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Trigeminal nerve injury induced thrombospondin-4 upregulation contributes to orofacial neuropathic pain states in a rat model

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Abstract

Background—Injury to the trigeminal nerve often results in the development of chronic pain states including tactile allodynia, or hypersensitivity to light touch, in orofacial area, but its underlying mechanisms are poorly understood. Peripheral nerve injury has been shown to cause upregulation of thrombospondin-4 (TSP4) in dorsal spinal cord that correlates with neuropathic pain development. In this study, we examined whether injury-induced TSP4 is critical in mediating orofacial pain development in a rat model of chronic constriction injury to the infraorbital nerve (CCI-ION).

Methods—Orofacial sensitivity to mechanical stimulation was examined in a unilateral infraorbital nerve ligation rat model. The levels of TSP4 in trigeminal ganglia and associated spinal subnucleus caudalis and C1/C2 spinal cord (Vc/C2) from injured rats were examined at time points correlating with the initiation and peak orofacial hypersensitivity. TSP4 antisense and mismatch oligodeoxynucleotides were intrathecally injected into injured rats to see if antisense oligodeoxynucleotide treatment could reverse injury-induced TSP4 upregulation and orofacial behavioral hypersensitivity.

Results—Our data indicated that trigeminal nerve injury induced TSP4 upregulation in Vc/C2 at a time point correlated with orofacial tactile allodynia. In addition, intrathecal treatment with TSP4 antisense, but not mismatch, oligodeoxynucleotides blocked both injury-induced TSP4 upregulation in Vc/C2 and behavioral hypersensitivity.

Conclusions—Our data support that infraorbital nerve injury leads to TSP4 upregulation in trigeminal spinal complex that contributes to orofacial neuropathic pain states. Blocking this pathway may provide an alternative approach in management of orofacial neuropathic pain states.

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• It is known that spinal nerve injury induced thrombospondin-4 upregulation in spinal cord that plays a critical role in pain processing.

• Findings from this study added that injury to the trigeminal nerve also induces thrombospondin-4 upregulation that contributes to the development of orofacial neuropathic pain states.

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Introduction

Neuropathic pain is a disorder of chronic pain resulting from injuries to the peripheral or central nervous systems and adversely affecting the life quality of a large patient population (Jensen *et al*., 2007; Fredheim *et al*., 2008). Chronic pain states include exaggerated painful sensations to stimuli that are normally innocuous (allodynia), or cause mild painful response (hyperalgesia), which tend to be long-term and, in some cases, refractory to conventional analgesic treatments (O'Connor & Dworkin, 2009). Orofacial neuropathic pain due to trigeminal nerve injury could be the most severe and debilitating neuropathic pain conditions with limited therapeutic options (Woda *et al*., 2005; Clark, 2006; Forssell *et al*., 2007). Unfortunately, the molecular mechanisms associated with orofacial neuropathic pain are not well understood (Clark, 2006), thus, target specific, effective pharmacological agents for orofacial neuropathic pain management are very limited.

Recently, we have reported that peripheral nerve injury (Kim *et al*., 2012) and spinal cord contusion injury (Zeng *et al*., 2013) induce upregulation of thrombospondin-4 (TSP4) in dorsal spinal cord that is critical in neuropathic pain state development. Blocking TSP4 upregulation can reverse neuropathic pain states in these models (Kim *et al*., 2012; Zeng *et al*., 2013), supporting that upregulated TSP4 in dorsal spinal cord is a common factor in mediating neuropathic pain states from different etiologies. Thrombospondin proteins belong to a large extracellular, oligomeric matrix glycoprotein family, and play important roles in cell migration, attachment, cytoskeletal dynamics and synaptogenesis (Bornstein *et al*., 2004; Christopherson *et al*., 2005). However, it is not clear whether trigeminal nerve injury also causes TSP4 dysregulation in the trigeminal spinal complex, and if so, whether this neuroplasticity contributes to orofacial hypersensitivity. In this study, we examined TSP4 expression in trigeminal ganglia (TG) and associated trigeminal spinal complex of dorsal spinal subnucleus caudalis and C1/C2 spinal cord (Vc/C2) in an orofacial neuropathic pain model derived from trigeminal nerve injury and whether altered TSP4 expression contributed to the development of orofacial neuropathic pain states.

Materials and methods

Experimental animals

Adult male Sprague–Dawley rats (160–250 g, 73 total) were acclimated in their cages for 3–4 days under a 12/12 h light-dark cycle with free access to food and water before any experiments. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of California Irvine.

Generation of animal model

The unilateral infraorbital nerve (ION, a branch of the trigeminal nerve) chronic constriction injury model (CCI-ION) was generated similarly to that described by Kernisant et al. (2008). Briefly, isoflurane anesthetized rats with hair shaved above the left eye were put in a prone position. An incision parallel to the curve of the frontal bone was made above the left eye. ION lying on the maxillary bone was exposed by gently pushing the fascia and muscles laterally so that the contents of the orbit could be laterally retracted. Fine forceps and a silk suture (5–0) loaded needle with a bended tip were used to place two loose ligation around the ION, 3–4 mm apart, after the nerve was freed from surrounding tissues. For sham surgeries, the same procedure was performed except that ION was not ligated. The rats were recovered on a warm heating pad after the incision was closed in layers with silk sutures (5–0).

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Intrathecal injection

An intrathecal catheter was implanted for intrathecal injection. Briefly, the inserting end of a PE-10 tubing (8 cm long) was blocked with superglue and holds approximately 1.0 cm from the blocked end were made with a 30G needle. This allowed the insertion of the distal end of the catheter into the subarachnoid space so that drugs could be delivered through the holes near the Vc/C2 region. Dura mater was exposed in isoflurane anesthetized rats after tissues were gently retracted caudally from the occipital bone. A small nick was cut with a 25G needle so that the catheter could be inserted. A sterilized catheter filled with saline was inserted gently and caudally (approximately 0.5 cm) until a loose overhand knot about 1.5 cm from the indwelling tip stopped the insertion. The catheter was securely sutured into surrounding muscle ligaments through another loose knot about 2.0 cm from the indwelling tip before closing muscle and skin layers with 5–0 silk sutures. Rats were housed individually after recovery from anesthesia on a warm pad.

Behavioral tests

Orofacial sensitivity to mechanical (von Frey filament) stimulation was tested blindly as described by Vos et al. (1994) one day before and at designated times after CCI-ION surgeries, or before each daily intrathecal injection and at designated times post the last injection. One day before testing, rats lightly anesthetized with isoflurane were shaved at and near the vibrissal pad. During the acclimatization period (at least one hr) in individual cages before the testing, the examiner reached slowly into the cage and touched the cage wall slightly with the handle of a von Frey filament. The acclimatization period would be extended if necessary until the rats were claim before the testing. Orofacial mechanical sensitivity was examined on both sides of free-moving rats in the same cages as 50% withdrawal thresholds to a series of graded von Frey filaments (Numbers 3.61, 3.84, 4.08, 4.31, 4.56, 4.74, 4.93 and 5.18; equivalent to: 0.4, 0.6, 1.0, 2.0, 4.0, 6.0, 8.0, and 15 g of force, respectively) as determined by the up–down method of Dixon (1980). Briefly, starting with the 4.31 one, the examiner slowly applied the von Frey filament near the center of the vibrissal pad so that the filament was slightly bent. One or more of the following behaviors were considered a positive response that would lead to the application of next filament with a lower stimulating force: (1) a brisk withdrawal reaction from a rat; (2) an escape/attack reaction in which a rat avoided the filament either by moving away from the filament to assume a crouching position against a cage wall or burying the head under the body, or attacked the filament by biting and grabbing; (3) asymmetric face grooming shown as at least 3 uninterrupted face-wash strokes directed to the facial area being stimulated, often preceded by a brisk withdrawal reaction. Absence of a positive response would lead to the application of next filament with a higher stimulating force. This testing paradigm continued for collecting six responses to von Frey stimuli, starting from the one before the first change (either negative or positive). The 50% withdrawal threshold values were calculated using the formula: $10^{(X+kd)}/10^4$, where X is the value of the final von Frey filament used in log units, k is the tabular value for the positive/negative response patterns from Chaplan et al. (1994), and d is the mean differences between stimuli in log units. Scores of 0.25 g or 15 g were assigned, respectively, when consecutive positive or negative responses were observed. A small percentage of injured rats displayed a bilateral drop in behavioral thresholds after three weeks of injury (Vos *et al*., 1994; Iwata *et al*., 2011) were excluded from the studies.

Western blots

Western blots were performed at designated times post injury in samples from CCI-ION rats displayed normal orofacial sensitivity in the contralateral side similar to that in the sham rats, and comparisons were made between the injury side and contralateral side. Since CCI-ION caused TSP4 dysregulation in the three-week time point post surgery, sham control

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samples were also collected at this time point for analysis. Briefly, TG and dorsal Vc/C2 were collected from deeply anesthetized, rapidly decapitated rats at designated time after behavioral test, either proceeded immediately for biochemical studies or kept at −80 °C until use. Frozen TG and dorsal Vc/C2 samples were extracted in 50 mM Tris buffer containing 0.5% Triton X-100, 150 mM NaCl, 1 mM EDTA and protease inhibitors (pH 8.0). Proteins from cell extracts were separated by electrophoresis in denaturing NuPAGE Tris-acetate gels (Invitrogen, Carlsbad, CA), then transferred to PVDF membranes electrophoretically. The membranes were incubated with 5% low-fat milk in Tris-buffered saline containing 0.1% Tween-20 to block nonspecific binding sites for at least 1 hr at room temperature, cut in half based on the molecular weights of proteins of interest, then incubated for 1 h at room temperature or overnight at 4°C with the guinea pig polyclonal TSP4 antibody (1:750, Kim *et al*., 2012), or mouse monoclonal β-actin antibody (for loading controls, Novus Biologicals, LLC, Littleton, CO, 1:10,000) in phosphate-buffered saline with 0.1% Tween-20. The antibody-protein complexes were visualized by chemiluminescent reagents after incubation with secondary antibodies conjugated to horseradish peroxidase at room temperature for 1 hr. Imaging quantification or densitometry within the linear range of the film sensitivity curve were used to quantify the band densities. Ratios of the TSP4 to β-actin band densities were calculated within each sample first before calculating the percentage changes in TSP4 protein levels for cross-sample comparisons. Band density variations in the contralateral side (control) were calculated by comparing each band density with the mean of the band densities from at least two different control samples run in the same Western blot after the TSP4 to β-actin band density ratios were calculated.

Oligodeoxynucleotide treatment

TSP4 antisense or mismatch oligodeoxynucleotides or saline were injected intrathecally through a catheter into allodynic rats for four consecutive days (50μg/10μL/rat/day), starting at least three weeks post CCI-ION, followed by flashing saline (5 μL to compensate for tubing death volume). The TSP4 antisense (CCATCATTGTTGCTATCTTCC) (GenBank Accession No. X89963) or mismatch control (ACCATCGTTGTTACTTTCTCC) oligodeoxynucleotides were synthesized commercially with phosphothioate modification on three nucleotides at each end (GeneLink, Hawthorne, NY), precipitated, washed in 75% ethanol, and dissolved in sterile saline before injections. Daily test for sensitivity to von Frey filaments was performed before the daily injection, and after the final injection. These oligodeoxynucleotides have been used in non-orofacial pain models and toxicity was not observed at the concentration level used (Kim *et al*., 2012).

Statistics

Variance analyses for multi-group comparisons were done by ANOVA analysis and pair-wise comparisons were done by unpaired Student's *t* tests as indicated. Significance was indicated by a two-tailed p value < 0.05.

Results

Infraorbital nerve injury induced TSP4 upregulation in Vc/C2 that correlated with orofacial tactile allodynia

We hypothesized that if infraorbital nerve injury leads to TSP4 dysregulation that is critical in orofacial neuropathic pain development, injury-induced TSP4 dysregulation should correlate temporally with the development of orofacial pain states. To test this hypothesis, we examined if TSP4 protein expression was altered in Vc/C2 and associated trigeminal ganglia in the CCI-ION orofacial neuropathic pain model (Vos *et al*., 1994), and if so, whether changes in TSP4 expression correlated temporally with the development of orofacial neuropathic pain states. As indicated in Fig. 1, unilateral CCI-ION injury resulted

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in a orofacial hypersensitive state (about 3 weeks post injury) shown as reduced orofacial thresholds to von Frey filament stimuli in the injury side compared with that from the non-injury side (n = 10) or sham (n = 8) operated rats, similar to that initially described by Vos et al (1994). The allodynic state in the injured rats lasted for about 7–8 weeks followed by a graduate recovery.

TSP4 expression levels were examined in Vc/C2 and TG samples collected from CCI-ION rats at different stages of orofacial allodynia development: 1) One-week post injury before the onset of allodynia; 2) Three-weeks post injury when the injured rats displayed severe allodynia; and 3) 10-weeks post injury when the injured animals recovered from allodynia. As shown in Fig. 2, TSP4 levels were similar between the injury and non-injury sides in both Vc/C2 and TG samples collected one-week post CCI-ION before the onset of orofacial allodynia (n = 6). However, CCI-ION injury induced TSP4 upregulation in Vc/C2, but down regulation in TG from the injury side at the orofacial hypersensitive stage (three-week post CCI-ION, n = 5), which were not observed from sham control samples (n = 5) collected at the same time point (data not shown). Both of these changes in the injury side were recovered to a level similar to that from the non-injury side when the injured rats were recovered from orofacial allodynia (10-weeks post CCI-ION, n = 4). This temporal correlation between orofacial hypersensitivity and injury-induced TSP4 upregulation in Vc/C2 suggests that increased TSP4 in Vc/C2 may play a critical role in orofacial pain processing.

Intrathecal TSP4 antisense oligodeoxynucleotide treatments resulted in reversals of Vc/C2 TSP4 upregulation and orofacial hypersensitivity in CCI-ION rats

To determine if CCI-ION-induced TSP4 upregulation in Vc/C2 played a critical role in mediating orofacial hypersensitivity, we injected intrathecally TSP4 antisense or mismatch oligodeoxynucleotides (50μg/rat/day in 10 μL) into the Vc/C2 region of CCI-ION rats through a catheter for four consecutive days, starting at least three weeks post CCI-ION when the injured rats had developed orofacial hypersensitivity. The same volume of sterile saline was also injected as a control. These treatments were followed by daily orofacial behavioral test. Western blot analysis was performed at one day after the last intrathecal treatment, which correlated with the peak anti-hyperalgesic effects of the antisense oligodeoxynucleotide treatment, to see if the antisense oligodeoxynucleotide treatments could block or diminish injury-induced orofacial behavioral hypersensitivity and elevated TSP4 levels. As indicated in Fig. 3, intrathecal treatments with TSP4 antisense oligodeoxynucleotides (n = 10), but not mismatch oligodeoxynucleotides (n = 7) or saline (n = 6), resulted in a time-dependent reversal of orofacial allodynia. The antisense effects had an onset time of three days, peaked approximately one day after the last injection, and lasted for over two days after the last injection. As shown in Fig. 4, data from Western blots indicated that CCI-ION induced TSP4 expression in Vc/C2 was blocked by intrathecal antisense oligodeoxynucleotide treatments (n = 4), but not by similar intrathecal treatments with mismatch oligodeoxynucleotides (n = 4), or saline (n = 4). This correlation in antisense oligodeoxynucleotide-mediated reversal between CCI-ION-induced orofacial hypersensitivity and Vc/C2 TSP4 upregulation supports that TSP4 upregulation in the Vc/C2 region post CCI-ION injury may play a critical role in mediating the development of orofacial hypersensitivity.

Discussion

Mechanisms underlying orofacial neuropathic pain are not well understood. We have shown here that trigeminal nerve injury leads to TSP4 upregulation in dorsal Vc/C2 spinal cord that correlates with orofacial neuropathic pain development. Intrathecal treatment with TSP4 antisense, but not mismatch, oligodeoxynucleotides blocked injury-induced TSP4

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upregulation in Vc/C2 and neuropathic pain states. Together, these findings support that injury-induced TSP4 upregulation in Vc/C2 is likely contributing to the development of orofacial hypersensitivity.

It is interesting that trigeminal nerve injury induces TSP4 upregulation in Vc/C2, but down regulation in TG at the time when nerve injured animals display severe neuropathic pain states. This observation is similar to that observed in a non-orofacial pain model in which spinal nerve injury-induced TSP4 upregulation in dorsal spinal cord, but down-regulation in dorsal root ganglia of the injury side at a time point correlating with peak hindpaw behavioral hypersensitivity (Kim *et al*., 2012). This indicates that injury-induced TSP4 dysregulation is tissue-type specific, and suggests that injury-induced TSP4 dysregulation in the peripheral and central nervous systems may be regulated by distinct mechanisms. In addition, elevated TSP4 expression is shown to correlate with neuropathic pain states across different models, including peripheral nerve injury (Kim *et al*., 2012), spinal cord contusion injury (Zeng *et al*., 2013), and trigeminal nerve injury (this study), and blocking injury-induced TSP4 upregulation can reverse neuropathic pain states in these models. Together, these findings support that upregulated TSP4 in dorsal spinal cord is a common factor in mediating neuropathic pain states derived from different pathological conditions.

The cellular mechanism underlying the contribution of Vc/C2 TSP4 to orofacial pain processing remains elusive. It has been reported that nerve injury induces TSP4 upregulation in spinal cord reactive astrocytes (Kim *et al*., 2012). Astrocyte-secreted TSP4 binds to the calcium channel alpha-2-delta-1 subunit proteins ($Ca_v\alpha_2\delta_1$) that mediates excitatory synaptogenesis in the central nervous system (Eroglu *et al*., 2009). In addition, Vc/C2 astrocytes have been shown to participate in central sensitization and orofacial pain processing (Piao *et al*., 2006; Chiang *et al*., 2007; Xie *et al*., 2007; Chiang *et al*., 2008; Okada-Ogawa *et al*., 2009; Itoh *et al*., 2011; Ren & Dubner, 2011; Tsuboi *et al*., 2011). Therefore, it is highly likely that trigeminal nerve injury induced TSP4, presumably secreted from astrocytes, in the Vc/C2 region interacts with $Ca_v\alpha_2\delta_1$ proteins, leading to abnormal excitatory synapse formation in the Vc/C2 region and causing sensitization of Vc/C2 neurons, which in turn contributes to orofacial neuropathic pain states. Experiments are underway to test this hypothesis.

Like originally reported by Vos et al. (1994) in this model, we also observed contralateral behavioral hypersensitivity, even though less severe compared with that from the injury side, in a small percentage of CCI-ION rats. This is consistent with the bilateral effects in ION or inferior alveolar nerve injury models observed in other studies (Iwata *et al*., 2011; Miyamoto *et al*., 2011; Cao *et al*., 2013). The bilateral effects could derive from dysregulation of circulating factors such as pro-inflammatory cytokines (Anderson & Rao, 2001), nerve growth factors (Anderson *et al*., 1998; Anderson & Rao, 2001), or descending facilitatory mechanisms (Shimizu *et al*., 2009; Chai *et al*., 2012) by unilateral nerve injury. Since changes in these bilateral factors might not be specifically correlating with the onset or duration of neuropathic pain states post CCI-ION (Anderson & Rao, 2001), we only used CCI-ION rats with unilateral behavioral hypersensitivity in our studies so that we could focus on TSP4 effects in behavioral hypersensitivity at the injury side. Arguably, this could be one of the limitations in our studies since bilateral effects were not studied. Other limitations in our studies include that the whole Vc/C2 region was used so that sub-regional changes, if any, were undetected. In addition, we only used male rats in our studies, so that the influence of female sex-hormones in pain processing, which has been shown to be prevalent (Unruh, 1996; Berkley, 1997; LeResche, 1997; Fillingim, 2000; Fillingim & Ness, 2000; Fillingim *et al*., 2009), was not evaluated.

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In conclusion, our findings support that CCI-ION injury induces TSP4 upregulation in Vc/C2 dorsal spinal cord that plays a critical role in mediating orofacial neuropathic pain states. Blocking this pathway may represent a novel therapeutic option for managing trigeminal nerve injury-induced orofacial neuropathic pain states.

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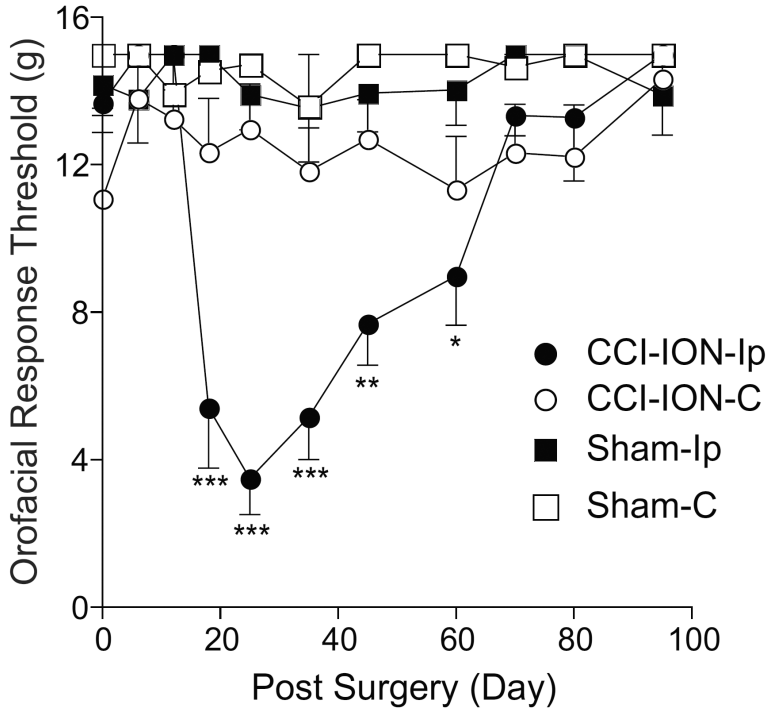

Fig. 1. Chronic constriction injury to the rat infraorbital nerve (CCI-ION) caused orofacial hypersensitivity to mechanical stimuli

Sensitivity to von Frey filament stimulation in both sides of the whisker pad area was tested in rats with unilateral sham or CCI-ION operations before and at designated times after the injury. Data presented are the Means ± SEM from eight (sham) to ten (CCI-ION) rats in each group. C - contralateral to injury; Ip - ipsilateral to injury. *$p < 0.05$; **$p < 0.01$, ***$p < 0.001$ compared with pre-injury level by two-way ANOVA with Bonferroni posttests.

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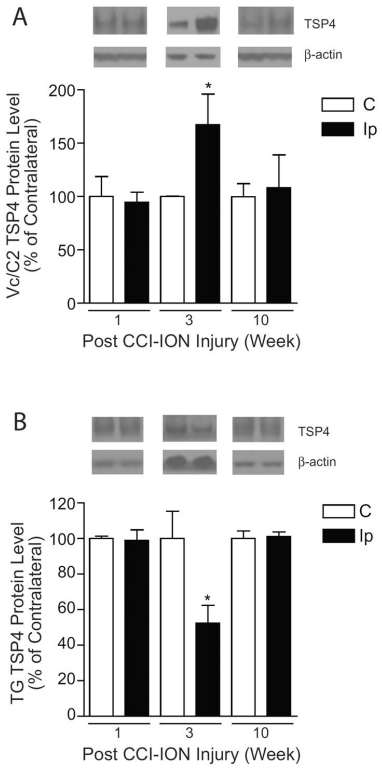

Fig. 2. TSP4 dysregulation in TG and Vc/C2 of CCI-ION rats with orofacial behavioral hypersensitivity

Western blot analysis was performed to examine TSP4 protein levels in dorsal Vc/C2 (A) and TG (B) at designated time points after CCI-ION. Representative Western blot bands from Vc/C2 or TG were shown on top of each bar graph summarizing respective Western blot data. For sample loading normalization, band density ratios of TSP4 to β-actin were taken within each sample before cross-sample comparisons between the injury side and non-injury side. Data presented are the Means ± SEM from four to six independent experiments. *$p < 0.05$ compared with non-injury side by Student's *t* test. C - contralateral to injury; Ip - ipsilateral to injury.

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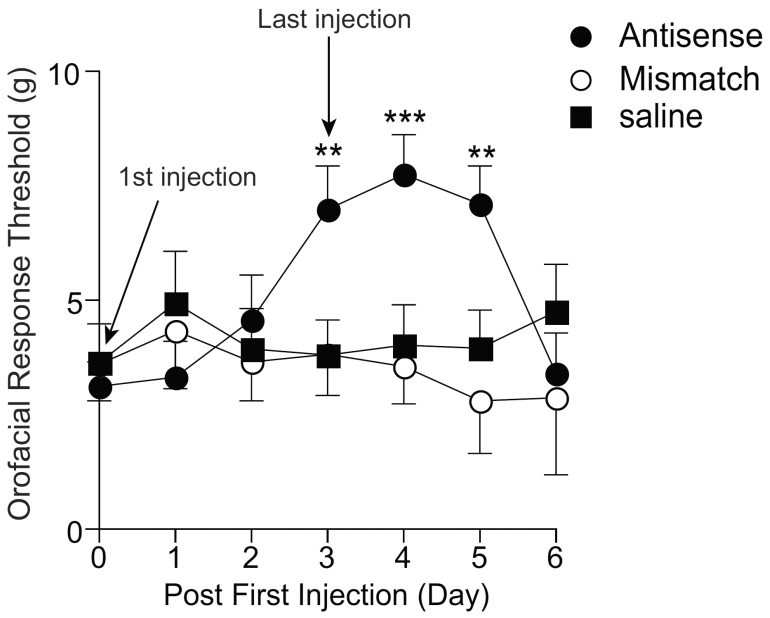

Fig. 3. Intrathecal treatment with TSP4 antisense, but not mismatch, oligodeoxynucleotides blocked CCI-ION-induced orofacial hypersensitivity

Rats with orofacial allodynia to mechanical stimuli were treated intrathecally with TSP4 antisense or mismatch oligodeoxynucleotides (50 μg/rat/day) for four consecutive days, starting at least three weeks after CCI-ION. Injection with the same volume of sterile saline was also used as a control. Orofacial sensitivity to von Frey filament stimulation was tested daily in the injury side before each injection and after the last injection. Data presented are the Means ± SEM from six to ten rats in each group. **$p < 0.01$, ***$p < 0.001$ compared with pre-treatment level by two-way ANOVA with Bonferroni posttests.

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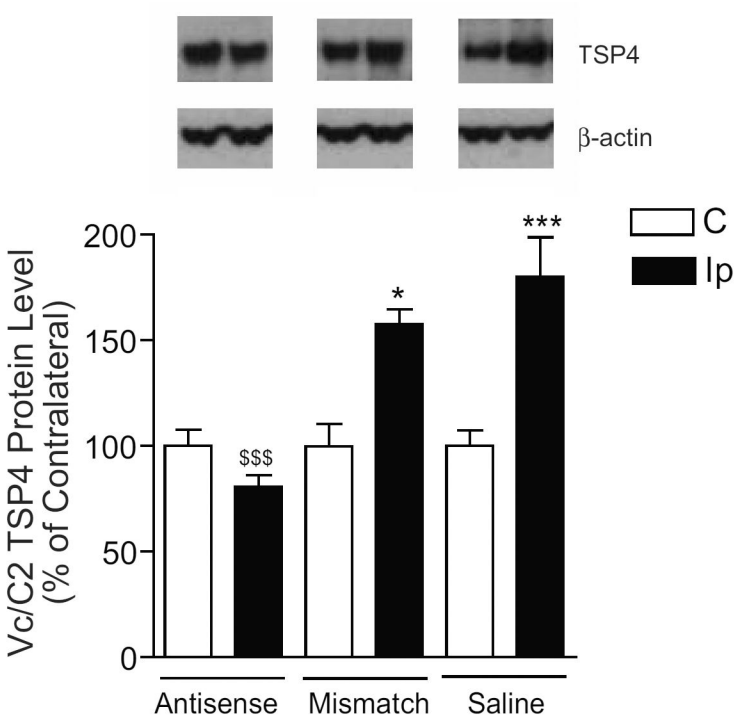

Fig. 4. Intrathecal treatment with TSP4 antisense, but not mismatch, oligodeoxynucleotides blocked CCI-ION-induced TSP4 upregulation in Vc/C2

Western blot analysis was performed to examine TSP4 protein levels in dorsal Vc/C2 samples collected from CCI-ION rats with orofacial allodynia one-day after the last intrathecal injection of the four consecutive daily treatments. Representative Western blot bands were shown on top of each bar graph summarizing respective Western blot data. For sample loading normalization, band density ratios of TSP4 to β-actin were taken within each sample before cross-sample comparisons between the injury side and non-injury side. Data presented are the Means ± SEM from four independent experiments in each group. *$p < 0.05$, ***$p < 0.001$ compared with non-injury side; $^{\$\$\$}p < 0.001$ compared with saline or mismatch oligodeoxynucleotide treated group by one-way ANOVA analysis. C - contralateral to injury; Ip - ipsilateral to injury.