

## Neuroinflammation

# Topography of Microglial Activation in Sensory- and Affect-Related Brain Regions in Chronic Pain

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Microglial activation in the spinal cord plays a central role in the development and maintenance of chronic pain after a peripheral nerve injury (PNI). There has not yet been a thorough assessment of microglial activation in brain regions associated with pain and reward. To this end, this study uses a mouse model of neuropathic pain in which the left sciatic nerve of male C57Bl/6J mice is loosely constricted (chronic constriction injury) to assess microglial activation in several brain regions 2 weeks after injury, a time point at which pain hypersensitivity is well established. We found significant microglial activation in brain regions associated with sensory pain transmission and affect, including the thalamus, sensory cortex, and amygdala. Activation was consistently most robust in brain regions contralateral to the side of injury. Brain regions not directly involved in either sensory or affective dimensions of pain, such as the motor cortex, did not display microglial activation. This study confirms that PNI induces microglial activation in regions involved with both sensory and affective components of pain. © 2016 Wiley Periodicals, Inc.

**Key words:** neuropathic pain; affect; inflammation; microglia activation; brain; RRID:AB\_839506

Chronic noncancer pain is defined as pain that persists for greater than 3 months, often in the absence of overt tissue damage (IASP, 1994). It is characterized by spontaneous, ongoing pain and heightened sensitivity to innocuous and painful stimuli. Microglia, the resident immune cells of the central nervous system, are critical players in the development of pain hypersensitivities that characterize chronic pain. Various animal models of chronic pain exhibit significant microglial activation in the superficial dorsal horn of the spinal cord, which directly contributes to the hypersensitivity of nociceptive afferents (Keller et al., 2007; Tsuda and Inoue, 2015). Blocking microglial activation alleviates pain hypersensitivity in several animal models of chronic pain (Raghavendra et al., 2003; Ledebuer et al., 2005; Mika et al., 2010).

Chronic pain conditions are also accompanied by significant affective and mood disorders, including depression and anxiety (Juurlink et al., 2004; Nicholson and Verma, 2004;

Asmundson and Katz, 2009; Elman et al., 2013). Microglial activation has been reported in brain regions of patients with major depressive disorder and in preclinical studies has been shown to contribute to the progression and severity of these conditions (for review see Bhattacharya et al., 2016). Although several animal and human studies have identified chronic pain-induced microglial activation in discrete regions of the brain, including the ventral tegmental area (VTA), nucleus accumbens, amygdala, and rostroventral medulla (Roberts et al., 2009; Huang et al., 2014; Sawada et al., 2014; Loggia et al., 2015; Taylor et al., 2015), systematic research to assess simultaneous microglial activation in brain regions involved in sensory and affective dimensions of pain has not yet been conducted. Therefore, this study assesses microglial activation in ipsilateral and contralateral brain regions associated with pain and affect in a mouse model of neuropathic pain.

## SIGNIFICANCE:

This study shows that a peripherally restricted chronic pain stimulus is capable of robustly activating central immune cells widely throughout the brain, particularly in regions associated with pain and affect. The potential effects of activated microglia are wide ranging with regard to chronic pain behaviors.

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## MATERIALS AND METHODS

### Animals

Thirty-four male, 8–10-week-old C57Bl/6 mice (The Jackson Laboratory, Bar Harbor, ME) were housed in groups of four on a 12-hr light/dark cycle, with food and water available ad libitum. No animals were excluded from analysis in any of the behavioral or molecular assays. All procedures were conducted in accordance with the National Institutes of Health *Guide for the care of use of laboratory animals* and were approved by the University of California, Los Angeles, and the University of California, Irvine, institutional animal care and use committees.

### Peripheral Nerve Injury

Animals were randomly assigned to one of two surgery groups, sham or peripheral nerve injury (PNI). Surgery was performed as described previously (Mosconi and Kruger, 1996; Taylor et al., 2015). All animals were anesthetized with gaseous isoflurane (induction at 5% and maintenance at 2.5% in oxygen). For the PNI, an incision was made in the lateral left thigh, followed by a blunt dissection of the left lateral thigh to expose the sciatic nerve. The nerve was encased with a polyethylene tube (PE50, 2 mm long). The control group (sham surgery) received a similar surgery without dissection of the nerve. Animals were allowed to recover in their home cages for 2 weeks.

### Behavior

Two weeks after nerve injury, mice were placed in a 10 × 10-cm Plexiglas box on a wire mesh floor ( $n = 6$  per group, based on previous experience with this technique; Taylor et al., 2015). After 30 min of acclimation to the environment, mechanical thresholds were assessed in the ipsilateral (left) paw of sham and PNI animals. von Frey filaments of incremental stiffness were applied, and a positive reaction was recorded after vigorous retraction of the hindpaw. The up-and-down method (Dixon, 1991) was used to measure the 50% withdrawal threshold (Chaplan et al., 1994). Withdrawal thresholds were averaged among groups and compared by Mann-Whitney test, and significance was set at  $P < 0.05$ .

### Immunocytochemistry

To assess PNI-induced microglial activation, tissue was processed for immunocytochemistry 2 weeks after nerve injury surgery. This time point was selected because we had previously shown that it correlates with peak pain behavior and robust microglial activation in the VTA (Taylor et al., 2014). Animals ( $n = 6$  per group) were transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer. The brain was dissected from the skull and postfixed in 30% sucrose before being cryo-sectioned at 50  $\mu\text{m}$ . Whole brains were divided into left and right hemispheres, and coronal sections were collected in 0.1 M phosphate-buffered saline (PBS) with 0.2% Triton X-100 (PBS-T) and incubated with blocking buffer (5% normal goat serum) for 1 hr prior to being immunolabeled for microglia with an anti-ionized calcium-binding adaptor molecule 1 (Iba-1) antibody (1:2,000; rabbit polyclonal; RRID:AB\_839506; catalog No. 016-20001; Wako, Osaka, Japan; previously

validated; Imai et al., 1996). Labeling was visualized with a goat anti-rabbit IgG conjugated to Alexa Fluor 488 (1:1,000; Invitrogen, Carlsbad, CA). For analysis, images were acquired on an Olympus (Tokyo, Japan) BX51 fluorescent microscope with a  $\times 20$  objective. Microglial activation was quantified by measuring cell body size in ImageJ. This technique has been validated against our previous methods with defined qualitative criteria to assess activation (Kozlowski and Weimer, 2012; Taylor et al., 2015, 2016). At least 20 cells over three separate slices per region per animal were measured and averaged to give a single value per animal. An experimenter blind to conditions performed the imaging and the quantification. For statistical analysis, mean and SEM were calculated per group and compared by two-way ANOVA, followed by Bonferroni post hoc analysis for comparing surgery and side. Differences were considered statistically different at  $P < 0.05$ .

### Quantitative Real-Time PCR

Brains were collected from sham and PNI mice (5/group) 8 weeks postsurgery, coronal sectioned via cryostat (150- $\mu\text{m}$  thick) at  $-20^\circ\text{C}$ , and mounted on charged Superfrost slides. Tissue punches (1 mm diameter) were taken by using a disposable biopsy plunger (Miltex, York, PA) for medial prefrontal cortex (mPFC), nucleus accumbens (NAc), bed nucleus stria terminalis (BNST), amygdala, hippocampus, thalamus, VTA, and dorsal raphe nucleus. Because the nerve injury was performed in only the left hind leg of the mouse, brain tissue regions from the left and right hemispheres were assessed separately.

Total RNA was collected from the brain tissue punches via Trizol extraction method (Ambion Life Technologies, Grand Island, NY). RNA was converted to cDNA with 100 U M-MuLV reverse transcriptase, 1  $\mu\text{M}$  oligo d(T)<sub>23</sub>VN, and 2 mM dNTP mix (New England Biolabs, Ipswich, MA); annealed at  $70^\circ\text{C}$ ; and inactivated at  $95^\circ\text{C}$ . Quantitative real-time PCR (qRT-PCR) was conducted with primer sets for Iba-1 (with primers for *Iba1* Iba1-F, ATC AAC AAG CAA TTC CTC GAT GA; Iba1-R, CAG CAT TCG CTT CAA GGA CAT A) and  $\beta$ -actin (with primers for *actb* as a control gene Actb-F, GGC TGT ATT CCC CTC CAT CG; Actb-R, CCA GTT GGT AAC AAT GCC ATG T). With 96-well optical plates (Applied Biosystems, Singapore), cDNA and PerfeCTa SYBR Green FastMix containing the primer sets (Quanta Biosciences, Gaithersburg, MD) were loaded and run on an ABI ViiA7 fast block qPCR machine with cycling conditions from the PerfeCTa SYBR Green FastMix manual. Cycle threshold outputs were calculated and normalized to the actin housekeeping gene to compute  $\Delta\text{Ct}$ . Relative expression levels were determined by normalizing sham and PNI groups to actin control via the  $\Delta\text{Ct}$  method. The results expressed as relative expression ( $2^{\Delta\text{Ct}}$ ). The mean and SEM were statistically analyzed by one-way ANOVA, with Bonferroni correction between sham and PNI brain regions.  $P < 0.05$  was considered significant.

## RESULTS

PNI resulted in a sustained pain state 2 weeks after surgery, as indicated by a significant reduction in mechanical

withdrawal thresholds (sham  $0.91 \text{ g} \pm 0.10$  vs.  $0.14 \text{ g} \pm 0.04$ ;  $P = 0.002$ , Mann-Whitney comparison;  $n = 6$  per group). Iba-1 gene expression was significantly increased in neuropathic pain compared with sham animals, especially in the hemisphere of the brain contralateral to the nerve-injured leg ( $n = 5/\text{group}$ ; Table I). Because primary nociceptive afferents decussate at the level of the spinal cord, injured afferent inputs are represented on the contralateral hemisphere of the brain. In particular, we observed significant increases in contralateral Iba-1 gene expression in the NAc (about twofold;  $F_{1,16} \text{ side} = 49.74$ ,  $P < 0.0001$ ;  $F_{1,16} \text{ injury} = 21.59$ ,  $P < 0.001$ ;  $F_{1,16} \text{ interaction} = 8.071$ ,  $P < 0.05$ ), VTA (3.6-fold;  $F_{1,16} \text{ side} = 6.1787$ ,  $P = 0.175$ ;  $F_{1,16} \text{ injury} = 41.74$ ,  $P < 0.01$ ;  $F_{1,16} \text{ interaction} = 2.912$ ,  $P = 2.91$ ), and thalamus (2.6-fold;  $F_{1,16} \text{ side} = 5.954$ ,  $P = 0.099$ ;  $F_{1,16} \text{ injury} = 45.84$ ,  $P < 0.001$ ;  $F_{1,16} \text{ interaction} = 17.19$ ,  $P < 0.01$ ) of the PNI group (Fig. 1).

**TABLE I. Iba-1 mRNA Levels in Isolated Brain Regions of Control (Sham) and PNI Groups\***

| Brain region | Sham        |               | PNI         |                              |
|--------------|-------------|---------------|-------------|------------------------------|
|              | Ipsilateral | Contralateral | Ipsilateral | Contralateral                |
| mPFC         | 0.13 (0.07) | 0.06 (0.03)   | 0.12 (0.12) | 0.17 (0.10)                  |
| NAc          | 0.15 (0.04) | 0.32 (0.03)   | 0.23 (0.07) | 0.62 (0.03) <sup>‡***</sup>  |
| BNST         | 0.14 (0.08) | 0.14 (0.05)   | 0.42 (0.02) | 0.74 (0.06) <sup>†***</sup>  |
| Amygdala     | 0.62 (0.05) | 0.54 (0.06)   | 0.71 (0.04) | 1.21 (0.07) <sup>‡,‡‡</sup>  |
| Hippocampus  | 0.31 (0.19) | 0.36 (0.02)   | 0.15 (0.05) | 0.12 (0.06)                  |
| Thalamus     | 0.35 (0.07) | 0.28 (0.05)   | 0.47 (0.07) | 0.74 (0.04) <sup>*,#††</sup> |
| VTA          | 0.14 (0.14) | 0.19 (0.07)   | 0.42 (0.11) | 0.67 (0.08) <sup>§</sup>     |

\*Data are relative expression normalized to  $\beta$ -actin ( $2^{\Delta Ct}$ ), with SEM in parentheses.

<sup>†</sup> $P < 0.01$  compared with ipsilateral side for same condition.

<sup>‡</sup> $P < 0.001$  compared with ipsilateral side for same condition.

<sup>§</sup> $P < 0.05$  compared with sham contralateral side.

<sup>|</sup> $P < 0.01$  compared with sham contralateral side.

<sup>#</sup> $P < 0.001$  compared with sham contralateral side.

<sup>\*\*</sup> $P < 0.05$  interaction between side and treatment.

<sup>††</sup> $P < 0.01$  interaction between side and treatment.

<sup>‡‡</sup> $P < 0.001$  interaction between side and treatment.

Similarly to the qRT-PCR data, significant increases in microglial cell body size as determined by Iba-1 immunolabeling were evident in many brain regions important for both sensory and affective pain transmission ( $n = 6$  per group; Fig. 2, Table II). In most regions, microglial cell body size was significantly greater on the contralateral but not the ipsilateral side of the PNI group. In particular, we noted significant increases in cell body size in several somatotopic regions of the primary sensory cortex, including the regions corresponding to hindlimb ( $F_{1,10} \text{ side} = 0.18$ ,  $P = 0.30$ ;  $F_{1,10} \text{ injury} = 64.48$ ,  $P < 0.0001$ ;  $F_{1,10} \text{ interaction} = 14.39$ ,  $P < 0.01$ ) and head ( $F_{1,10} \text{ side} = 6.26$ ,  $P = 0.09$ ;  $F_{1,10} \text{ injury} = 0.58$ ,  $P = 0.46$ ;  $F_{1,10} \text{ interaction} = 7.13$ ,  $P < 0.05$ ). We also noted significant unilateral microglial activation in habenula ( $F_{1,10} \text{ side} = 13.72$ ,  $P < 0.01$ ;  $F_{1,10} \text{ injury} = 5.85$ ,  $P < 0.05$ ;  $F_{1,10} \text{ interaction} = 2.55$ ,  $P = 0.14$ ) and VTA ( $F_{1,10} \text{ side} = 26.96$ ,  $P < 0.01$ ;  $F_{1,10} \text{ injury} = 3.98$ ,  $P = 0.07$ ;  $F_{1,10} \text{ interaction} = 10.68$ ,  $P < 0.008$ ). The nucleus accumbens showed microglial activation on both contralateral and ipsilateral sides in the PNI group ( $F_{1,10} \text{ side} = 0.29$ ,  $P = 0.59$ ;  $F_{1,10} \text{ injury} = 10.25$ ,  $P < 0.01$ ;  $F_{1,10} \text{ interaction} = 0.33$ ,  $P = 0.57$ ). The motor cortex did not show any change in microglial morphology on either side in the PNI group ( $F_{1,10} \text{ side} = 2.45$ ,  $P = 0.15$ ;  $F_{1,10} \text{ injury} = 0.07$ ,  $P = 0.79$ ;  $F_{1,10} \text{ interaction} = 0.56$ ,  $P = 0.47$ ). No change in microglial cell body size was observed in either the ipsilateral or the contralateral side of the sham group. Total numbers of microglial cells did not differ between sham and PNI groups or between ipsilateral and contralateral sides in any of the brain regions measured (data not shown).

**DISCUSSION**

This study shows that a peripheral nerve lesion results in significant microglial activation in brain regions associated with pain and affect, including thalamus, sensory cortex, and several limbic regions. This complements previous studies that have demonstrated robust microglial activation in the spinal cord after nerve injury and suggests that pain-induced neuroinflammation occurs widely

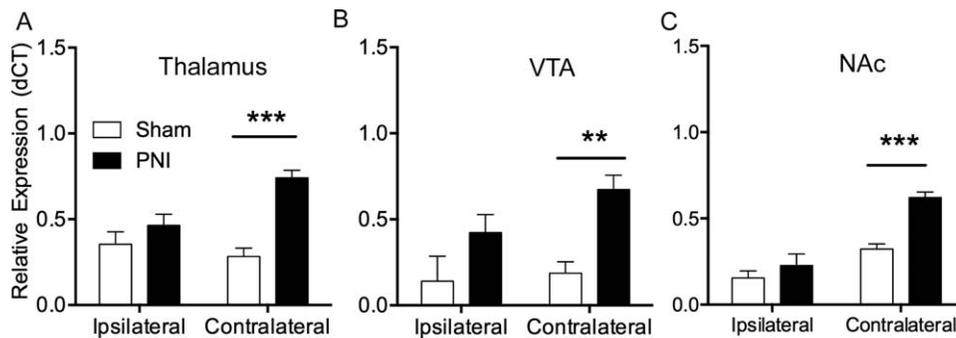


Fig. 1. Representative brain regions showing upregulation of Iba-1 mRNA after PNI. Expression levels of the Iba-1 mRNA were measured by qRT-PCR from brain regions both ipsilateral and contralateral to surgery. Data are transformed to relative expression ( $2^{\Delta Ct}$ ). Error bars show SEM. **\*\*** $P < 0.01$ , **\*\*\*** $P < 0.001$ .

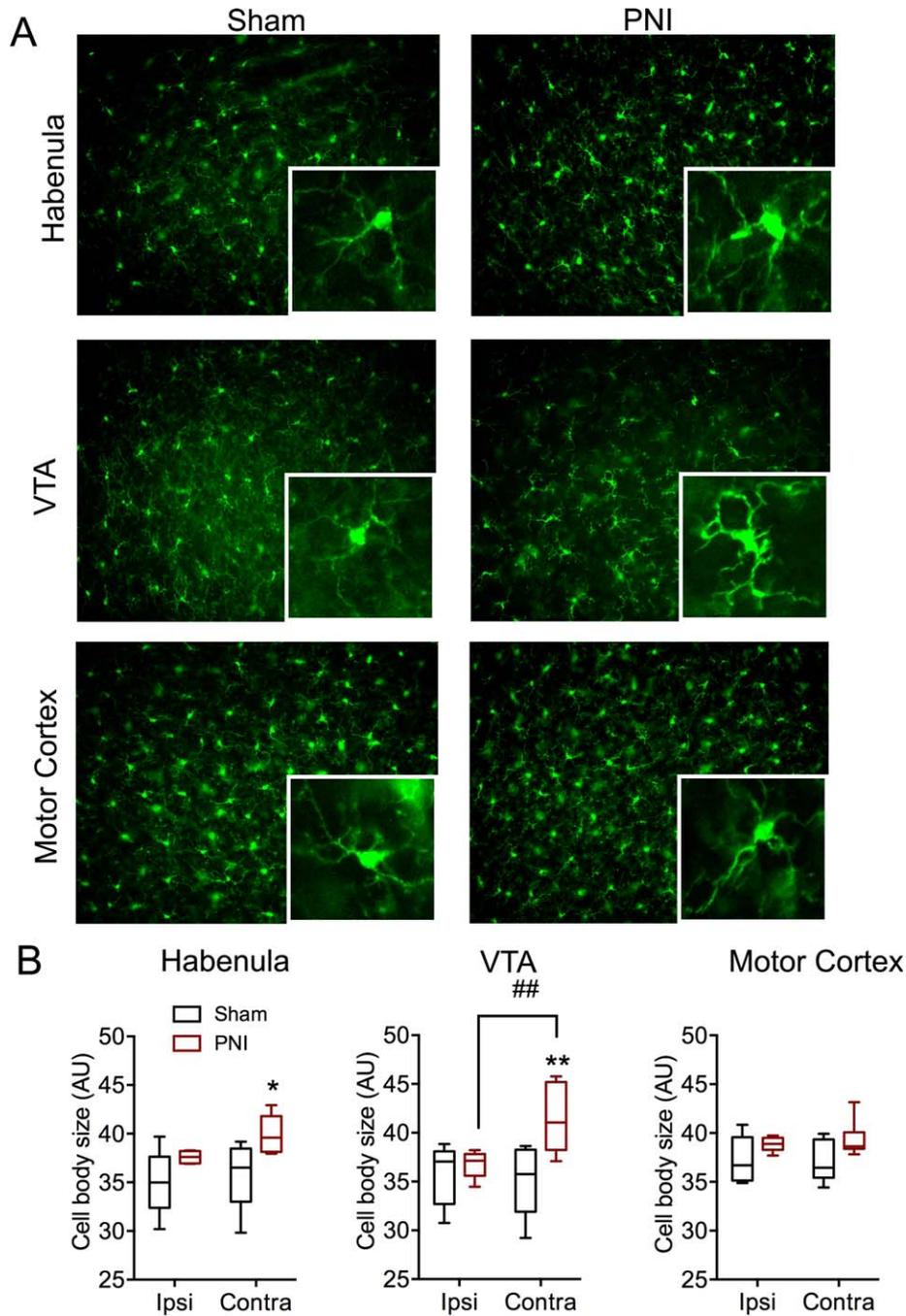


Fig. 2. Representative brain regions show changes in microglial morphology after PNI. **A**: Representative fluorescent micrographs taken with a  $\times 20$  objective from contralateral habenula, VTA, and motor cortex sections of sham and PNI animals labeled with Iba-1. **Insets**: Magnifications of larger images; inset box dimensions =  $100 \times 100 \mu\text{m}$ . **B**: Quantification of average microglial cell body size from sham

and PNI groups in select brain regions. Data are expressed as box and whiskers plots, with whiskers representing minimum and maximum values. Ipsi, ipsilateral; contra, contralateral. \* $P < 0.05$ , \*\* $P < 0.01$  compared with ipsilateral side of same group; ## $P < 0.01$  compared with contralateral side of sham group.

throughout the neuroaxis (Li et al., 2016; Taves et al., 2016). This premise is supported by a previous study showing that chemotherapy-induced pain resulted in significant microglial activation throughout many brain

regions, including the periaqueductal gray (PAG), thalamus, anterior cingulate cortex, secondary sensory cortex, and medium forebrain bundle (Di Cesare Mannelli et al., 2013). The current study extends these results by showing

TABLE II. Microglial Cell Body Size in Isolated Brain Regions of Control/Sham and PNI Groups\*

| Brain region       | Sham        |               | PNI                       |                           |
|--------------------|-------------|---------------|---------------------------|---------------------------|
|                    | Ipsilateral | Contralateral | Ipsilateral               | Contralateral             |
| S1-J <sup>†</sup>  | 36.7 (0.64) | 38.0 (1.37)   | 36.25 (0.67)              | 38.84 (0.63)**:‡‡         |
| S1-HL <sup>‡</sup> | 35.5 (0.76) | 34.1 (0.74)   | 39.7 (0.70)               | 42.3 (0.50)#,§§           |
| MC-J <sup>§</sup>  | 36.9 (0.75) | 37.2 (0.79)   | 38.8 (0.59)               | 39.3 (0.59)               |
| MC-HL <sup>‡</sup> | 36.0 (0.44) | 32.5 (0.77)   | 33.7 (0.64)               | 31.6 (1.01)               |
| NAc                | 35.7 (0.67) | 35.6 (0.37)   | 37.7 (0.72) <sup>††</sup> | 38.5 (0.69) <sup>††</sup> |
| Amygdala           | 38.1 (0.30) | 35.1 (0.80)   | 35.6 (0.52)               | 38.45 (1.56)              |
| Habenula           | 35.0 (1.51) | 35.9 (1.60)   | 37.6 (0.24)               | 39.9 (0.79)**:††          |
| PAG                | 35.5 (2.14) | 36.2 (1.34)   | 38.4 (0.45)               | 40.3 (0.83)#,††           |
| VTA                | 35.7 (1.41) | 35.2 (1.67)   | 36.8 (0.56)               | 41.4 (1.47)**:§§          |

\*Data are average cell body size in arbitrary units, with SEM in parentheses.

<sup>†</sup>Primary sensory cortex (jaw).

<sup>‡</sup>Primary sensory cortex (hindlimb).

<sup>§</sup>Motor cortex (jaw).

<sup>‡</sup>Motor cortex (hindlimb).

# $P < 0.05$  compared with ipsilateral side for same condition.

\*\* $P < 0.01$  compared with ipsilateral side for same condition.

†† $P < 0.05$  compared with sham contralateral side.

‡‡ $P < 0.05$  interaction between side and treatment.

§§ $P < 0.01$  interaction between side and treatment.

that even pain of a peripherally restricted origin is capable of activating microglia in diverse regions of the central nervous system. A recent study that examined chronic pain patients showed evidence for microglial activation in several of the same brain regions that are described in the current study, including the thalamus and sensory cortical regions (Loggia et al., 2015). Taken together, these studies form an emerging perspective that recognizes brain microglia as a key mechanism in the progression of chronic pain phenotypes.

It should be noted that an earlier study indicated no microglial activation in higher cortical regions after PNI (Zhang et al., 2008). The lack of microglial activation in this study could be due to the use of transgenically labeled microglia (CX3CR1-GFP); because this line exhibits only one functional copy of the CX3CR1 receptor, impairments in pain-induced microglial activation might be expected (Jung et al., 2000). This would be particularly true in the brain, in which the degree of microglial activation is not as robust as that observed in the ipsilateral spinal cord.

The wide range of brain regions in which microglial activation was detected raises the question of what signals or mechanisms are responsible for pain-induced microglial activation at sites far removed from the site of injury. The observation that the PNI did not induce microglial activation in all brain regions, such as the motor cortex, is notable. Moreover, in regions in which significant microglial activation was observed, the effect was always highest on the contralateral side. Because nociceptive afferents decussate at the level of the spinal cord, injured afferent inputs would be expected on the contralateral side. These observations suggest that microglial activation is driven by cues relating to injured-afferent input, although what these specific signals are is a question that remains unanswered.

The breadth of microglial activation throughout the brain suggests a potential for influencing pain progression in a variety of brain regions. Research delineating the mechanisms by which microglia influence pain behavior in a region-specific manner is in its infancy. We have recently shown that microglial activation in the VTA impairs dopamine neuron activity and opioid reward via a BDNF-KCC2 pathway (Taylor et al., 2015). In the amygdala, chemokine receptor type 2 and interleukin-1 $\beta$  were implicated in the development of pain-induced anxiety (Sawada et al., 2014). Given the robust activation of microglia that we observed in several brain regions, additional research is warranted to identify novel areas in which microglia contribute to the development and progression of chronic pain.

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## CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest.

## ROLE OF AUTHORS

AMWT and CMC conceived and designed the experiments. SL performed the qRT-PCR. AJT, SM, and AMWT performed the immunocytochemistry and analysis. AMWT and CMC wrote the article.

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