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Effects of chronic constriction injury and spared nerve injury, two models of neuropathic pain, on the numbers of neurons and glia in the rostral ventromedial medulla

Mai Lan Leong, Ph.D.^{*}, Rebecca Speltz^{**}, and Martin Wessendorf, Ph.D.[†]

Department of Neuroscience, University of Minnesota, 6-145 Jackson Hall, 321 Church Street SE, Minneapolis, MN 55455

Abstract

In previous studies we have reported that spinal nerve ligation (SNL), a model of neuropathic pain, results in the loss of over 20% of neurons in the rostral portion of the ventromedial medulla (RVM) in rats, 10 days after SNL. The RVM is involved in pain modulation and we have proposed that loss of pain inhibition from the RVM, including loss of RVM serotonin neurons, contributes to the increased hypersensitivity observed after SNL. In the present study we examined whether RVM neuronal loss occurs in two other models of neuropathic pain, chronic constriction injury and spared nerve injury (SNI). We found no evidence for neuronal loss 10 days after either nerve injury, a time when robust tactile hypersensitivity is present in both CCI and SNI. We conclude that loss of RVM neurons appears not to be required for expression of tactile hypersensitivity in these models of neuropathic pain.

Keywords

pain; neuropathy; medulla; nociception; apoptosis; glia

Introduction

Pain is modulated by descending systems, including projections from brain stem neurons in the rostral portion of the ventromedial medulla (RVM). Activation of the RVM inhibits nociception potently and it has been proposed that the RVM mediates both stimulation-produced analgesia and opiate analgesia (see [3] for review). However, the RVM also facilitates nociception [5, 30, 47, 48] and it appears to play a crucial role in maintaining hypersensitivity after spinal nerve ligation [9]. The RVM contains serotonergic neurons [14,

[†]Author for correspondence: martinw@umn.edu.

^{*}Present address: OMS IV Des Moines University 3200 Grand Ave. Des Moines, IA 50312

^{**}Present address: Department of Diagnostic and Biological Science, School of Dentistry, University of Minnesota, Minneapolis, MN 55455

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25, 41] and there is evidence that these neurons may have both pro-nociceptive and antinociceptive effects [27, 28, 49].

Peripheral nerve injury frequently results in chronic neuropathic pain [11]. Changes in both the periphery and the CNS underlie the development of neuropathic pain, including altered expression of neurotrophic factors and ion channels [1, 8, 20], activation of astrocytes and microglia [26, 44], and activity of bulbospinal neurons in the RVM [9, 23, 37]. Previous studies have suggested that neuronal death in dorsal root ganglia, spinal cord and brain contributes to neuropathic pain [10, 22, 40, 43, 45], (although see also [35, 36]). We have reported that spinal nerve ligation (SNL), a model of neuropathic pain, also results in a significant decrease in the total number of neurons in the RVM [32]. Pharmacologically promoting neuronal survival in the RVM reduces hypersensitivity after nerve injury [32], suggesting that neuronal death contributes to the phenotype of neuropathic pain. Moreover, previous studies have reported that lesioning descending brain stem axons after SNL causes nociceptive thresholds to increase [9]. These findings suggested that neuropathic pain results at least part from nerve injury selectively killing antinociceptive RVM neurons [32].

In the present study, we examine whether RVM neurons are lost in other models of neuropathic pain. We examined two such models, chronic constriction injury (CCI) and spared nerve injury (SNI). Like SNL, both CCI and SNI involve injury to a nerve innervating the hindpaw and result in tactile hypersensitivity [15, 31]. We found that no neuronal loss was observed in either model 10 days after nerve injury. These findings suggest that cutaneous hypersensitivity can develop in response to nerve lesions without significant loss of RVM neurons.

Materials and Methods

Animals

Male Sprague-Dawley rats (150-175 g; Harlan, Madison, WI) were used for these studies. Animals were housed in pairs and allowed ad lib access to food and water. All experiments and procedures were performed using protocols approved by the University of Minnesota Institutional Animal Care and Use Committee.

Animal surgeries

All survival surgeries were performed using isoflurane anesthesia. Anesthesia was induced using 4% isoflurane in oxygen and maintaining using 1.5-2.5%.

Chronic constriction injury—The common sciatic nerve was exposed at the level of the middle of the thigh by blunt dissection through bicep femoris. Proximal to the trifurcation of the sciatic, about 1 cm of nerve was freed of adhering tissue and 4 ligatures (4.0 chromic gut) were tied loosely around it with about 1 mm spacing [7]. The length of nerve thus affected was 4-5 mm long. Great care was taken to tie the ligatures such that the diameter of the nerve was just barely constricted when viewed with magnification. In sham-operated animals, the sciatic nerve was exposed without being ligated.

Spared nerve injury—The sciatic nerve was exposed and its three terminal branches (the sural, common peroneal and tibial nerves) were identified. The tibial and common peroneal nerves were ligated with 5.0 silk and cut distal to the ligation, leaving the sural nerve intact [15]. Great care was taken to avoid any contact with or stretching of the sural nerve. In sham-operated controls, the sciatic nerve and its branches were exposed but were not ligated or sectioned.

Perfusion fixation

Rats were euthanized 10 days after surgery. Rats were deeply anesthetized with a mixture of ketamine (67.5 mg/kg), xylazine (22.5 mg/kg) and acepromazine (1 mg/kg) and perfused via the ascending aorta with 180 ml oxygenated Ca²⁺-free Tyrode's solution (pH 7.2) followed by 500 ml of 4% formaldehyde (freshly made from paraformaldehyde) in 0.16 M phosphate buffer (pH 6.9). Immediately after fixation, brains were removed and stored in a 5% sucrose solution prior to sectioning.

Histology and Immunocytochemistry

Previous studies using transient markers such as TUNEL or caspase-3 have not successfully demonstrated apoptosis in the RVM after peripheral nerve injury [32, 44], possibly due to the very short duration of apoptosis [34]. For that reason we used neuronal counting, a cumulative method for determining cell loss that is not sensitive to the speed at which neurons die. The RVM was sectioned using a freezing microtome (Leica, SM2400) at a nominal thickness of 50 µm. The free-floating sections were washed in phosphate-buffered saline (PBS) for three 5-minute intervals. To identify serotonergic neurons, we stained for tryptophan hydroxylase (TPH), the enzyme catalyzing the rate-limiting step in serotonin synthesis. RVM sections were incubated overnight at 4°C in a solution containing mouse anti-TPH; (catalog #T0678, Sigma, Saint Louis, MO, 1:1000). Sections were then washed in PBS and incubated for 4 h with Cy5-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA, 1:500). All sections were counterstained with the fluorescent Nissl stain ethidium bromide (30 nM, Sigma; [39]) [39].

Microscopy and Quantification

Conventional microscopy was used to collect images of the RVM. An Olympus BX50 fluorescence microscope (Tokyo, Japan) equipped with a 40×, 0.85 NA objective and correction collar was used; the filter sets were designed to allow selective visualization of Cy5 and ethidium bromide (Chroma Technology, Bellows Falls, Vermont). Microscopic images were collected with a Scion 1346 digital camera.

We estimated the number of neurons and glia in the RVM using methods similar to those previously described [32]. The RVM was defined as an isosceles triangle that lies at the level of the facial nucleus with a base having a width equal to that of the combined pyramidal tracts, and a height equal to half the width of the base [32]. The RVM extended from the rostral end of inferior olive to the caudal end of the trapezoid body. It was divided into ipsilateral and contralateral sides by the midline.

Nissl staining was used to count neurons and glia. Neurons had brightly fluorescent cytoplasm, a vacuuous nucleus, and single distinct nucleolus. By contrast, glia had very lightly-stained cytoplasm, a dark nucleus, and nuclear granulations rather than a nucleolus (Figure 1). We previously have obtained nearly identical results for cell counting using Nissl staining and NeuN staining [32]. However, we chose to use Nissl staining for cell counting for three reasons. Firstly, some neurons are not stained by NeuN (e.g., neurons of the facial nucleus, adjacent to the RVM). Secondly, the top of the nucleus is used as the counting point for NeuN-stained neurons, whereas the top of the nucleolus is used with Nissl-stained neurons. The nucleolus is smaller and thus allows greater precision in localizing the top, which would be expected to result in more accurate counts. Thirdly, with regard to counting glia, glial stains such as GFAP and OX42 are induced by glial activation [18, 42] and unlike Nissl staining (which shows the presence of nucleic acids) would not be expected to label all glia.

Systematic random sampling was used to choose the sections and the points within the RVM that were to be evaluated [29]; the entire RVM was sampled. Stereologically unbiased methods for cell counting (including use of a counting frame and the optical disector method) were used to count cells at those points and to estimate the total numbers of RVM neurons ipsilateral and contralateral to the lesion [32]. For counting neurons, a volume of $50\text{ }\mu\text{m} \times 50\text{ }\mu\text{m} \times 50\text{ }\mu\text{m}$ of RVM was counted per sampling point. Because glia were denser than neurons in the RVM, a smaller counting frame ($35\text{ }\mu\text{m} \times 35\text{ }\mu\text{m} \times 50\text{ }\mu\text{m}$) was used for counting glia.

Von Frey Testing for Tactile Hypersensitivity

Mechanical sensitivity was determined by measuring the paw withdrawal threshold in response to the application of von Frey filaments, using the up-down method of Chaplan [13]. Filaments above 15 g were not used; if a rat didn't respond to the 15g filament the threshold was listed as 15g. Withdrawal thresholds were measured bilaterally; baseline values were determined prior to surgery and experimental values were determined ten days after surgery. Observers were unaware of the experimental group to which the animals belonged.

Statistics

Cell counts were shown to be normally distributed except in the case of the SNI sham-operated group, in which the N (which was 6) was sufficiently small that no conclusion could be drawn from a normality test. Given that the data from all the other experimental groups were normally distributed, it appeared likely that the sham-SNI population was also normally distributed, and therefore parametric statistical tests were used. Differences among experimental groups were identified by 2-way analyses of variances (ANOVA). Statistical tests were performed using the GraphPad Prism software.

Results

CCI and SNI induced tactile hypersensitivity ipsilateral to the surgery

As previously reported [7, 15], CCI and SNI resulted in hypersensitivity to tactile stimuli ipsilateral, but not contralateral, to the surgery. Ten days after CCI surgery, the withdrawal threshold for the hindpaw ipsilateral to the surgery was significantly lower than that in sham-operated rats. Thresholds in the ipsilateral paws of rats subjected to CCI were also significantly lower than those in their contralateral hindpaws (Figure 2A)

Similar results were found when comparing SNI experimental groups. At day 10 post-surgery, the withdrawal threshold for the hindpaw ipsilateral to the lesion had significantly decreased in SNI-treated rats when compared to sham-operated rats. In addition, ipsilateral paw thresholds of animals receiving SNI were significantly lower than those in their contralateral hindpaws (Figure 2B).

No decrease in the number of RVM neurons, including TPH-ir neurons, 10 days after CCI—Ten days after surgery, CCI resulted in no significant decrease in the total number of RVM neurons ipsilateral to the lesion (Figure 3A). In rats that had received CCI, the number of Nissl-stained neurons in the half of the RVM ipsilateral to the lesion was $12,622 \pm 1,885$ (mean $\pm$ SEM), which was not significantly different from the number found in the ipsilateral side of the RVM in sham-operated rats ($14,412 \pm 1,270$). Additionally, there was no significant difference between the number of neurons in the ipsilateral and contralateral sides of the same CCI-treated animals. Similarly, no differences were observed among TPH-ir neurons after CCI (Figure 3B). We also determined the proportion of all RVM neurons expressing TPH. On average, 9.4% of all RVM neurons expressed TPH-ir in the CCI experiments. The proportion ranged from 8.8% (contralateral sham-CCI) to 10.1% (ipsilateral sham-CCI); no significant differences were observed among treatment groups (2-way ANOVA; $p > .42$).

No decrease in the number of RVM neurons 10 days after SNI—As with CCI, SNI induced no significant differences in the total number of RVM neurons, either when compared to sham-operated rats or compared to the contralateral sides of rats receiving SNI (Figure 4A). Similarly, we found no differences if we examined only RVM TPH-ir neurons (Figure 4B). In the SNI experiments, an average of 12.5% of neurons expressed TPH-ir, ranging from 10.9% (ipsilateral sham-SNI) to 13.6% (ipsilateral SNI). Again, no significant differences in proportion were observed among treatment groups (2-way ANOVA; $p > .64$).

Glial number unchanged after CCI and SNI—Our previous experiments found that the number of RVM glia increased by over one-third, after SNL [32]. Therefore, we counted the number of RVM glia ten days after CCI, SNI, and their respective sham operations. Using Nissl-stained tissue, we found no increases in numbers of glia in CCI-treated animals compared to sham-operated animals, in either side of the RVM (Figure 5A). In addition, we found no significant differences in numbers of glia between animals receiving SNI or sham-SNI surgery (Figure 5B).

Discussion

The main finding of this study is that neither CCI nor SNI resulted in significant RVM neuronal loss within 10 days after surgery. Whereas SNL resulted in loss of over 20% of RVM neurons ipsilateral to the lesion and profound gliosis [32], neither neuronal loss nor glial proliferation were observed after CCI or SNI. We had hypothesized that neuronal loss in the RVM was necessary for the development of long-lasting hypersensitivity after peripheral nerve injury, but this appears not to be the case.

The RVM has been proposed to play a crucial role in opioid antinociception, electrical stimulation-induced analgesia, and placebo-induced analgesia [3, 19]. Electrical stimulation of the RVM results in powerful antinociception in experimental animals [21, 24, 38]. Similarly, microinjection of opioids into the RVM also results in antinociception [2, 16, 17, 33]. Lesions of the RVM or the dorsal portion of the lateral funiculus (DLF: in which RVM axons descend to the spinal cord) reduce the antinociceptive effects of both systemic morphine and opioids microinjected into the periaqueductal gray matter [4, 6, 38, 46]. However, the RVM has also been proposed to have a crucial role promoting hypersensitivity in neuropathic pain. Previous studies have suggested that the hypersensitivity observed after SNL results from increased activity of RVM ON-cells [12], and that selectively lesioning their somata significantly reduces the hypersensitivity [37].

It is not entirely clear how the RVM facilitates nociception after nerve injury. Activation of RVM glia appears to mediate some of the hypersensitivity seen after CCI [44], but it is unclear to what extent this plays a role in other models of neuropathic pain. Interestingly, though, the hypersensitivity observed after SNL is not initially mediated by the RVM [9]. The first phase of hypersensitivity, which occurs from one to four days after surgery, is unaffected by DLF lesions. In contrast, lesioning the DLF reduces hypersensitivity starting five days after SNL [9]. We suspect that this four-day delay may reflect the time needed for death of RVM neurons. The neuronal loss that we have observed after SNL was accompanied by extensive gliosis [32], consistent with neuronal death. Moreover, the neuronal loss was completely blocked by administration of tauroursodeoxycholic acid, a drug that inhibits apoptosis and promotes cell survival [32]. Based on these and other findings, we have concluded that antinociceptive RVM neurons selectively die after SNL, leaving behind RVM neurons that facilitate pain.

Although it plays a significant role in generating the tactile hypersensitivity observed after SNL, RVM neuronal loss appears not to characterize all models of neuropathic pain. In the present study, we found neither RVM neuronal loss nor gliosis (which would be expected in response to neuronal death) 10 days after CCI or SNI. These findings suggest that loss of RVM neurons is not necessarily required for development of long-lasting neuropathic pain. Both CCI and SNI damage nerves further from the spinal cord than SNL and it is possible that RVM cell loss takes longer to develop in these models. Thus these experiments should be repeated using longer survival times.

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Abbreviations

CCI	chronic constriction injury
CNS	central nervous system
DLF	dorsal portion of the lateral funiculus
PBS	phosphate-buffered saline
RVM	rostral ventromedial medulla
SNI	spared nerve injury
SNL	spinal nerve ligation
TPH	tryptophan hydroxylase

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Highlights

- We examined whether RVM neurons were lost in two models of neuropathic pain, chronic constriction injury (CCI) and spared nerve injury (SNI).
- Tactile hypersensitivity was observed after both CCI and SNI.
- RVM neuronal loss was observed ten days after neither CCI nor SNI.
- RVM neuronal death would be expected to result in glial proliferation (gliosis). Gliosis was not observed 10 days after either CCI or SNI.
- We conclude that loss of RVM neurons is not required for development of hypersensitivity after CCI or SNI.

Figure 1.

Nissl staining of RVM neurons and glia. Neurons and glia have distinct morphologies in the RVM. A: Lower-magnification overview of the medial RVM, showing the region from which the higher magnification image was obtained. The pyramidal tracts (pt) are highlighted with dotted lines. B: Higher-magnification images of neurons (arrows) and glia (arrowheads). Note that neurons have stained nucleoli, vacuolated nuclei, and stained cytoplasm, and generally are larger than glia. Also note the sparse cytoplasm and stained

nuclei of glia, and that glia in the RVM resemble the glial labeling in the pyramidal tracts.
Bar = 100 μm in A and 50 μm in B.

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Figure 2.

CCI and SNI result in tactile hypersensitivity ten days after surgery. In both cases, tactile sensitivity was measured as von Frey thresholds. A: Chronic constriction injury (CCI) induced tactile hypersensitivity. Paw withdrawal thresholds ipsilateral to the injury were significantly decreased compared the contralateral side and to thresholds in sham-operated rats (* $p < 0.0001$, $n = 9$ animals receiving CCI, $n = 8$ sham-operated rats, $F_{(1,15)} = 29.9$ for experimental treatment; $F_{(1,15)} = 30.17$ for side; 2-way ANOVA). B: Spared nerve injury (SNI) induces mechanical hypersensitivity. Paw withdrawal thresholds were again

significantly decreased compared both to the contralateral side and to sham-operated animals (* $p < 0.0001$; $n = 8$ in both groups; $F_{(1,14)} = 286.6$ for experimental treatment and $F_{(1,14)} = 357.3$ for side; 2-way ANOVA).

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Figure 3.

No significant differences in RVM neuronal number were observed ten days after CCI. Ten days after CCI surgery, rats were killed and their brains were sectioned. The RVM was counterstained with the fluorescent Nissl stain ethidium bromide and immunocytochemically stained for TPH. (A) CCI resulted in no change in the number of Nissl-stained neurons ($n=9$ animals in each group, $p>0.973$ and $F_{(1,16)} = 0.00114$ for the effect of experimental treatment, and $p>0.864$ and $F_{(1,16)} = 0.03013$ for the effect of side; 2-way ANOVA). (B) CCI resulted in no significant difference in the number of TPH-ir

neurons observed in the RVM ($p > 0.93$ and $F_{(1,16)} = .0062$ for the effect of experimental treatment and $p > .490$ and $F_{(1,16)} = .4996$ for the effect of side; 2-way ANOVA).

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Figure 4.

No significant differences in RVM neuronal number were observed ten days after SNI. As with CCI, rats were allowed to survive 10 days after surgery, after which their brains were fluorescently Nissl-stained and immunocytochemically stained for TPH. (A) SNI did not significantly change the number of Nissl-stained neurons ($N=6$ for sham surgery and $N=8$ for SNI. $F_{(1,12)} = 1.127$ for the effect of experimental treatment; $F_{(1,12)} = 3.415$ for the effect of side; $P > .05$ in both cases.) (B) Similarly, SNI had no significant effect on the number of

TPH-ir neurons (N=6 for sham surgery and N=8 for SNI. $F_{(1,12)} = 1.43$ for the effect of treatment and $F_{(1,12)} = .822$ for the effect of side, $p > 0.25$ in both cases; 2-way ANOVA).

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Figure 5.

Neither CCI nor SNI significantly altered the number of glia counted in the RVM ten days after surgery. A: CCI resulted in no significant difference in the number of RVM glia (N=8 animals in each group; $F_{(1,14)} = .513$ for the effect of experimental treatment and $F_{(1,14)} = .221$ for the effect of side; $p > 0.4$ in both cases.) B: In addition, SNI had no significant effect on the number of RVM glia (N =8 animals receiving SNI, n=6 sham-operated animals;

$F_{(1,12)} = .0445$ for the effect of experimental treatment and $F_{(1,12)} = .321$ for the effect of side; $p > 0.5$ in both cases.)

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