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LOSS OF NEURONS IN ROSTRAL VENTROMEDIAL MEDULLA THAT EXPRESS NEUROKININ-1 RECEPTORS DECREASES THE DEVELOPMENT OF HYPERALGESIA

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Abstract—It is well known that neurons in the rostral ventromedial medulla (RVM) are involved in descending modulation of nociceptive transmission in the spinal cord. It has been shown that activation of neurokinin-1 receptors (NK-1Rs) in the RVM, which are presumably located on pain facilitating ON cells, produces hyperalgesia whereas blockade of NK-1Rs attenuates hyperalgesia. To obtain a better understanding of the functions of NK-1R expressing neurons in the RVM, we selectively ablated these neurons by injecting the stable analog of substance P (SP), Sar^{9}, Met(O$_2$)^{11}-Substance P, conjugated to the ribosomal toxin saporin (SSP–SAP) into the RVM. Rats received injections of SSP–SAP (1 μM) or an equal volume of 1 μM of saporin conjugated to artificial peptide (Blank–SAP). Stereological analysis of NK-1R- and NeuN-labeled neurons in the RVM was determined 21–24 days after treatment. Withdrawal responses to mechanical and heat stimuli applied to the plantar hindpaw were determined 5–28 days after treatment. Withdrawal responses were also determined before and after intraplantar injection of capsaicin (acute hyperalgesia) or complete Freund's adjuvant (CFA) (prolonged hyperalgesia). The proportion of NK-1R-labeled neurons in the RVM was 8.8 ± 1.3% in naïve rats and 8.1 ± 0.8% in rats treated with Blank–SAP. However, injection of SSP–SAP into the RVM resulted in a 90% decrease in NK-1R-labeled neurons. SSP–SAP did not alter withdrawal responses to mechanical or heat stimuli under normal conditions, and did not alter analgesia produced by morphine administered into the RVM. In contrast, the duration of nocifensive behaviors produced by capsaicin and mechanical and heat hyperalgesia produced by capsaicin and CFA were decreased in rats pretreated with SSP–SAP as compared to those that received Blank–SAP. These data support our earlier studies using NK-1R antagonists in the RVM and demonstrate that RVM neurons that possess the NK-1R do not play a significant role in modulating acute pain or morphine analgesia, but rather are involved in pain facilitation and the development and maintenance of hyperalgesia.

Key words: capsaicin, inflammation, descending facilitation, ON cells, saporin, pain modulation.

INTRODUCTION

It is well established that nociceptive processing in the spinal cord is modulated by descending projections from the brain stem. Neurons in the rostral ventromedial medulla (RVM), a brain stem region that includes nucleus raphe magnus, nucleus gigantocellularis pars alpha, and lateral paragigantocellular nucleus, project to the spinal cord (Watkins et al., 1980, 1981) and can inhibit (antinociception) or facilitate (pronociception) nociceptive transmission (Basbaum and Fields, 1978, 1984; Ren and Dubner, 1996, 2002; Urban and Gebhart, 1999; Porreca et al., 2002; Heinricher et al., 2009). Accumulating evidence suggests that neurons in the RVM that express neurokinin-1 receptors (NK-1Rs) (Ljungdahl et al., 1978; Nakaya et al., 1994; Budai et al., 2007) play a role in descending facilitation of nociceptive transmission. Behavioral studies showed that microinjection of substance P (SP) into the RVM produced hyperalgesia (Lagraize et al., 2010) whereas injection of NK-1R antagonists into the RVM reduced the hyperalgesia produced by intraplantar capsaicin injection (Pacharinsak et al., 2008) and hind paw inflammation (Hamity et al., 2010).

Neurons in the RVM are classified electrophysiologically as ON, OFF, NEUTRAL, and serotonergic cells (Fields et al., 1983; Fields and Heinricher, 1985; Gao and Mason, 2000). ON cells are considered to be pronociceptive because they are excited by noxious stimulation, exhibit a burst-like increase in discharge rate just prior to a withdrawal reflex, and are inhibited by morphine. OFF cells, which are believed to be antinociceptive, respond with a pause in ongoing discharge during noxious stimulation and are excited by morphine (reviewed by Mason, 2001; Heinricher et al., 2009). The role of NEUTRAL cells in nociceptive processing is unclear. These cells are not affected by noxious cutaneous stimuli but may modulate nociceptive transmission of visceral (Brink and Mason, 2003, 2004; Brink et al., 2006) and trigeminal (Ellrich et al., 2000)

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Abbreviations: ANOVA, analysis of variance; CCK, cholecystokinin; CFA, complete Freund's adjuvant; NDS, normal donkey serum; NeuN-ir, NeuN-immunoreactive; NK-1R, neurokinin-1 receptor; NK-1R-ir, NK-1R-immunoreactive; NMDA, *N*-methyl-D-aspartate; PBS, phosphate-buffered saline; RVM, rostral ventromedial medulla; SAP, saporin; SP, substance P; SSP, Sar^{9},Met(O$_2$)^{11}-Substance P.

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inputs. Serotonergic cells are a separate group of RVM neurons defined by slow, regular discharge and distinct neurochemistry that appears to modulate autonomic activities (Potrebic et al., 1994; Mason, 1997, 2012).

In earlier studies (Budai et al., 2007) we proposed that NK-1Rs are located on ON cells because iontophoretic application of the selective NK-1R agonist, Sar^{9},$Met(O_2)^{11}$-Substance P excited only ON cells and enhanced their responses to iontophoretic application of *N*-methyl-D-aspartate (NMDA), effects that were blocked by an NK-1R antagonist. We also showed that sensitization of ON cells to cutaneous stimulation following capsaicin (Brink et al., 2012) or prolonged inflammation (Khasabov et al., 2012a,b) was reduced by an NK-1R antagonist. Although these studies showed that activation of NK-1Rs can enhance activity of ON cells and thereby contribute to facilitation of nociceptive transmission, this approach has limitations to understanding the role of NK-1R expressing RVM neurons because NK-1Rs are only a part of neurochemical mechanisms that regulate activity of these neurons. For example, we showed that almost all RVM neurons that express NK-1Rs also express NMDA receptors (Budai et al., 2007). Also, it is possible that RVM neurons that express NK-1Rs are a subpopulation of ON cells with specific functions while other ON cells (without NK-1Rs) may have different functions, such as modulating acute nociception. To further determine the role of NK-1R expressing neurons in the RVM in pain modulation, we selectively ablated these neurons by injection of a stable analog of SP, Sar^{9}, $Met(O_2)^{11}$-Substance P, conjugated to the ribosomal toxin saporin (SSP–SAP) (Wiley and Lappi, 1999, 2001, 2003; Lappi and Wiley, 2004; Wiley et al., 2007; Wiley, 2008) into the RVM. The loss of NK-1R expressing neurons following injection of SSP–SAP into the RVM was quantified using stereological methods, and the effects of loss of NK-1R expressing neurons in the RVM on withdrawal responses and on the development of hyperalgesia were determined. Our results demonstrate that a relatively small proportion of RVM neurons express NK-1Rs but these neurons play an important role in the development of hyperalgesia.

EXPERIMENTAL PROCEDURES

Animals

One hundred twenty-seven adult, male, Sprague–Dawley rats (Harlan, Indianapolis, IN, USA) weighing 250–360 g were used. Rats were maintained in a climate-controlled room on a 12-h dark/light cycle, and food and water were available *ad libitum*. All experimental procedures were performed in accordance with the guidelines recommended by the International Association for the Study of Pain and were approved by the Institutional Animal Care and Use Committee at the University of Minnesota.

Injection of SSP–SAP and Blank–SAP into the RVM

Rats were anesthetized with ketamine (67.5 mg/kg) and xylazine (22.5 mg/kg) and placed in a stereotaxic apparatus. Body temperature was maintained at 37 °C using a feedback-controlled heating blanket (Harvard Apparatus, Holliston, MA, USA). A skin incision (~10 mm) was made on the top of the cranium to expose the skull and a small hole (~1.5-mm diameter) was made through the skull over the RVM (anterior–posterior interaural stereotaxic coordinate −2.3 mm). Rats received two injections into the RVM (one each into the left and right sides) of either 1 μM of SSP–SAP in 0.3 μl or an equal volume of 1 μM of saporin conjugated to artificial peptide (Blank–SAP). Both conjugates were dissolved in phosphate-buffered saline (PBS). Interaural stereotaxic coordinates for these injections were: anterior–posterior = −2.3 mm; dorsal–ventral = −0.5 mm; lateral = ±0.5 mm according to the atlas of Paxinos and Watson (1998). Injections were made over a period of 2 min each using a glass micropipette with ~ 50-μm tip diameter attached to a 1-μl Hamilton microsyringe (Hamilton Company, Reno, NV, USA) placed in microinjection unit (KOPF, Tujunga, CA, USA, Model 5001). After injection the micropipette was kept in place for 1 min to allow drug diffusion through the RVM. The micropipette was then removed from the brain and the skin was closed with silk ligature. Rats were returned to their home cage for recovery and were used for behavioral and immunohistochemical studies 24–28 days later.

Chronic cannula implantation into the RVM

Rats were implanted stereotaxically with a chronic stainless steel cannula into the RVM for behavioral studies of morphine analgesia. Before cannula implantation, SSP–SAP or Blank–SAP was injected into the RVM as described above. Following a 2-week recovery period, animals were anesthetized with ketamine (67.5 mg/kg) and xylazine (22.5 mg/kg) and placed in a stereotaxic apparatus. After craniotomy, a guide cannula (17.5 mm in length, 26 gauge; Plastics One, Roanoke, VA, USA) was inserted toward the RVM. The interaural stereotaxic coordinates for implantation were: anterior–posterior = −2.3 mm; dorsal–ventral = +0.5 mm; lateral = 0 mm according to the rat brain atlas of Paxinos and Watson, 1998. Three stainless steel screws were inserted into the skull in near proximity, and the guide cannula and screws were secured with dental resin (Duralay Dental Mfg. Co., Worth, IL, USA). To maintain patency of the guide cannula, a dummy cannula of the same length (33 gauge; Plastics One) was inserted into the guide cannula. Animals were allowed to recover for 7 days before the behavioral studies.

Immunohistochemistry

Rats were deeply anesthetized with ketamine (67.5 mg/kg) and xylazine (22.5 mg/kg). Perfusions were made through the ascending aorta with 100 ml of PBS at room temperature followed by 800 ml of cold 4% paraformaldehyde in PBS (pH 7.4) for 40 min. After fixation, brains were removed, post-fixed in the same