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Calcitonin gene-related peptide contributes to peripheral nerve injury-induced mechanical hypersensitivity through CCL5 and p38 pathways

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Abstract

The role of calcitonin gene related peptide (CGRP) in neuropathic pain was investigated in a mouse model of neuropathic pain, spinal nerve L5 transection (L5Tx). Intrathecal injection (i.t) of CGRP_{8–37}, a CGRP antagonist, significantly reduced L5Tx-induced mechanical hypersensitivity and lumbar spinal cord CCL5 expression. i.t. injection of a CCL5 neutralizing antibody significantly inhibited L5Tx-induced mechanical hypersensitivity. Further, pre-treatment with a p38-inhibitor, SB203580, was able to reduce CGRP-induced mechanical hypersensitivity, but not CGRP-induced CCL5 production. Our data indicate that CGRP can play its pro-nociceptive role through both a spinal cord CCL5-dependent, p38-independent pathway, and a p38-dependent, CCL5-independent pathway.

Graphical abstract

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Keywords

Calcitonin gene-related peptide (CGRP); CCL5; Neuropathic pain; Spinal nerve L5 transection; p38; Spinal cord

1. Introduction

Defined as pain caused by a lesion or disease of the somatosensory system, neuropathic pain is one of the most devastating kinds of chronic pain. Neuroimmune interaction is believed to play an important role in the development of neuropathic pain (DeLeo et al., 2004; Ji and Suter, 2007; Milligan and Watkins, 2009; Tsuda et al., 2005).

Calcitonin gene-related peptide (CGRP) is a 37-amino acid neuropeptide within the calcitonin peptide family, and is widely distributed in both the peripheral and the central nervous systems. Produced largely by medium and small diameter primary afferent neurons, CGRP is important in modulating pain perception. Despite some controversy, studies have provided evidence that CGRP plays a pro-nociceptive role under various conditions. For example, in rodents intrathecal injection of CGRP produced significant hyperalgesia in rodents to both mechanical and thermal stimuli (Cridland and Henry, 1989; Oku et al., 1987). Administration of a CGRP antagonist has been shown to reduce both thermal and mechanical hypersensitivity in non-injured rats and rats with inflammatory pain or neuropathic pain-like behaviors (Bennett et al., 2000a; Lee and Kim, 2007; Yu et al., 1996a; Yu et al., 1996b). Further, spinal nerve ligation (SNL) markedly enhanced capsaicin-evoked CGRP release in lumbar spinal cord tissue in rats, suggesting a potentially increased functional activity of CGRP+ nociceptors following-nerve injury (Gardell et al., 2003). Previously, we have observed a significant increase of CGRP expression in the lumbar spinal cord in mice following spinal nerve L5 transection (L5Tx), a murine model of neuropathic pain. In the current study we set out to assess the role of CGRP in neuropathic pain-like behaviors following L5Tx.

It has been established that numerous chemokines are involved in the development of neuropathic pain (Gao and Ji, 2010b; White et al., 2007). For example, CCR2 knockout mice (receptor for CCL2) displayed impaired neuropathic pain responses (Abbadie et al., 2003). Blocking CCL2 (monocyte chemoattractant protein-1 (MCP-1)) signaling via a neutralizing antibody prevented the development of SNL-induced sensory hypersensitivities (Gao et al., 2009). Further, mice either lacking CCL5 (regulated on activation, normal T cell expressed and secreted (RANTES)) or peritoneally administered with a selective CCL5 receptor antagonist, Met-RANTES, showed reduced hypersensitivity following partial sciatic nerve ligation (Liou et al., 2013; Liou et al., 2012). Injections of CCL5 either peripherally (Oh et al., 2001) or centrally (Benamar et al., 2008) induced hypersensitivities in rat. In addition, CX3CL1/CX3CR1 signaling and other chemokines, such as, CXCL1, CCL21 have also been implicated in the development of neuropathic pain (Clark and Malcangio, 2014; Zhang et al., 2013). In this study, the involvement of several chemokines in CGRP-mediated pain-like behaviors was further investigated using a chemokine multiplex assay. Result indicated a CCL5 mediated CGRP-induced mechanical hypersensitivity.

Mitogen activated protein Kinase (MAPK) pathways (including Jun N-terminal kinase (JNK), extracellular signal-regulated kinases (ERKs), and p38 MAPK) have been linked to chemokine signaling during the development of pain-like behaviors (Gao and Ji, 2010b). Associations between the p38 pathway and microglial activation, the JNK pathway and astrocyte activation, and the ERK pathway and neuronal responses, have been suggested during the development of pain state (Gao and Ji, 2010a; Gao and Ji, 2009; Ji and Suter, 2007; Jin et al., 2003; Li et al., 2010). Therefore, the involvement of MAPK pathways was also examined, which led to the detection of both CCL5-dependent/p38-independent and CCL5-independent/p38-dependent pathways mediated CGRP's pro-nociceptive effects.

2. Material and Methods

2.1. Animals

Male and female adult BALB/c mice (7–8 weeks old) were purchased from National Cancer Institute (NCI, Frederick, MD) and were allowed to habituate to the institutional animal facility for at least one week before experimental use. All mice were group-housed with food and water *ad libitum* and maintained on a 12-h light/dark cycle. The Institutional Animal Care and Use Committee (IACUC) at the University of New England (UNE) approved all experimental procedures used in this study.

2.2. L5Tx and drug treatment

Spinal nerve L5 transection (L5Tx) and sham surgeries were performed on 8-week-old BALB/c mice following our previously published procedures (Cao and DeLeo, 2008). Approximately the same numbers of male and female mice were used in each group. CGRP is 37 amino acids in length. The N-terminal amino acids 1–7 are required for receptor activation and signal transduction, whereas the remainder (amino acids 8–37) of CGRP is necessary for receptor binding; therefore a polypeptide that contains only amino acids 8–37 of CGRP (CGRP_{8–37}) acts as a CGRP receptor antagonist (Arulmani et al., 2004). To test the effects of CGRP and CCL5 in the development of L5Tx-induced behavioral

hypersensitivities, we utilized CGRP₈₋₃₇ (Sigma Aldrich, St. Louis, MI; 1 µg/mouse) and a monoclonal neutralizing antibody against CCL5 (R&D Systems, Minneapolis, MN; 0.5 µg or 2.5 µg/mouse), respectively. Mice were randomly assigned to different treatment groups, and then received intrathecal injections (5 µl/mouse) (see Table 1) daily from days 6 to 13 post-surgery. This time period was chosen based on the spinal cord level of CGRP expression (peaked at day 7 and day 14 post-L5Tx) (Malon et al., 2011) and previous report (Yu et al., 1996a; Yu et al., 1996b). Intrathecal injections were performed as described previously (Malon et al., 2011). Besides vehicle-injected mice, animals that underwent L5Tx but received no i.t. injections were also used as controls. An isotype control antibody was not used as a control due to its potential “specific antibody-like” effects as shown in a previous study (Arruda et al., 2000). If behavioral tests were conducted on the same day of injection, injection was given after the behavior tests on that day. Following conclusion of the study, spinal cord tissues from treated animals were collected and sent out for chemokine analysis (Quansys Biosciences, Logan, UT) (see below section 2.5).

2.3. CGRP and SB203580 treatment

To test the involvement of p38 MAPK pathway in CGRP-mediated mechanical hypersensitivity, CGRP and a p38 inhibitor, SB203580 (Sigma Aldrich) were i.t. injected to naïve animals (Table 1). SB203580 (5 ng/5 µl/mouse) was administered first and CGRP (2 µg/5 µl/mouse) was given 30 minutes later. Animals receiving corresponding vehicles were used as controls. i.t. injections were given as described above. Behavioral test started one hour after the second injection.

2.4. Behavioral tests

As previously described (Cao et al., 2012), mechanical sensitivity was determined via von Frey test following the up-down method (Chaplan et al., 1994) and the heat sensitivity was measured via Hargreaves test using an IITC thermal plantar analgesia instrument for mice (IITC Life Science, Woodland Hills, CA). Both tests were performed with mice under non-restrained conditions. The 50% threshold force needed for paw withdrawal was calculated and used to represent mechanical sensitivity. Average of latencies (at least two tests with an interval of at least 10 min) for paw withdrawal was recorded and used to represent heat sensitivity. Mice were baseline tested twice before day 0 (surgery or CGRP injection depending on the experiment). When von Frey up-down and Hargreaves tests were performed on the same day, mice were given 1 hour to acclimate between tests to avoid potential influence between tests. The person performing the behavioral tests was blinded to the experimental groups.

2.5. Measurement of chemokine levels

Chemokine multiplex assay was performed by Quansys Biosciences. Briefly, lumbar spinal cords were collected following previously published methods (Cao et al., 2009). First, tissues were sonicated in PBS with 1:100 dilution of protease inhibitor cocktail (Sigma). Then supernatants were collected following centrifugation ($10000 \times g$ at 4°C for 15 min) and stored at -80°C until assay. Prior to data collection, technicians at Quansys performed extensive pilot analyses to determine the assay buffer and sample dilutions. Total of 8 chemokines were included in the manufacturer’s pre-designed multiplex assay: CXCL1

(KC), CCL1 (TCA-3), CCL2 (MCP-1), CCL3 (MIP-1 α), CCL5 (RANTES), CCL11 (Eotaxin), CCL17 (TARC), and CCL22 (MDC). In follow-up experiments, levels of CCL5 were determined via the enzyme-linked immunosorbent assay (ELISA) using a mouse DuoSet ELISA kits (R&D Systems, Minneapolis, MN, USA) following the manufacturers' protocols. The standard series ranged from 0 – 1000 pg/ml for CCL5. Tissue levels of chemokines were normalized to the total protein levels of each sample, as determined by BCA assays (ThermoFisher Scientific, Waltham, MA).

2.6. Cytometric Bead Array (CBA) assay

Lumbar spinal cord tissue homogenates and cell lysates were collected following manufacturers recommendations (BD Biosciences, San Jose, CA). CBA assay was run following manufacturer's guidelines with kits that contained: capture beads, detection reagent, and standards for: phospho-p38 (T180/Y182), phosphorylated-JNK1/2 (T183/Y185), and phospho-ERK1/2 (T202/Y204) flex sets. An Accuri C6 flow cytometer (BD Biosciences) was used to analyze all samples. Flow cytometry files were further analyzed using the FCAP Array™ software (version 3.0) provided by the manufacturer. Data were recorded and normalized to the total protein of each sample, which was determined through BCA assay (ThermoFisher Scientific, Rockford, IL). The CBA assay was chosen instead of Western blotting because of it being a quantitative assay and due to difficulties in interpreting Western blots of spinal cord samples (due to the overwhelming non-specific bands generated by these complex tissue homogenates). The CBA assay offers a sensitive and high-throughput approach to examine signaling pathways (Elshal and McCoy, 2006; Morgan et al., 2004) and is highly comparable to Western blotting (Schubert et al., 2009).

2.7. Primary adult spinal cord glial cultures

Primary adult mixed glia cultures were established using 8-week-old BABL/c NCI mice and maintained in complete Dulbecco's Modified Eagle Media (cDMEM) (Lonza, Walkersville, MD) containing 50 μ M 2-mercaptoethanol (2-ME, Sigma-Aldrich) in 24-well plates as we have previously described (Malon et al., 2011). Media was replaced on days 2, 4, 8, and 12 after the establishment of each culture. All cells were maintained at 37°C in a humidified atmosphere with 5% CO₂. Mixed glia were treated at day 14 following the establishment of each culture with CGRP at 25, 100, 1000, and 4000 ng/ml as determined from previous experiments (Malon et al., 2011). Supernatants and cell lysate were collected 24 and 48 hours after treatment for CCL5 ELISAs and CBA respectively (see above).

2.8. Statistical analysis

All statistical analyses were performed using SigmaStat 3.5 (Systat Software, Inc.). Appropriate analysis of variance (ANOVA) with treatment and time as factors were performed followed by Student-Newman-Keuls (SNK) *Post hoc* analysis. All data are presented as mean $\pm$ SEM. $p < 0.05$ was considered as statistically significant.

3. Results

3.1. Changes of L5Tx-induced sensory hypersensitivity following the treatment of CGRP₈₋₃₇

Previously, we have shown that L5Tx induced a significant increase in CGRP expression in the lumbar spinal cord (Malon et al., 2011). To further determine the role of CGRP in the development of L5Tx-induced hypersensitivity, a CGRP antagonist, CGRP₈₋₃₇ was i.t. injected (at 1 µg/5 µl) into WT BALB/c mice daily from days 6 to 13 post-L5Tx. Both mechanical and heat sensitivities were measured via the up-down method and Hargreaves test respectively. Both male and female mice were used and gender differences were also determined during data analysis. For the mechanical sensitivity, no significant gender effect was detected (Figure 1A, three-way analysis of variance (ANOVA) with time, treatment, and sex as factors, $p_{\text{sex}} = 0.656$), thus all data were pooled together for further analysis. Consistent with our previous report (Malon et al., 2011), compared to both non-injected and vehicle injected controls, CGRP₈₋₃₇ significantly reduced L5Tx-induced mechanical hypersensitivity beginning at day 7 and continued until the end of the experiment (day 20) (Figure 1A, Two-way ANOVA, $p_{\text{treatment}} < 0.001$, $p_{\text{time}} < 0.001$ and $p_{\text{interaction}} < 0.001$; lumbar spinal cords were collected from selected mice at various time points for chemokine analysis below, thus here $n = 9-45$). For the Hargreaves test, initial analysis suggested a gender difference (Figure 1B, three-way ANOVA with time, treatment and sex as factors, $p_{\text{sex}} = 0.023$), therefore, data from male and female mice were analyzed separately. In both male and female mice CGRP₈₋₃₇ did not significantly affect L5Tx-induced heat hypersensitivity (male Two-way RM ANOVA, $p_{\text{treatment}} = 0.937$, $p_{\text{time}} < 0.001$ and $p_{\text{interaction}} = 0.068$; and female Two-way RM ANOVA, $p_{\text{treatment}} = 0.222$, $p_{\text{time}} < 0.001$ and $p_{\text{interaction}} = 0.168$). Since no differences were detected in these separate analyses for males vs. for females, all data were pooled together to be shown in Figure 1B. Together, this set of data combined with our previous report, points to CGRP's involvement in the maintenance of mechanical, but not heat, hypersensitivities following L5Tx.

3.2. Reduction of L5Tx-induced lumbar spinal cord CCL5 following the treatment of CGRP₈₋₃₇

To determine whether chemokines are involved in CGRP-mediated mechanical hypersensitivity, lumbar spinal cords were collected from individual mice in the above three groups: i.t. with CGRP₈₋₃₇, i.t. with vehicle, and non-treated group, and were then analyzed for selected chemokine expression via a multiplex assay. Of the eight chemokines tested, CGRP₈₋₃₇ only significantly reduced L5Tx-induced increase of spinal cord CCL5 (Figure 2A, two-way ANOVA comparing data from day 7, 14 and 20, $p_{\text{group}} = 0.041$, $p_{\text{time}} = 0.054$, and $p_{\text{interaction}} = 0.427$). Although levels of spinal cord CCL2 and CCL11 were also significantly increased by L5Tx, they were not affected by CGRP₈₋₃₇ treatment (Figure 2B, one-way ANOVA for “no i.t.” groups $p < 0.001$; Figure 2C, one-way ANOVA for “no i.t.” groups $p = 0.049$). Therefore, our data indicate that CGRP can play its pro-nociceptive role through the induction of CCL5 in lumbar spinal cord following L5Tx.

3.3. Reduction of L5Tx-induced mechanical hypersensitivity following the treatment of CCL5 neutralizing antibody

To directly test the effects of CCL5 in L5Tx-induced mechanical hypersensitivity, WT mice were i.t. treated with a CCL5 neutralizing Ab (0.5 or 2.5 μg per 5 μl per mouse) daily from days 6 to 13 post-L5Tx. For all treatment groups, L5Tx induced significant mechanical hypersensitivity. Both doses of anti-CCL5 significantly reduced L5Tx-induced mechanical hypersensitivity at day 7, 10, 14, and 20 post-surgery when compared to either PBS injected or non-injected groups (Figure 3, two-way RM ANOVA, $p_{\text{group}} < 0.001$, $p_{\text{time}} < 0.001$, and $p_{\text{interaction}} < 0.001$). Together with the data presented in Figure 2, these data further support the involvement of spinal cord CCL5 in CGRP-mediated pro-nociceptive role.

3.4. L5Tx-induced activation of MAPK pathway

MAPK pathways, including p38, JNK and ERK pathways have been shown to be involved in the chronic pain state in animal models (Chen et al., 2013; Gao and Ji, 2010a; Ji and Suter, 2007; Ji et al., 2007; Li et al., 2010). We then examined the involvement of these pathways in L5Tx-induced behavioral hypersensitivity. Spinal cord tissues were collected from injured and sham-operated animals at days 0 (naïve), 1, 3, 7, 10 and 14 post-surgery and processed for the analysis of phosphorylated p38, JNK and ERK (pho-p38, pho-JNK and pho-ERK) via CBA assay. Spinal cord levels of pho-p38 gradually increased following L5Tx, which peaked at day 7 post-L5Tx, and returned to baseline by day 14 post-L5Tx (Figure 4A, two-way ANOVA, $p_{\text{group}} = 0.045$, $p_{\text{time}} = 0.018$, and $p_{\text{interaction}} = 0.599$). L5Tx did not significantly change the levels of pho-JNK (Figure 4B, two-way ANOVA, $p_{\text{group}} = 0.460$, $p_{\text{time}} = 0.665$, and $p_{\text{interaction}} = 0.947$). The levels of pho-ERK were too low to be detected in the majority of tissue samples.

3.5. Reduction of CGRP-induced mechanical hypersensitivity by pre-treatment with a p38 inhibitor

To further test whether the p38 MAPK pathway is involved in CGRP mediated mechanical hypersensitivity, WT mice were i.t. treated with a p38 inhibitor SB203580 30 min prior to CGRP i.t. injection. Mice were randomly assigned to one of the following 4 groups: vehicle-vehicle, p38 inhibitor-vehicle, vehicle-CGRP, and p38 inhibitor-CGRP injected. We observed that one injection of CGRP significantly increased mechanical sensitivity at 1, 5, and 24 hours after CGRP injection, and injection of the p38 inhibitor before CGRP was able to reduce the CGRP-induced mechanical hypersensitivity seen at 1, 5, and 24 hours post-CGRP injection (Figure 5A, two-way RM ANOVA, $p_{\text{group}} = 0.009$, $p_{\text{time}} < 0.001$, and $p_{\text{interaction}} = 0.001$). Furthermore, to test whether the p38 MAPK pathway is involved in CGRP-induced CCL5 production, levels of spinal cord CCL5 were quantified in mice from the above 4 groups. Single CGRP i.t. injection induced a slight increase of CCL5 in the lumbar spinal cord and this increase was not affected by SB203580. Unexpectedly, SB203580 induced a slight increase of CCL5 in the lumbar spinal cord 24 hours post-i.t. injection (Figure 5B, two-way ANOVA, $p_{\text{group}} = 0.404$, $p_{\text{time}} < 0.001$, and $p_{\text{interaction}} = 0.782$). Thus, data suggest that CGRP/CCL5 mediated pro-nociceptive pathway is p38-independent and an additional p38-dependent/CCL5-independent pathway is possible.

CGRP receptors have been detected on CNS glia, including both astrocytes and microglia (Moreno et al., 2002; Parsons and Seybold, 1997). To further identify the cellular source of CGRP-induced CCL5 and *pho-p38*, primary mixed glial cells established from adult mouse spinal cords were treated with various doses of CGRP *in vitro*. *pho-p38*, *pho-JNK* and *pho-ERK* in the cells, and CCL5 in the supernatants were measured via CBA assay and ELISA respectively, 24 and 48 hours post-treatment. Under the treatment condition we used, CGRP did not induce a significant increase in the expression of any of the MAPK pathway proteins or CCL5 release (data not shown).

4. Discussion

In this current study, we investigated the role of CGRP in peripheral nerve injury-induced neuropathic pain-like behaviors using a well-established mouse model, L5Tx. Our results indicate a novel neuroimmune pathway involved in the development of neuropathic pain behavior: peripheral nerve injury -> increased spinal cord CGRP expression (shown in our previous study (Malon et al., 2011)) -> increased spinal cord CCL5 production -> mechanical hypersensitivity (Figure 6, thick lines). It should be noted that we have performed pilot study with CGRP₈₋₃₇ at both 0.5 and 1 µg/mouse and CGRP₈₋₃₇ at 0.5 µg/mouse was able to reduce L5Tx-induced mechanical hypersensitivity up to day 14 post-L5Tx; however, this reduction declined at days 17 and 20 post-L5Tx. Thus, we think the effects of CGRP₈₋₃₇ are dose-dependent and the effects of 1 µg of CGRP are not permanent. Interestingly, this pathway does not seem to be involved in L5Tx-induced heat hypersensitivity as measured by the Hargreaves test. Although unexpected, similar phenomena have been observed by others. For example, in a rat model of sleep disruption, mechanical and heat hypersensitivity exhibited differential sensitivity to several pharmacological agents, suggesting differing and dissociable mechanisms in mechanical and heat sensory responses (Wodarski et al., 2014). A study with mice lacking various types of prostaglandin receptors also showed disparities in the responses following mechanical vs. heat stimuli (Popp et al., 2009). Together with the current study, these studies suggest possible modality-specific sensory signal transduction pathways in nociception. Although it is well known that different types of noxious stimuli activate different transient receptor potential channels (TRP channels) (Nilius et al., 2007), studies have indicated that the interaction between different TRP channels expressed by a sensory neuron, rather than the existence of a specific TRP channel, determines the sensory neuron's response to a certain stimulus (Belmonte and Viana, 2008). CGRP may influence the composition of TRP channels expressed by the sensory neurons and thus modify their response to mechanical stimulus specifically. CGRP may also target particular inflammatory pathways (*p38*- or CCL5-mediated) that control the development of mechanical hypersensitivity. One study of interest in rats found that bee venom-induced inflammation during the neonatal stage modified the mechanical, but not thermal responses, in adulthood. This modification was associated with changes in IL-1 β and cyclooxygenase 2 (COX-2) expression in the spinal cord (Li et al., 2014). Another study, which used a mouse model of second-degree burn injury, demonstrated that the voltage-gated sodium channel Na(v)1.7 was essential for lowering the threshold for the heat but not the mechanical sensitivity response after burn injury (Shields et al., 2012). It is possible that certain sodium channels are critical for

mechanical sensitivity, and these channels can be modified by CGRP. Delineating the differences within these pathways may result in more specific and individualized treatment strategies for neuropathic pain patients, a patient population that exhibits a wide range of varying responses to noxious and non-noxious stimuli (Baron, 2006).

In the current study, we also examined the involvement of various MAPK pathways in the effects mediated by CGRP. Although JNK, ERK and p38 MAPK pathways have all been associated with the development of neuropathic pain (Gao and Ji, 2010a; Gao and Ji, 2009; Gao and Ji, 2010b; Ji and Suter, 2007), we only observed a significant upregulation of spinal cord p38 following L5Tx. Using a p38 pathway inhibitor, we demonstrated that CGRP's effect on mechanical hypersensitivity can be mediated through the activation of the p38 pathway. Despite this, our data suggest that CGRP-mediated CCL5 production following L5Tx was p38-independent. Thus, at least two separate pathways downstream of CGRP, a p38-dependent/CCL5-dependent pathway and a CCL5-dependent/p38 independent pathway are involved in mediating L5Tx-induced mechanical hypersensitivity (Figure 6 all solid lines). In addition, based on the previous reports on the role of CCL5 in neuropathic pain (Liou et al., 2013; Liou et al., 2012) and the fact that CCL5 was still notably elevated at day 20 post-L5Tx (Figure 2A), we expect that CCL5 is still contributing to L5Tx-induced mechanical hypersensitivity at day 20, although it may be less tightly regulated by CGRP at this time.

Previously, we have shown that microglial CD40 (a co-stimulatory molecule whose expression is increased by activated microglia) is critical in maintaining the upregulation of CGRP, i.e., CD40 signaling acts upstream of CGRP signaling (Figure 6). In that study, we also showed that CD40 supported CCL2 production in the spinal cord following L5Tx; however, CGRP and CCL2 appeared to act independently to promote L5Tx-induced mechanical hypersensitivity ((Malon et al., 2011) and current observation) (Figure 6 dash lines on the right side). In summary, we have identified several interconnected neuroimmune pathways that all contribute to the development of L5Tx-induced neuropathic pain-like behaviors (Figure 6). It is very clear that a multifactorial neuroimmune network is regulating these behaviors and that further dissecting this network will lead to a better understanding of peripheral nerve injury-induced pain-like behaviors.

The involvement of CGRP in the development of neuropathic pain may depend on the peripheral nerve injury model being examined. In a previous study (Zheng et al., 2008), changes in CGRP expression were examined in three different sciatic nerve injury models: crush (SNC), ligation (SNL), and transection combined with subsequent neurotomy (SNT). Both SNC and SNT induced significant increase in CGRP expression in the lumbar spinal cord, while SNL induced sustained reduction of lumbar spinal cord CGRP expression. Similar reduction was also observed with L5 spinal nerve ligation model (Kaku et al., 2007). One may predict that CGRP is more likely to contribute to the development of neuropathic pain in peripheral nerve injury models that induce significant upregulation of CGRP compared to models that result in decrease of CGRP expression.

Consistent with the above discussion regarding mechanical vs. heat sensitivity, distinct pathways may be involved in mediating different neuropathic pain-like behaviors. Many

details within the neuroimmune network require further investigation. The underlying mechanisms through which CD40-expressing microglia interact with primary sensory neurons to sustain CGRP expression in the spinal cord is particularly interesting, as it may provide a target for modulating neuronal response early after peripheral nerve injury, thus reducing or even preventing injury-induced central sensitization. CX3CL1/CX3CR1 signaling has been recognized as a critical link between the primary sensory neurons and spinal cord microglia during the central sensitization of neuropathic pain (Clark and Malcangio, 2014). It has been shown that peripheral nerve injury-activated microglia could further stimulate neuronal release of CX3CL1 (fractalkine) through microglial production of lysosomal protease cathepsin S (CatS) (Clark and Malcangio, 2014). Elevated CatS mRNA levels have been associated with increased CD40 mRNA levels in the plasma of patients with coronary atherosclerosis (Cerme et al., 2011). Thus, it is possible that microglia-expressed CatS serves as one of the mediators and facilitates the interaction between CD40-expressing microglia and nociceptors. Specifically, CD40 signaling may act upstream and promote microglial expression of CatS, which could further enhance CGRP release from nociceptors and the subsequent production of CCL5 in the spinal cord.

In our study, L5Tx significantly induced the production of CCL2, CCL5 and CCL11 in the lumbar spinal cord. It was somewhat unexpected that CGRP was not involved in the upregulation of CCL2 and CCL11, and its effects were specific to CCL5. Nevertheless, others have reported similar phenomena regarding CGRP's effects. For example, CGRP was observed to specifically stimulate the production of CXCL8, but not CCL2 or CCL5 by human corneal epithelial cells (Tran et al., 2000). Conversely, CGRP was found to inhibit the production of several chemokines by human dermal microvascular endothelial cells upon lipopolysaccharide stimulation (Huang et al., 2011). Thus, CGRP's effects on chemokines appear to be cell/tissue type-specific and these differential effects are likely to be mediated by different signaling pathways. This CGRP-specific effect on chemokine production may also explain the aforementioned sensory modality-specific response mediated by CGRP. In addition, due to the limitation of the pre-assembled multiplex assay, we only examined eight chemokines. It is possible that CGRP also regulates the production of other chemokines, such as CXCL10, CXCL12 and CXCL21, which have been implicated in the development of neuropathic pain. As such, we will investigate CGRP's effects on other chemokines in the future.

Interestingly, CCL5 has been identified as one of the potential biomarkers for lumbar disc degeneration in humans (Grad et al., 2016). Considering that disc degeneration can often cause nerve-root damage and subsequent neuropathic pain, this recent observation may suggest that the pathophysiological changes identified in our L5Tx model could be translated to disc degeneration-associated neuropathic pain in humans. Besides targeting on CCL5, the role of CGRP in low back pain should be further explored, as appropriate blocking of CGRP responses may lead to alleviation of discogenic neuropathic pain. It is also important to note that CGRP blocking has been shown to be effective in a chronic central neuropathic pain model, spinal cord hemisection (Bennett et al., 2000b).

Glial cell activation and the subsequent inflammatory responses are well-known to be critical in the central sensitization process during the development of neuropathic pain

(DeLeo et al., 2004; Ji and Suter, 2007; Milligan and Watkins, 2009; Tsuda et al., 2005). Interestingly, under our experimental conditions, we did not see a significant increase of CCL5 when CGRP was used to stimulate mixed adult glial cells *in vitro*. Although further experiments are needed to determine the contribution of glial cells to the CCL5 production we observe post L5Tx, other cellular sources such as neurons, should also be considered. For example, increased expression of several chemokines (CCL2, CCL5 and CXCL10) has been observed in dorsal root ganglia neurons in different rodent models of neuropathic pain (Bhangoo et al., 2007; Bhangoo et al., 2009).

Altogether, our current study demonstrated a CGRP-CCL5 mediated pathway in peripheral nerve injury-induced mechanical hypersensitivity. Together with our previous studies, we have identified a neuroimmune network that is involved in the development of peripheral nerve injury-induced mechanical hypersensitivity (summarized in Figure 6). Further delineation of the molecular/cellular mechanisms within these pathways may lead to new treatments for neuropathic pain. It should be noted that many components in this neuroimmune network are involved in maintaining normal physiological functions. For example, chemokines and CD40 are vital mediators in immune responses. Global inhibition of these factors can cause severe unwanted side effects. Based on our observations, in order to effectively inhibit the progression of neuropathic pain, multiple factors may need to be targeted simultaneously in order to completely block the advancement of pain pathways. Inhibition of CCL5 and CCL2 together, or blocking CCL2 while treating with a CGRP antagonist would be more effective in providing pain relief than inhibiting any one of these molecules alone. Further, the potential synergistic effects between individual treatments may help to reduce the side effects from each treatment alone, which has been shown when the combination treatment with clonidine (an alpha-2 adrenoceptor agonist) and p38 MAPK inhibition were used in an oxaliplatin-induced neuropathic pain model (Yeo et al., 2016). In summary, potential effective treatment for neuropathic pain should target multiple pro-nociceptive mediators and ideally, in order to reduce the side effects, should be known to specifically affect the injury or disease-induced function of these mediators.

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Abbreviations

ANOVA	analysis of variance
CatS	cathepsin S
cDMEM	complete Dulbecco's Modified Eagle Media
CGRP	Calcitonin gene-related peptide
COX-2	cyclooxygenase 2

ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal-regulated kinase
IACUC	Institutional Animal Care and Use Committee
JNK	Jun N-terminal kinase
L5Tx	spinal nerve L5 transection
MAPK	Mitogen activated protein Kinase
MCP-1	monocyte chemoattractant protein-1
NCI	National Cancer Institute
RANTES	regulated on activation, normal T cell expressed and secreted
SNK	Student-Newman-Keuls
SNL	spinal nerve ligation
TRP	transient receptor potential
UNE	University of New England.

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Highlights

- CGRP contributes to spinal nerve L5 transection-induced mechanical hypersensitivity.
- CGRP can play its pro-nociceptive role through both a spinal cord CCL5-dependent, p38-independent pathway, and a p38-dependent, CCL5-independent pathway.

Figure 1. Effects of CGRP₈₋₃₇ on L5Tx-induced behavioral hypersensitivities in mice
Wild type BALB/c mice were i.t. injected with either a CGRP antagonist (CGRP₈₋₃₇, 1 µg/5 µl/mouse) or vehicle control daily from day 6 to 13 post-L5Tx. Mice were tested for mechanical (A) and heat (B) sensitivities via von Frey filaments using the up-down method and the Hargreaves test respectively. Data are presented as mean ± SEM (in A: n = 9–45 per group; in B: n = 8–12 per group). Note that in both A and B, L5Tx induced statistically significant behavioral hypersensitivities in all groups (not marked in the graphs). In addition, * indicates $p < 0.05$, compared between the indicated group and all other treatment groups at the same time point.

Figure 2. Effects of CGRP₈₋₃₇ on lumbar spinal cord chemokine production in mice post-L5Tx Lumbar spinal cords were collected from mice that received no i.t. injection, CGRP₈₋₃₇ (1 μ g/5 μ l/mouse) i.t. injection or saline i.t. injection. Chemokines, CXCL1 (KC), CCL1 (TCA-3), CCL2 (MCP-1), CCL3 (MIP-1 α), CCL5 (RANTES), CCL11 (Eotaxin), CCL17 (TARC), and CCL22 (MDC) within these spinal cord samples were determined via a multiplex assay. The three chemokines, CCL5 (A), CCL2 (B) and CXCL11 (C) that were significantly induced by L5Tx are shown here. Data are presented as mean $\pm$ SEM (n = 9–45 per group). * indicates $p < 0.05$ compared between the indicated group and all other treatment groups at the same time point. # indicates $p < 0.05$ compared between the indicated group and the “Day 0” (naïve) group.

Figure 3. Effects of anti-CCL5 neutralizing antibody on L5Tx-induced mechanical hypersensitivity in mice

Wild type BALB/c mice were i.t. injected with either one of the two doses of anti-CCL5 neutralizing antibody (α CCL5 at 0.5 and 2.5 μ g/5 μ l/mouse) or a vehicle control (PBS) daily from day 6 to 13 post-L5Tx. Mice were tested for mechanical sensitivities via von Frey filaments using the up-down method. Data are presented as mean $\pm$ SEM ($n = 8$ per group). Note that L5Tx induced statistically significant behavioral hypersensitivities in all groups at all selected times post-L5Tx (not marked in the graphs). In addition, * indicates $p < 0.05$, compared between the indicated group and both non-injected and PBS injected groups at the same time point.

Figure 4. Lumbar spinal cord levels of MAPK pathway proteins post-L5Tx in mice

Lumbar spinal cords were collected from mice that underwent either L5Tx or sham surgery. MAPK pathway proteins, pho-p38 (A), pho-JNK (B), and pho-ERK (too low to be detected in most samples, data not shown) within lumbar spinal cord were determined via the CBA assay. Data are presented as mean $\pm$ SEM ($n = 6-7$ per group). * indicates $p < 0.05$ compared between the indicated group and the sham group at the same time point. # indicates $p < 0.05$ compared between the indicated group and the “Day 0” (naïve) group.

Figure 5. Effects of a p38-inhibitor on CGRP-induced mechanical hypersensitivity and lumbar spinal cord levels of CCL5

Wild type BALB/c mice were randomly assigned to the following treatment groups: vehicle/vehicle, SB 203580 (a p38-inhibitor, 5 ng/5 μ l/mouse)/vehicle, vehicle/CGRP (2 μ g/5 μ l/mouse), and SB 203580 (5 ng/5 μ l/mouse)/CGRP (2 μ g/5 μ l/mouse). In A, Mechanical sensitivity was tested via von Frey filaments using the up-down method. Data are shown as mean $\pm$ SEM (n = 8 per group). * indicates $p < 0.05$, compared between the indicated group and all other treatment groups at the same time point. # $p < 0.05$, compared between the indicated group and the corresponding “before i.t.” group. In B, lumbar spinal cord levels of CCL5 following i.t. injections were measured via ELISA. Data are shown as mean $\pm$ SEM (n = 8 per group). # indicates $p < 0.05$, compared between the indicated group and the “naive” group. \$ indicates $0.05 < p < 0.1$, compared between the indicated group and the “naive” group.

Figure 6. Summary of neuroimmune pathways that mediate L5Tx-induced mechanical hypersensitivity

Pathways marked with solid lines are those identified in the current study. Pathways marked with dash lines are those identified in our previous studies (Malon et al., 2011).

Table 1

List of solutions and their corresponding vehicles used in intrathecal injections.

Solution	Drug dosage (per mouse in 5 µl)	Corresponding Vehicle
CGRP ₈₋₃₇	1 µg	Saline
α-CCL5	0.5µg and 2.5µg	1xPBS
p38 inhibitor (SB203580)	5 ng	1xPBS w/0.1% DMSO
CGRP	2 µg	1xPBS