the level of the spinal cord, it has long been appreciated that spinal nociceptive transmission is subject to descending modulatory influences from supraspinal sites, including the brainstem

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rostral ventromedial medulla (see Fields and Basbaum, 1994 for overview). Whereas earlier work focused on the inhibitory nature of these descending influences, recent studies have demonstrated potent facilitatory influences as well. These descending facilitatory influences from the rostral ventromedial medulla have been shown to significantly contribute to hyperalgesia in a number of studies involving inflammatory and neuropathic models of somatic pain (see Urban and Gebhart, 1999 for recent overview). Accordingly, in the work described here, we investigated the role of NMDA receptors and NOS in both the spinal cord and rostral ventromedial medulla in the mediation of visceral hyperalgesia.

This report summarizes work described in full elsewhere (Coutinho et al., 1996, 1998; Coutinho and Gebhart, 1999).

2. Materials and methods

2.1. Animals

Adult male Sprague–Dawley rats (400–425 g, Harlan, Indianapolis, IN) were used throughout. They were housed one to two per cage with food and water available ad libitum and maintained on a 12:12 h light/dark cycle (lights on between 06.00 and 18.00 h). The Institutional Animal Care and Use Committee of The University of Iowa approved the experimental protocols.

2.2. Surgical preparation

Rats were anesthetized with pentobarbital sodium (45 mg/kg, i.p., Nembutal, Abbott Labs, North Chicago, IL) and electrodes (Teflon-coated stainless steel wire, Cooner Wire Sales, Chatworth, CA) were sewn into the external oblique musculature immediately superior to the inguinal ligament for electromyographic (EMG) recording. The electrode leads were tunneled subcutaneously and exteriorized at the base of the neck, where they were anchored to the neck musculature with a suture.

In some experiments, an intrathecal (i.th.) catheter (PE-10 tubing, 8.5-cm long) was inserted through the dura overlying the alanto-occipital junction into the subarachnoid space and guided to the lumbar enlargement. The catheter was surgically anchored to the musculature at the neck and externalized with the EMG leads.

In other experiments, a stainless-steel microinjection guide cannula (26 gauge) was stereotaxically implanted 3 mm dorsal to the rostral ventromedial medulla. Following implantation, each cannula was secured by dental acrylic cement, held in place by two stainless-steel skull screws. To prevent the cannula from becoming clogged, it was fitted with a 33-gauge stylette.

2.3. Colorectal distension

The response that was quantified was the visceromotor response, a contraction of the abdominal and hind limb musculature during colorectal distension in awake rats (Ness and Gebhart, 1988). The descending colon and rectum were distended by pressure-controlled air inflation of a 7–8-cm-long flexible latex balloon tied around a flexible tube (Tygon). The balloon was lubricated, inserted intraanally and positioned such that the end of the balloon was 1 cm proximal to the anus. It was secured in place by taping the balloon catheter to the base of the tail. Constant pressure colorectal distension (typically 80 mm Hg, 20 s) was given by opening a solenoid gate to a constant pressure air reservoir; intracolonic pressure was continuously monitored. The EMG activity in the external oblique musculature was quantified by recording the number of spikes exceeding the resting activity in the muscle.

2.4. Experimental protocols

On the day of testing, typically 4–5 days after the surgeries described above, control responses to colorectal distension (80 mm Hg, 20 s) were obtained prior to any treatment. Rats were then briefly anesthetized with halothane and either saline or zymosan (1 ml, 25 mg/ml), an inflammogen from the cell wall of *S. cerevisiae*, was instilled into the colon through a gavage needle inserted intraanally to a depth of about 7–8 cm. Three hours following intracolonic treatment, responses to colorectal distension were obtained again to determine the magnitude of hyperalgesia. Drugs were then administered i.th. or directly into the rostral ventromedial medulla and colorectal distension trials at 4-min intervals followed to assess both the magnitude and duration of drug action.

2.5. Drugs

Drugs used included NMDA, the NMDA receptor antagonists MK-801 and DL-2-amino-5-phosphonovaleric acid (APV), nitric oxide synthase inhibitors *N*-nitro-L-arginine methyl ester (NAME) or ARL17477 (a generous gift from Astra Arcus, Rochester, NY) or vehicle.

For i.th. administration, drugs were given in a volume of 5 μl followed by a flush with 10 μl of preservative-free saline. For intrarostral ventromedial medulla administration, drugs were given in volumes of either 0.5 or 1.0 μl over a period of 30 s.

2.6. Data analysis

All experimental groups consisted of at least four to six rats and data are expressed as mean ± S.E.M. Data for visceromotor responses are normalized as percentage of control, calculated as percent of the mean response of two

responses to colorectal distension obtained in the same animal, 3 h post-intracolonic treatment, but prior to i.th. or intrarostral ventromedial medulla drug administration. Changes in the visceromotor response following drug administration were statistically analyzed by repeated measures analysis of variance. $P < 0.05$ was considered statistically significant in all tests.

3. Results

3.1. Spinal cord experiments

Intracolonic instillation of zymosan produced a robust increase in responses to colorectal distension, as illustrated in Fig. 1. The stimulus–response function to graded intensities of colorectal distension (10–80 mm Hg) following intracolonic instillation of zymosan was shifted leftward relative to rats that received intracolonic saline. Although not illustrated (see Coutinho et al., 1996), the stimulus–response function to colorectal distension in saline-treated rats did not differ from rats in which no prior intracolonic treatment was given. Fig. 1 also illustrates that i.th. MK-801 (10 nmol) significantly attenuated the exaggerated (hyperalgesic) responses to colonic distension. In these experiments (Coutinho et al., 1996), we also found that i.th. administration of the non-NMDA receptor antagonist, 6,7-dinitroquinoxaline-2,3-dione (DNQX), but not the metabotropic glutamate receptor antagonist, D,L,-2-amino-3-phosphonopropionic acid (AP-3), was anti-hyperalgesic. A specific role for spinal NMDA and non-NMDA receptors in visceral hyperalgesia was supported by the fact that these receptor antagonists were without effect in rats that had received intracolonic saline.

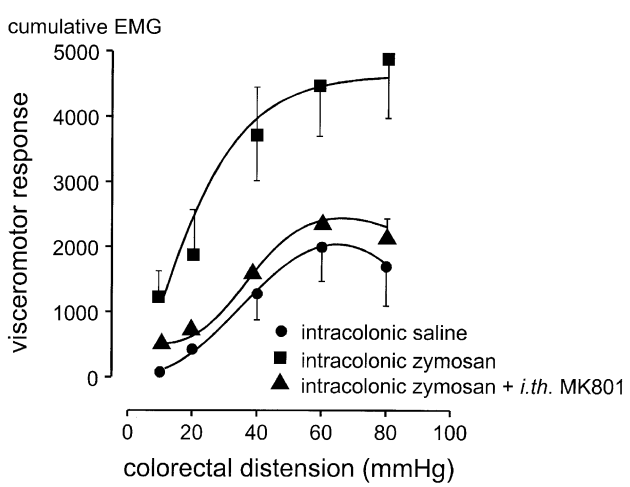

Fig. 1. Summary of the effect of intrathecal (i.th.) administration of MK801 (10 nmol) on the intensity coding of the visceromotor response to colorectal distension. In contrast to rats that had received intracolonic saline (●), rats that had received intracolonic zymosan in the colon 3 h earlier exhibit greater responses to colorectal distension (■). This hyperalgesia is reversed by i.th. MK801 (▲).

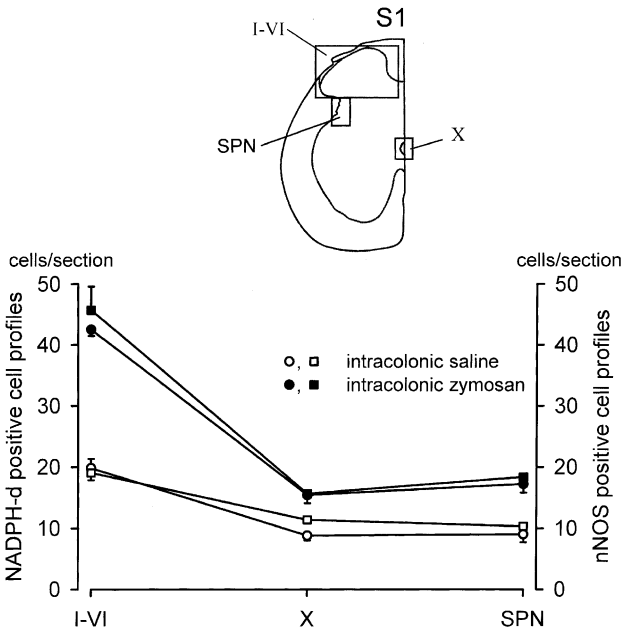

Fig. 2. Summary of the quantitative analysis of NADPH-d positive cell profiles (left axis; ○, ●) and nNOS immunostained cell profiles (right axis; □, ■) in the lumbosacral spinal cord. Intracolonic instillation of zymosan significantly increased the number of stained cells in laminae I–VI, lamina X and the sacral parasympathetic nucleus (see cartoon inset).

We also examined the potential contribution of spinal $NO^{\cdot}$ to zymosan-produced visceral hyperalgesia in two ways. We performed NADPH-d histochemistry and nNOS immunocytochemistry in lumbosacral spinal cord sections from saline-treated and zymosan-treated rats. In other experiments, we gave non-selective and selective nNOS inhibitors into the intrathecal space in rats previously treated with saline or zymosan intracolonically. Fig. 2 summarizes the data from the histochemistry and immunocytochemistry experiments. We examined the spinal dorsal horn (laminae I–VI), the sacral parasympathetic nucleus and the area around the central canal (lamina X). As illustrated, the number of NADPH-d positive cell profiles and nNOS positive cell profiles were significantly increased in all three areas of the spinal cord in zymosan-treated rats relative to saline-treated rats. The greatest increases occurred in the spinal dorsal horn (laminae I–VI). Additionally, the patterns of NADPH-d histochemistry and nNOS immunostaining in the lumbosacral spinal cords were virtually identical. Although we did not double-stain the same spinal sections, those cell profiles that were histochemically stained for NADPH-d appeared in adjacent sections to be the same cells which were immunocytochemically stained for nNOS.

Experiments wherein drugs were given i.th. to saline- or zymosan-treated rats revealed that the non-selective inhibitor of NOS, L-NAME, but not its inactive stereoisomer D-NAME, significantly and dose-dependently attenuated exaggerated responses to colorectal distension. I.th. admin-

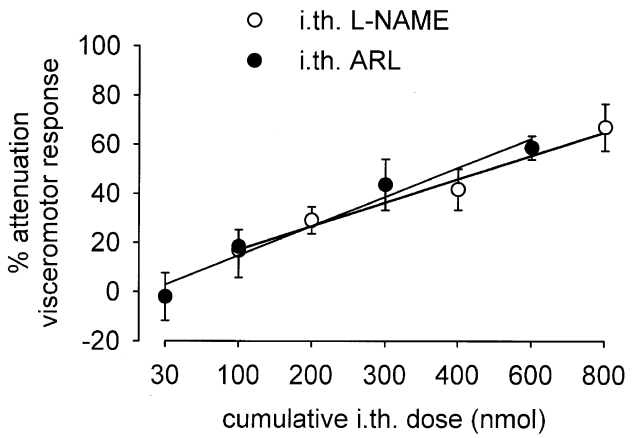

Fig. 3. Dose-dependent effects of NOS inhibitors given intrathecally (i.th.) on zymosan-produced visceral hyperalgesia. Responses to colorectal distension (80 mm Hg, 20 s) were recorded before and 3 h after intracolonic instillation of zymosan. L-NAME or ARL17477 was administered in cumulative doses 3 h after treatment with zymosan. Data are presented as mean ± S.E.M., calculated as percentage attenuation of the response to colorectal distension 3 h after intracolonic treatment with zymosan, but before i.th. drug administration.

istration of the selective nNOS inhibitor ARL17477 produced virtually overlapping dose-dependent effects to those seen with L-NAME (Fig. 3), confirming that the isoform of NOS in the spinal cord that is upregulated in zymosan-treated rats is the neuronal isoform. Because these NOS inhibitors had no effect in rats that had received intracolonic saline, these results support a specific role for spinal NOS in mediating visceral hyperalgesia.

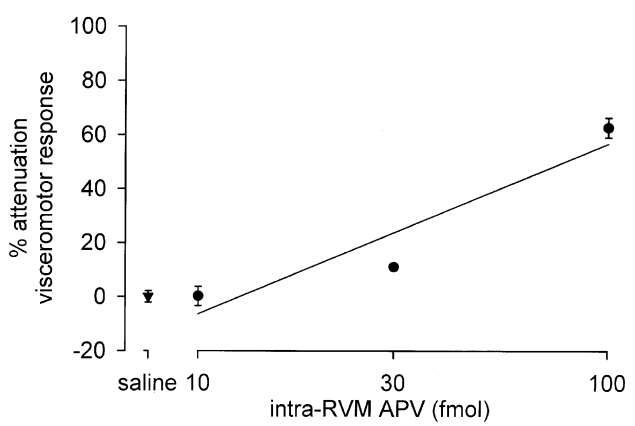

Fig. 4. Summary of the effect of intra-rostral ventromedial medulla administration of APV on zymosan-produced visceral hyperalgesia. Data are plotted as mean ± S.E.M., calculated as percentage attenuation of the response to colorectal distension 3 h after intracolonic instillation of zymosan, but prior to intra-rostral ventromedial medulla APV or saline administration. APV significantly and dose-dependently attenuated the enhanced visceromotor response in animals that received intracolonic zymosan; intra-rostral ventromedial medulla saline had no effect on zymosan-produced visceral hyperalgesia.

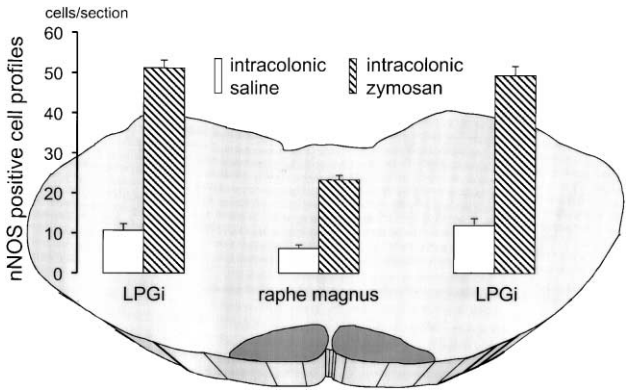

Fig. 5. Summary of the quantitative analysis of nNOS immunostaining in the rostral ventromedial medulla 3 h following intracolonic instillation of either saline or zymosan. Data are presented on a histological representation of a coronal section through the rostral ventromedial medulla. Data are presented as mean ± S.E.M. Zymosan produced significant increases in nNOS immunostaining bilaterally and in nucleus raphe magnus.

3.2. Rostral ventromedial medulla experiments

Experiments complementary to those described above were carried out in the rostral ventromedial medulla in saline- and zymosan-treated rats. Intra-rostral ventromedial medulla administration of APV (Fig. 4) or L-NAME, but not D-NAME, significantly attenuated exaggerated responses to colorectal distension (80 mm Hg, 20 s) in zymosan-treated rats, but had no effect on responses to colorectal distension in saline-treated rats. Correspondingly, intra-rostral ventromedial medulla administration of NMDA (10 pmol) produced a time-limited and significant enhancement of the visceromotor response to colorectal distension in saline-treated (uninflamed) rats (see Coutinho et al., 1998).

As in the spinal cord, nNOS immunocytochemical staining revealed significant increases in the number of positively stained cell profiles in the rostral ventromedial medulla 3 h after intracolonic instillation of zymosan compared to saline treated controls (Fig. 5). In rats previously treated with intracolonic saline, NADPH-d cell profiles were apparent in the midline nucleus raphe magnus and bilaterally in the adjacent lateral paragigantocellular areas. The number of NADPH-d positively stained cell profiles was significantly increased 3 h after intracolonic instillation of zymosan (and comparable to nNOS immunocytochemistry) in all areas of the rostral ventromedial medulla.

4. Discussion

The experiments summarized here illustrate that intracolonic instillation of zymosan results in a significant

enhancement of responses to colorectal distension across the dynamic range of distending pressures tested. This visceral hyperalgesia, like cutaneous hyperalgesia, can be attenuated significantly by intrathecal administration of antagonists of NMDA receptor function. At the level of the spinal cord, intracolonic zymosan also produces significant increases in cells in the lumbosacral spinal cord that stained positively for NADPH-diaphorase or nNOS. Similar to effects produced by intrathecal administration of NMDA receptor antagonists, inhibition of the neuronal isoform of NOS also attenuates, dose-dependently, the visceral hyperalgesia that develops 3 h following intracolonic instillation of zymosan. Entirely complementary results were found at the level of the rostral ventromedial medulla: intra-rostral ventromedial medulla NMDA enhanced responses to colorectal distension in normal rats; staining for NADPH-d and nNOS was significantly increased 3 h after intracolonic instillation of zymosan; and intra-rostral ventromedial medulla APV or L-NAME significantly attenuated visceral hyperalgesia produced by intracolonic instillation of zymosan.

There exists ample evidence supporting the involvement of excitatory amino acid receptors in mediating activity-dependent changes in neuron excitability and synaptic plasticity. Noxious stimulation or peripheral inflammation causes the release of glutamate and aspartate in the spinal cord dorsal horn (Skilling et al., 1988; Sorkin et al., 1992). Further, intrathecal administration of excitatory amino acid receptor agonists produces spontaneous nociceptive behaviors and hyperalgesia (e.g., Aanonsen and Wilcox, 1987; Kolhekar et al., 1993). The present and other results (Coutinho et al., 1996; Rice and McMahon, 1995) reveal that both NMDA and non-NMDA receptors in the spinal cord attenuate exaggerated responses to visceral stimulation in the rat. Relatively fewer studies examining the role of excitatory amino acid receptors in visceral hyperalgesia have been undertaken than in more widely studied models of cutaneous hyperalgesia or neuropathic pain. Modulation of nociceptive input from the viscera may differ from cutaneous nociceptive input, not only because of a difference of origin of input to the CNS, but also because of possible differences at the receptor level with respect to type, location and functional mechanisms involved. In an earlier study, Kolhekar and Gebhart (1994) reported that NMDA administered to the lumbar spinal cord produced dose-dependent facilitation of responses to noxious colorectal distension. In a subsequent electrophysiological study, they found that local application of NMDA onto spinal neurons that received colonic input enhanced responses of those neurons to colorectal distension and also increased convergent cutaneous receptive field size, effects that were blocked by APV (Kolhekar and Gebhart, 1996).

These outcomes are consistent with results reported here for modulation of visceral hyperalgesia by inhibition of NOS in the lumbosacral spinal cord. Because activation of the $NO^{\cdot}$ cascade is known to occur secondary to NMDA receptor activation, evidence has accumulated implicating a role for spinal $NO^{\cdot}$ in models of somatic hyperalgesia (e.g., Lawland et al., 1997; Meller et al., 1994; Roche et al., 1996; Yonehara et al., 1997). The current report (see also Rice, 1995; Pandita et al., 1997) documents an analogous role for spinal $NO^{\cdot}$ in mediating the enhanced responses to colonic distension from the inflamed colon.

Although nNOS is the predominant isoform of NOS in the spinal cord, the inducible isoform of NOS may also contribute to hyperalgesia (e.g., Grzybicki et al., 1996; Wu et al., 1998). In preliminary experiments, however, we did not observe iNOS induction in the lumbosacral spinal cord 3 h after intracolonic zymosan, probably because induction of iNOS follows a longer time course (e.g., Grzybicki et al., 1996; Wu et al., 1998). That nNOS was the isoform involved in colonic hyperalgesia was confirmed by use of the selective nNOS inhibitor ARL17477, a compound reported to be 100-fold more selective for nNOS than other isoforms of this enzyme (Zhang et al., 1996). ARL17477 produced a significant dose-dependent and reversible attenuation of zymosan-produced visceral hyperalgesia and the slope of its dose–response function overlapped that for L-NAME. We further established the involvement of spinal $NO^{\cdot}$ histochemically and immunocytochemically. Three hours after intracolonic instillation of zymosan, there was a significant increase in cell profiles that stained positively for NADPH-d as well as nNOS.

Complementary results establish a role for NMDA receptors and $NO^{\cdot}$ in the rostral ventromedial medulla in the modulation of visceral hyperalgesia. As indicated above, the majority of studies have focused on cutaneous models of hyperalgesia and the underlying spinal mechanisms. A number of recent studies, however, have reported that descending facilitatory influences from the rostral ventromedial medulla significantly contribute to cutaneous hyperalgesia in models of inflammatory and neuropathic pain (see Kovelowski et al., 2000; Urban et al., 1996, 1999b; Urban and Gebhart, 1999 for review). Additionally, we have previously shown that cutaneous hyperalgesia following topical mustard oil (C-fiber excitant) involves activation of descending facilitatory influences mediated by NMDA receptors and NOS in the rostral ventromedial medulla (Urban et al., 1999a). In comparison to cutaneous hyperalgesia, the potential involvement of the rostral ventromedial medulla in the maintenance of visceral hyperalgesia has received very little attention. In the present study, the robust hyperalgesia produced by zymosan was dose-dependently attenuated by intra-rostral ventromedial medulla APV. The onset of effect was within 3–6 min following drug administration and, at the greatest dose tested (100 fmol), lasted about 30 min. Intra-rostral ventromedial medulla administration of this same dose of APV was without effect in animals that had previously received saline intracolonically. Accordingly, these data suggest that visceral hyperalgesia following colonic inflammation involves endogenous activation of descending facilitatory

influences mediated by NMDA receptors in the rostral ventromedial medulla. This interpretation is supported by the fact that administration of NMDA into the rostral ventromedial medulla of naive, uninflamed rats produced a short-duration, receptor-mediated facilitation of the visceromotor response to noxious colorectal distension. That NMDA receptors in the rostral ventromedial medulla mediate descending facilitation of visceral nociception is consistent with several studies which have shown that intra-rostral ventromedial medulla injection of glutamate or NMDA facilitates cutaneous nociceptive responses (Urban et al., 1999a; Zhuo and Gebhart, 1997). Additionally, glutamate and NMDA receptors are localized in the rostral ventromedial medulla, and ascending spinal projections that terminate in a variety of supraspinal sites contain glutamate (Beitz, 1990; DiBiasi et al., 1994). Thus, a spinal–bulbospinal loop likely contributes to the NMDA receptor-mediated activation of descending facilitatory influences from the rostral ventromedial medulla following colonic inflammation, and contributes significantly to visceral hyperalgesia.

The present data also suggest that production of $NO^{\cdot}$ in the rostral ventromedial medulla secondary to activation of NMDA receptors enhances descending facilitation of visceral hyperalgesia, but does not mediate normal responses to noxious colorectal distension. We found that intra-rostral ventromedial medulla administration of L-NAME, but not its inactive stereoisomer D-NAME, attenuated, but did not abolish, visceral hyperalgesia in zymosan-treated rats. Additionally, L-NAME was without effect in uninflamed rats that had previously received intracolonic saline. These data are wholly consistent with those described above for modulation of visceral hyperagesia at the NMDA receptor. As in the spinal cord, we further investigated the involvement of $NO^{\cdot}$ using NADPH-d histochemistry and nNOS immunocytochemistry. As reported previously by Vincent and Kimura (1992), we also found large, stained cells in the midline raphe magnus and bilaterally in the lateral paragigantocellular areas in uninflamed rats (see Coutinho et al., 1998 for photomicrographs). Three hours following intracolonic instillation of zymosan, there was a significant increase in NADPH-d and nNOS staining in the rostral ventromedial medulla compared to saline treated controls. Again, we did not double-label tissue sections, but the morphology and numbers of stained cells suggest that cells stained for NADPH-d or nNOS largely overlap. In support, we have previously shown that intra-rostral ventromedial medulla injection of an NOS inhibitor attenuates cutaneous hyperalgesia following topical mustard oil, and supraspinal or intra-rostral ventromedial medulla injection of $NO^{\cdot}$ donors produce cutaneous hyperalgesia (Shibuta et al., 1995; Urban et al., 1999a).

Taken together, the results summarized here are consistent with a role for $NO^{\cdot}$ in the spinal cord and rostral ventromedial medulla, produced as a consequence of activation of NMDA receptors and induction of nNOS, in mediating activation of descending facilitatory influences that significantly contribute to visceral hyperalgesia. Based on these and other studies (see Discussion in Coutinho et al., 1998; Urban and Gebhart, 1999), visceral hyperalgesia in this model is contributed to and maintained by spinal and supraspinal mechanisms.

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