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Profiling of Dynamically Changed Gene Expression in Dorsal Root Ganglia Post Peripheral Nerve injury and A Critical Role of Injury-Induced Glial Fibrillary Acetic Protein in Maintenance of Pain Behaviors

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Abstract

To explore cellular changes in sensory neurons after nerve injury and identify potential target genes contributing to different stages of neuropathic pain development, we used Affymetrix oligo arrays to profile gene expression patterns in L5/6 dorsal root ganglia (DRG) from the neuropathic pain model of left L5/6 spinal nerve ligation at different stages of neuropathic pain development. Our data indicated that nerve injury induced changes in expression of genes with similar biological functions in a temporal specific manner that correlates with particular stages of neuropathic pain development, indicating dynamic neuroplasticity in the DRG in response to peripheral nerve injury and during neuropathic pain development. Data from post-array validation indicated that there was a temporal correlation between injury-induced expression of the glial fibrillary acidic protein (GFAP), a marker for activated astrocytes, and neuropathic pain development. Spinal nerve ligation injury in GFAP knockout mice resulted in neuropathic pain states with similar onset, but a shortened duration compared with that in age, and gender-matched wild-type littermates. Intrathecal GFAP antisense oligonucleotide treatment in injured rats with neuropathic pain states reversed injury-induced behavioral hypersensitivity and GFAP upregulation in DRG and spinal cord. Together, these findings indicate that injury-induced GFAP upregulation not only serves as a marker for astrocyte activation, but it may play a critical, but yet identified, role in the maintenance of neuropathic pain states.

Keywords

Dorsal root ganglia; plasticity; peripheral nerve; injury; neuropathic pain; GFAP

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Introduction

Management of neuropathic pain, a chronic pain state derived from nerve injuries, is less satisfactory with conventional drugs such as non-steriodal anti-inflammatory drugs and opioid derivatives, suggesting that mechanisms underlying neuropathic pain differ from that of other pain states. It is appreciated that neuropathic pain is a multi-mechanism disorder that may derive from different etiologies and pathologies. Unfortunately, neuropathic pain pathways are not well understood, which prevents the development of target specific agents for better neuropathic pain management.

Enhanced excitability and/or suppressed inhibitory tone of sensory circuits at the level of spinal cord and associated afferent fibers originated from dorsal root ganglion (DRG) neurons are critical in neuropathic pain development. In addition to a rapid modulation of protein functions at the post-translational level, such as through phosphorylation and dephosphorylation, that can alter cell excitability and trigger long-term changes to occur, altered gene expression in spinal cord and DRG after nerve injury may contribute to long-term modulation of sensory excitability, leading to neuropathic pain development [30,50]. Therefore, identifying differentially expressed genes in DRG post nerve injury that are correlated with neuropathic pain development will help to accelerate the discovery of neuropathic pain targets.

One challenge in this task is to separate potential neuropathic pain-related genes from nerve injury responsive genes that may not relate directly to abnormal sensation development. Our previous studies have indicated that development of tactile allodynia, a neuropathic pain state, in spinal nerve ligated Harlan Sprague Dawley rats consists of three stages. The first stage is from nerve injury to the onset of tactile allodynia, the second stage is the duration of tactile allodynia that is followed by the third stage in which nerve injured animals are recovering from the neuropathic pain state [26,28,29]. These stages provide us with a useful tool to identify nerve injury-responsive genes that also correlate with neuropathic pain development and maintenance. However, several studies of DRG gene expression profiling in neuropathic pain models were performed either at a single time point post nerve injury [4,25,46,48], or without a time point correlating with the pain-free stage in nerve injured animals [23,51], or in non-DRG tissue [23]. Thus, these studies had limited detection power in separating pain-related genes in DRG from those related to nerve injury only.

In this study, we analyzed gene expression profiles in DRG from spinal nerve ligated rats at time points correlated with three stages of neuropathic pain processing. In addition, we further characterized the potential contribution of injury-induced glial fibrillary acidic protein (GFAP) to neuropathic pain development because it is considered a “marker” for injury-induced astrocyte activation, but its functional contribution to neuropathic pain is not known. Our data indicated that there is a dynamic change in DRG gene expression post nerve injury in which concurrent regulations of subsets of genes with similar physiological/pathological functions occur at a temporal specific manner correlating with different neuropathic pain stages. Injury-induced GFAP expression in DRG may play a contributory role in the maintenance, but not initiation, of nerve injury-induced pain states.

Materials and Experimental Procedures

Animals

Male Harlan Sprague Dawley rats (100-150 g, Harlan Industries Indianapolis, IN), and GFAP knockout mice (The Jackson Laboratory, Bar Harbor, Maine) were housed in separate cages, exposed to a 12/12 h day/night cycle and allowed free access to food and water. All animal care and experiments were carried out according to protocols approved by the Institutional Animal Care Committee of the University of California, Irvine.

Surgery and behavioral testing

Peripheral nerve injury was induced in isoflurane-anesthetized animals with tight ligation of the left L5/6 spinal nerves between DRG and the beginning of the spinal nerve [22]. To assess tactile allodynia, paw withdrawal threshold (PWT) was determined using the up-down method of Dixon [8] with von Frey filaments (Stoelting, Wood Dale, IL, USA) as described previously [1]. Briefly, animals were allowed to acclimate for 30 min in a clear plastic cubicle with a wire-mesh base. A 2 g (for rat) or 0.4 g (for mouse) calibrated filament was first applied to the left hindpaw plantar surface with a pressure causing the filament to bend. If no paw lifting was detected after 5s the next filament with increasing weight was used. If a paw lifting was observed, the next weaker filament was used. This paradigm was used for a total of six measurements, starting from the one before the first change in response, or five consecutive negative responses (assigned a score of 15 g for rat, or 3.0 g for mouse) or four consecutive positive responses (assigned a score of 0.25 g for rat and 0.01 g for mouse) were reached. The 50% response thresholds were calculated as described previously [28].

Intrathecal antisense oligodeoxynucleotide treatment

Data from our laboratory and others have indicated that antisense oligodeoxynucleotides against the translation initiation region of different genes are effective in knocking down the expression of these genes [20,21,26,47], thus, we designed antisense oligodeoxynucleotide: GCCCCTGACCATCGTCTCGG, which was complementary to the region containing the initiation codon ATG of the GFAP gene [11] (Genbank accession #: AF028784). Mismatch oligodeoxynucleotide: CGCCTCGCACTACTGCTGCG, was used as controls.

These antisense and mismatch sequences were not complementary to any sequence of other rat genes as indicated by BLAST search results. It had been shown that oligodeoxynucleotides with phosphorothioate modification have increased potential for crossing lipid bilayers, and reduce nuclease cleavage [6]. Even though extensive phosphorothioate modification of oligodeoxynucleotides might cause local inflammatory reactions [5,6,40], data from our previous experiments had indicated that limited phosphorothioate modification on only three nucleotides at both ends of the oligodeoxynucleotides does not cause inflammatory side effects [26], thus, the antisense and mismatch oligodeoxynucleotides were modified with phosphorothioate on three nucleotides at both ends.

Oligodeoxynucleotides were sterilized with ethanol precipitation and dissolved in sterile saline, and directly injected through the L5/6 spinal region of an isofluorane anesthetized rat in a total volume of 10 μL, once daily, using a microinjector connected to a 30 G needle.

RNA extraction and gene chip processing

Total RNA was extracted from previously frozen L5/6 DRGs from each side of nerve-ligated rats (9 per each Affymetrix microarray experiment) using TRIzol (Life Technologies, Gaithersburg, MD), then the RNeasy Mini Kit (Qaigen, Valencia, CA) according to the manufacturer's protocol. Since gene expression profiles are similar between tissues contralateral to the nerve ligation and that from sham animals [48], we used tissues contralateral to the nerve injury as controls. Affymetrix rat Genome U34A GeneChip experiments were performed by the Gene Chip Core of the University of California, Irvine according to the Affymetrix standard protocol. The rat U34A GeneChip contains 8799 oligonucleotide probe sets representing approximately 7000 full-length sequences and approximately 1000 expressed sequence tags (EST) clusters from UniGene database (Build 34). References and quality control indicators are included on each chip therefore enabling quantitative comparison between different data sets. Each gene transcript is examined by 16 pairs of specific oligonucleotide probe sets containing a perfect match and a corresponding mismatch sequence

against different regions of the transcript. This allows detection of splice variants of individual genes while minimizing the background signal and non–specific hybridization.

After reverse transcription from RNA to cDNA, then to cRNA in the presence of biotinylated nucleotides followed by fragmentation, cRNA was hybridized to a gene chip in the GeneChip Fluidics Station at 45°C for 16 h. After a series of stringent washes, the probe arrays were stained with a streptavidin-conjugated fluorescent stain that was excited at 488 mm and detected by an epifluorescence confocal microscopy based Agilent GeneArray Scanner. Fluorescent signals collected by a photomultiplier were first converted to electric currents, then to digital intensity values by the scanner (image data files), followed by a conversion to cell-intensity file (.CEL) for each probe cell after analyzed by the Microarray suite software. Iobion ArrayAssist software (Stratagene, La Jolla, CA) was used to generate a gene-expression analysis profile for each gene using the GC Robust Multi-array Average (GC-RMA) algorithm.

Gene chip data analyses

Pair-wise, cross-comparisons of Affymetrix gene-chip data were performed between contralateral side and injury side (3 chips each side, a total of nine comparisons) using GC-RMA algorithm in ArrayAssist software. Further inter-experimental comparisons were performed using Microsoft Access database function. The following criteria were used to define a gene that was differentially expressed in injured versus non-injured DRG samples: (i) the gene expression level from the treatment group had to show a greater than or equal to a two-fold change compared to that in the control group as determined by the GC-RMA algorithm; and ii) these changes occurred in at least six out of nine cross-comparisons [46].

Western blot analysis

To measure protein expression levels, previously frozen DRG and spinal cord tissues were extracted in 50 mM Tris buffer, pH 8.0, containing 0.5% Triton X-100, 150 mM NaCl, 1mM EDTA and protease inhibitors. Cell extracts were subjected to denaturing NuPAGE Bis-Tris gel (Invitrogen, Carlsbad, CA) electrophoresis under reducing conditions and then electrophoretically transferred to nitrocellulose membranes. 5% low-fat milk in TBS containing 0.1% Tween 20 (TBS-T) was used to block nonspecific binding sites. Antibodies against the proteins of interest were then used to probe membranes in TBS-T for 1 hr at room temperature. After washing, the antibody-protein complexes were probed using appropriate secondary antibodies linked to horseradish peroxidase and detected with chemiluminescent reagents. The nitrocellulose membranes were stripped with Re-Blot Western blot recycling kit (Chemicon, Temecula, CA), incubated with 5% low-fat milk in TBS-T then blotted with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or β-actin antibodies for loading normalization.

Immunohistochemistry

Immunohistological staining as described [9,29] with minor modifications was used to localize GFAP proteins in DRG sections from injury- and non-injury-side of SNL rats two weeks post injury. Briefly, animals were perfused intracardially with 4% paraformaldehyde and tissue samples are removed, postfixed and transversely sectioned (20 μm). For GFAP staining (mouse anti-GFAP, Chemicon), these sections were immersed in citrate buffer (pH 6.0) and heated to 90°C for 5 min, cooled, and then treated with 3% H_2O_2 in PBS for 5 min. After washed in PBS containing 0.3% Triton X-100, and blocked with 10% normal goat serum, these sections were incubated with the GFAP antibodies for 45 hours at 4 °C. After thorough rinsing, sections were incubated with biotinylated secondary antibody and then with avidin-biotin complex solution. After rinses, sections were developed in diaminobenzidine-H_2O_2 solution, washed, and mounted on slides, air-dried, dehydrated and coverslipped with Permount. In control sections,

the staining procedures were the same except that GFAP antibodies were omitted. The positive staining was examined under a light microscope.

Statistics

Unpaired Student's t tests were performed and significance was indicated by two-tailed p values. *$p < 0.05$; **$p < 0.005$; ***$p < 0.0005$.

Results

Neuropathic pain development and maintenance in relation to spinal nerve injured rats

As shown in Fig. 1, spinal nerve ligation (SNL) caused significant tactile allodynia in the rat left hind paw (injury side) as indicated by decreased paw withdrawal thresholds (PWT) to mechanical stimulation. Paws contralateral to the ligated side did not show significant tactile allodynia. Approximately 10 weeks after the injury, the PWT in the injury side gradually returned to that seen in the contralateral side (control level) even in the continuously presence of the ligation injury. The temporal changes in the neuropathic pain state enabled the selection of three time points for gene chip experiments that allowed the detection of factors regulated temporally in correlation with the development of neuropathic pain states. These time points were i) two days post injury but before the onset of allodynia, ii) two weeks post injury when the injured rats displayed severe allodynia and iii) 20 weeks post injury when the injured animals recovered from allodynia.

Temporal and dynamic changes in DRG gene expression post nerve-injury

Nine animals were assigned to each treatment group, and total RNA from contralateral or ipsilateral L5/6 DRG of three injured rats was pooled at the designated time point for one gene chip analysis. The criterion for determining significant changes in gene expression post nerve injury was that the mRNA levels of identified genes in DRG ipsilateral to the injury had to be at least two-fold different from that in DRG contralateral to the injury in at least six out of nine comparisons as specified in the Experimental Procedures [46]. The two-fold detection criterion is commonly used in gene chip studies to determine significantly regulated genes. Admittedly, this selection would not identify genes that were up or down regulated at lower levels, some of which might be important regulators or mediators of pain processing. As shown in Fig. 2, the most dramatic change in gene expression occurred two days post injury (309 genes total) that was followed by two weeks of injury (224 genes total). While most upregulated genes were detected two-days after injury (202 genes total), most down-regulated genes were detected at the two week time point (135 genes total). By 20 weeks, the number of regulated genes decreased dramatically (54 genes total), especially in the up-regulated gene group (14 genes total).

To determine the cellular response in DRG to nerve injury, we defined the functional groups of altered genes based on their known functions (Fig. 2). Interestingly, there is a dynamic change in expression of genes related to different cellular functions. For example, genes related to cell-cell communication and signaling pathways showed the most dramatic changes in all time points, including the largest number of these genes down-regulated 20-week post ligation. A lot of genes related to synaptogenesis and growth were highly up- and down-regulated at the two-day and two-week, but not the 20-week, time points after injury. In addition, while genes related to immediate early responses to injury and inflammatory/immune functions were mainly up-regulated at the early time points, most genes encoding membrane ion channels or receptors appeared to be down-regulated in the early time points.

Determining genes with altered expression post-nerve ligation that correlated with neuropathic pain onset and maintenance

To focus on identifying genes with altered expression post injury that may also play a role in the initiation and/or maintenance of neuropathic pain, we divided positively identified genes (up- or down-regulated) into groups correlated to the onset and/or maintenance of neuropathic pain. This strategy allowed us to reveal important relationship among co-regulated genes that may form important pathways in neuropathic pain processing, and obtain critical information in identifying potential target genes that may contribute to a particular stage of neuropathic pain (Supplemental data Table 1). We expected that genes related to neuropathic pain induction should be modulated significantly at the initial days before the onset of neuropathic pain (day 2 post SNL), with or without changes at the two-week time point, but returned to a basal level by the 20-week time point. Genes related to neuropathic pain maintenance could be modulated in the early time points, but not so in the last time point, except that expression of genes with inhibitory roles in chronic pain processing could be increased in SNL but allodynia free rats. Among the 309 genes (202 up-regulated, 107 down-regulated) identified at the two-day time point before the onset of tactile allodynia, the mRNA level of interleukin 18 (IL18) was shown to be up-regulated 4.4 ± 0.3 fold, but was back to the basal level at both the two-week and 20-week time points that correlated with severe allodynia and recovery from allodynia, respectively (Supplemental data Table 1). This suggests a potential role for this gene in neuropathic pain induction. In contrast, the mRNA level of protein kinase C epsilon was down-regulated at both the two-day (8.6 ± 1.2 fold) and two-week (5.8 ± 1.8 fold) times points, but returned to the basal level 20 weeks post injury when the animals recovered from allodynia (Supplemental data Table 1), suggesting that this gene could be a potential modulator in the induction and/or maintenance of neuropathic pain.

Contribution of injury-induced GFAP to neuropathic pain maintenance

GFAP is one of the identified genes that showed dramatic upregulation of its mRNA at two-days and two-weeks, but not 20-weeks, post the ligation injury (Fig. 3), suggesting a potential role of these changes in neuropathic pain initiation and maintenance. GFAP is known as a marker of astrocyte activation in different pain models, but whether changes in expression of this protein contribute to pain processing is not known. To address this question, we first examined if the increased mRNA expression translated to elevated protein levels. As indicated in Fig. 4, compared with that in the non-injury side, GFAP proteins were also increased in injured DRG at two-days and two-weeks post nerve injury, which returned to a level close to that observed in non-injured DRG 20-weeks post nerve injury. The two protein bands detected were identical to the reported GFAP protein band pattern in Western blots in which the upper band matched the size of expected intact GFAP proteins, and the lower band represented presumably degradation product [31]. The pattern of increased GFAP protein expression, however, differed from that of GFAP mRNA. While nerve injury induced GFAP mRNA upregulation was much higher at two-days than that in two-weeks post injury (Fig. 3), injury-induced GFAP protein upregulation was similar between two-days and two-weeks post injury (Fig. 4), suggesting an unparallel regulation between GFAP mRNA and proteins in DRG post nerve injury. Data from immunohistostaining experiments indicated that the increase of GFAP proteins was mainly in DRG non-neuronal cells (Fig. 5).

To test if GFAP induction is critical in neuropathic pain processing, we generated the SNL model in GFAP knockout mice and their age-, gender-matched wild type littermates, and compared behavioral hypersensitivity to mechanical stimulation between the GFAP knockout and wild type mice. Similar to SNL in rats, left L5 spinal nerve ligation in wild-type mice resulted in the development of tactile allodynia that had a quick onset and lasted for about one-month followed by a gradual recovery (Fig. 6). Interestingly, SNL injury caused an allodynia state in GFAP knockout mice with a similar onset, but shortened duration compared with that

in SNL wild-type mice (Fig. 6), suggesting a role of injury-induced GFAP in the maintenance, but not initiation, of the neuropathic allodynia states.

To further confirm this observation, we applied GFAP antisense oligodeoxynucleotides intrathecally into SNL rats after nerve injury when the SNL rats had developed allodynia, followed by behavioral testing to see if GFAP antisense treatment could reverse injury-induced allodynia. Intrathecal injection of GFAP antisense, but not mismatch, oligodeoxynucleotides (50 μg/rat/day) for four days, starting after six weeks of nerve injury, resulted in an allodynia reversal in the injury side without affecting the paw withdrawal sensitivity in the non-injury side (Fig. 7). The antisense effects in allodynia reversal started after two days of treatment, peaked after three-four days of treatment, and lasted over two days after the last injection (Fig. 7). Similar antisense treatment started two weeks post nerve injury was not effective in allodynia reversal (data not shown). To determine if the antisense effects in allodynia reversal were mediated through down-regulation of injury-induced GFAP expression, we examined the GFAP protein levels in L5/6 DRG and dorsal spinal cord one day after the last antisense injection, when treated animals still showed near the maximal allodynia reversal. Data from Western blot analysis indicated that nerve injury not only induced upregulation of GFAP in DRG, but also in corresponding dorsal spinal cord segments. Antisense, but not mismatch, oligodeoxynucleotide treatments reversed injury-induced GFAP upregulation in DRG and dorsal spinal cord, without affecting endogenous GFAP protein levels at the non-injury side (Fig. 8). This finding is consistent with reported findings from other groups and our previous studies that intrathecally delivered antisense oligodeoxynucleotides can down regulate proteins of interest in DRG and spinal cord [24,26,36]. Together with the behavioral data from SNL GFAP knockout and wild-type mice, these findings support that injury-induced GFAP expression plays a critical role in the maintenance of neuropathic allodynia.

Discussion

Using the strategy of detecting gene expression profiles at different stages of neuropathic pain development, we found that peripheral nerve injury induces a dynamic change in DRG gene expression such that altered expression of selective groups of genes correlates with different stages of neuropathic pain development. These findings provide us with an insight into the molecular changes in DRG in response to peripheral nerve injury, and helpful information in separating genes that may play a role in neuropathic pain development from those genes that are mainly responsive to injury per se, but are not involved directly in neuropathic pain processing. Since the gene encoding GFAP, a marker of astrocyte activation, is one of the identified genes correlated with neuropathic pain development, but its contribution to neuropathic pain is not clear, we have used biochemical, antisense, genetically modified mouse approaches to validate GFAP expression post injury, and its potential contribution to neuropathic pain states. We found that injury-induced GFAP expression in DRG and dorsal spinal cord contributes mainly to maintenance of injury-induced tactile allodynia.

Our data indicate that changes in gene expression post injury, which is considered one of the potential causal factors in modulating different disease states including pain, is not static, but dynamically changed over time. This suggests that temporally coordinated cellular responses occur in DRG post injury, and unique interaction of factors and pathways at a temporal specific manner may play distinct roles in pain modulation at different stages. It appears that the most dramatically regulated group of genes includes those encoding factors related to cell-cell communication and signaling pathways. A lot of these genes are up- or down-regulated at the early time points indicating that there is an active interaction among different cell types and pathways in the early stages post nerve injury that may program the cells to undergo adaptive changes. In addition, this group also has the largest number of genes down regulated at the 20-week time point, indicating long-term plasticity in signaling pathways related to altered

expression of these genes. As expected, most of the alternatively expressed genes related to immediate-early-response gene families are up-, only few are down-, regulated at the early time points. This indicates the activation of these genes and related pathways in DRG in response to nerve injury, which in turn may modulate expression of down-stream target genes. Similarly, most genes related to inflammation/immune functions are also upregulated at the early time points post injury, suggesting the activation of the defensive system in the early stage post injury. All these changes could lead to a long-term plasticity in injured DRG, which may underlie the development of chronic pain states. Interestingly, a lot of genes related to synaptogenesis are also up- or down-regulated at the two-day and two-week, but not the 20-week, time points. This suggests that modulation of synapses after nerve injury may play a critical role in the initiation and/or maintenance of neuropathic pain. One potential pain modulator in this group is the gene encoding thrombospondin-4 (TSP4). This gene is upregulated dramatically at the two-day, and two-week time points post injury, prior to the onset and correlating with the presence of tactile allodynia in the injured rats. A recent study has indicated that astrocyte-secreted thrombospondins are inducers of synaptogenesis in vitro and in vivo [2]. Thus, it is possible that nerve injury-induced TSP4 plays a critical role in neuropathic pain through modulation of synaptogenesis. Experiments are undertaken to test this hypothesis.

One of the limitations in the microarray approach is that a functional linkage between the genes of interest and the disease states under investigation is not known. It is therefore critical to further validate the expression of genes of interest with alternative approaches and determine if altered expression of these genes contributes to the disease state under investigation, such as pain processing in this study. This is supported by our findings that while a temporal correlation between injury induced GFAP expression and allodynia suggests an involvement of GFAP in the initiation and maintenance of neuropathic pain, data from our further validation have suggested that injury-induced GFAP expression may contribute mainly to neuropathic pain maintenance.

GFAP is a member of the cytoskeletal protein family, and the principal 8-9 nm intermediate filaments expressed in mature astrocytes of the central nervous system, and satellite glial cells (SGC) of sensory ganglia [17]. GFAP is important in modulating astrocyte motility by providing structure stability to astrocytic processes [10]. It has been reported that GFAP expression is enhanced in response to neuronal activity, suggesting that it may play an important role in neuron-astrocyte interactions [41]. Other GFAP functions include modulation of long-term potentiation in the central nervous system through presumably synaptic modulation [32,39,45] and long-term maintenance of myelination [27,35]. However, a functional role of GFAP upregulation in pain processing has not yet been demonstrated. It has been known for a long time that astrocytes become reactive in some pathological, including pain-inducing, conditions [10,14,16,33]. This is characterized by a rapid synthesis of GFAP, or termed astrogliosis, since there is no evidence for the proliferation of astrocytes after peripheral nerve injury [15,42]. Increased GFAP immunoreactivity in spinal cord and DRG has been reported in different pain models, including that of peripheral nerve injury [3,12,13, 34,37,49], and cancer pain [19,38]. Our data indicate that injury-induced GFAP upregulation not only serves as a marker for astrocyte or SGC activation, but it also likely contributes to the maintenance of neuropathic pain states at least at the spinal level. This is supported by the findings that lack of GFAP induction by ligation injury in GFAP knockout mice results in a shortened duration of tactile allodynia without affecting its initiation significantly. In addition, intrathecal antisense, but not mismatch, oligodeoxynucleotide treatments can reverse injury-induced tactile allodynia at a later (after six weeks post injury), but not early (2 weeks post injury) stage of neuropathic pain development. Currently, we do not know if injury-induced GFAP expression in other locations, such as peripheral and supra-spinal locations, also contributes similarly to neuropathic pain maintenance. More detail experiments are required

to determine the relative contribution of altered GFAP expression in different locations to neuropathic pain processing.

The mechanism underlying GFAP-mediated neuropathic pain maintenance remains elusive. Our data have indicated that GFAP mRNA level is much higher at day 2 than that in day 14 post injury, but the GFAP protein level at both time points are similar, suggesting that the expression levels of GFAP mRNA and proteins are not tightly correlated. It was to our surprise that antisense oligodeoxynucleotide treatment could revere injury-induced GFAP protein levels in DRG/spinal cord and established tactile allodynia after three days of treatment in a temporal-specific manner. This relatively rapid onset of the antisense effects is inconsistent with the relative long half-life of endogenous GFAP proteins in mouse spinal cord [7]. In addition to possible species-specific differences in GFAP turn over rates, it is possible that injury-induced GFAP in activated astrocytes or SGC has a shorter turn-over rate than that of endogenous GFAP, which would make the induced GFAP more susceptible to antisense blockade.

The lack of a significant role of induced GFAP in allodynia initiation suggests that GFAP induction by injury may play a permissive, but not determinant, role in neuropathic pain development. That is, injury-induced GFAP is not a causal factor in neuropathic pain development, but it is necessary for modulating long-term changes, either alone or with other factors, that are critical in maintaining neuropathic pain states. It is reported that SGC from embryonic and neonatal rats can transform into oligodendrocytes, astrocytes, and Schwann cells in vitro [43]. Prolonged nerve injury may activate this pathway in DRG, leading to differentiation of a subpopulation of satellite cells into glial cells with different biochemical and functional properties [17], and changes in neuron-satellite cell communications; Nerve injury enhances coupling of satellite cells from that surrounding a single neuron under control conditions to that enveloping adjacent neurons [18], leading to abnormal spreading of sensory signals among DRG neurons and sensory hyperexcitability. In addition, GFAP levels are increased in the central projection territories of peripherally axotomized sensory neurons [44], which may promote interactions between reactive astrocytes and damaging axonal endings at the presynaptic terminals [44], and modulate pre-synaptic neurotransmission post injury. Further more, continuous activation of N-methyl-D-aspartate receptors, a major contributor of central sensitization in pain processing, regulates enhanced spinal cord GFAP expression post injury [12], which in turn may enhance neuron-glial cell communications under this pathological condition. Together, it is possible that blocking GFAP upregulation post injury may help to shorten the duration of chronic pain states.

In conclusion, we have demonstrated that peripheral nerve injury causes dynamic changes in DRG gene expression in a temporal specific manner in different stages of neuropathic pain development. In addition, injury-induced GFAP expression in DRG and dorsal spinal cord plays a critical role in the maintenance of neuropathic pain. These data, together with other findings, support that GFAP induction is not only a marker of astrocyte or SGC activation, but also actively involved in neuropathic pain maintenance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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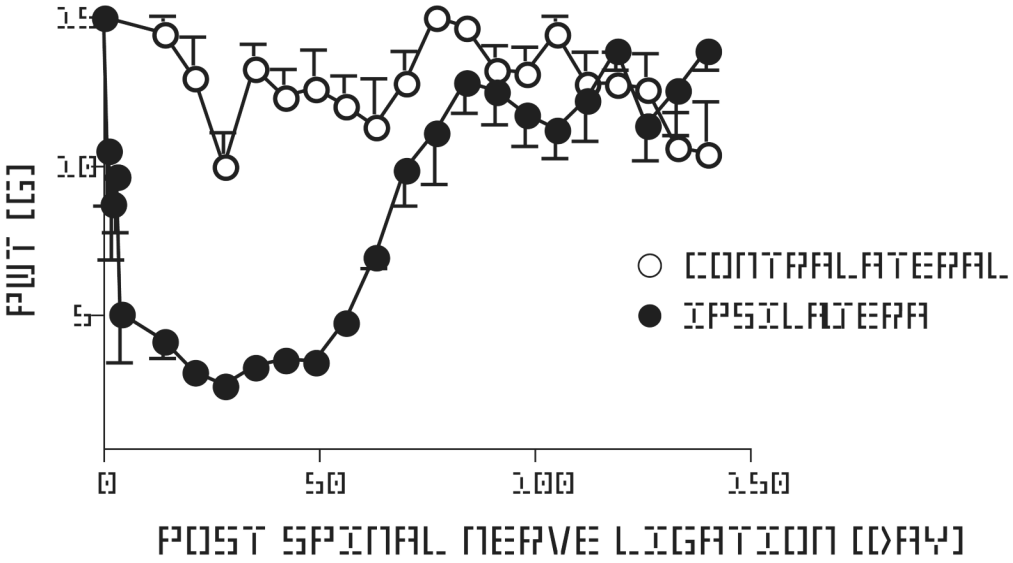

Fig. 1. Spinal nerve ligation injury in rats induced tactile allodynia that was followed by a recovery phase

Behavioral response to mechanical stimulation was tested in left L5/6 spinal nerve ligated rats at designated time points after injury as described in the Experimental Procedures. Nerve injured rats developed tactile allodynia as indicated by reduced paw withdrawal thresholds (PWT) to von Frey filament stimulation at the injury (ipsilateral) side compared with that at the non-injury (contralateral) side. Data presented are the means ± SEM from ten rats in each group.

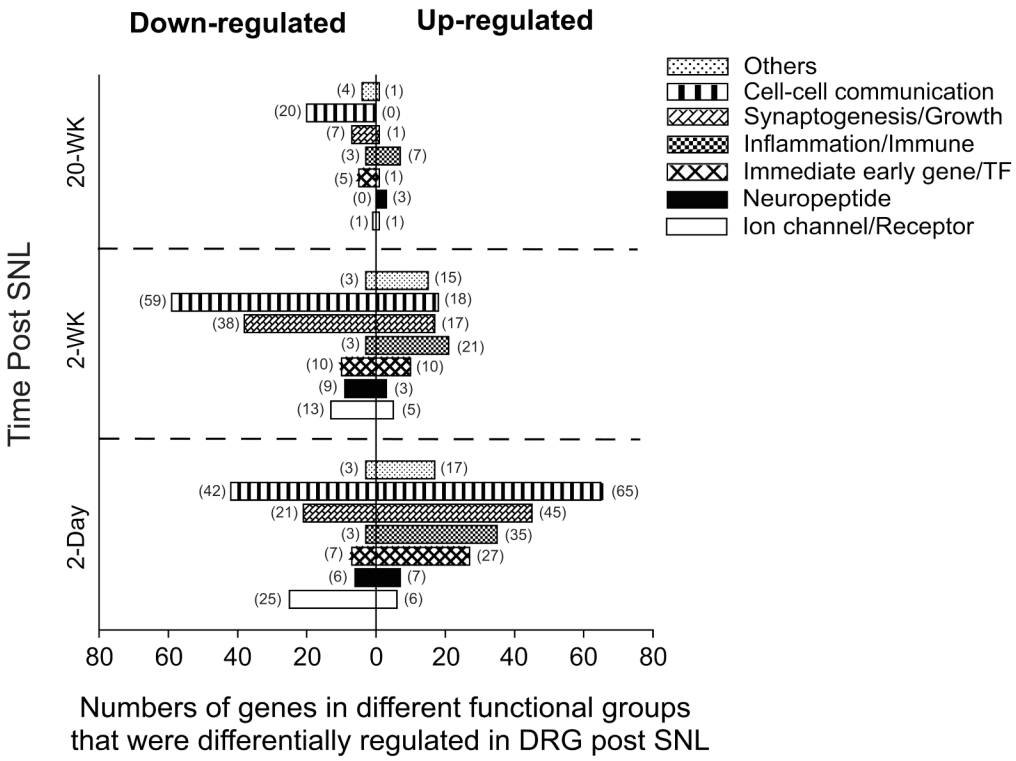

Fig. 2. Spinal nerve ligation injury in rats induced dynamic and temporal changes in DRG expression of genes with different functions

Gene expression profiling with Affymetrix rat genome U34A GeneChip as described in Experimental Procedures indicated that left L5/6 spinal nerve ligation injury (SNL) induced differential expression of genes in DRG of injury side, compared to that of the non-injury side, in a temporal specific manner correlating to different stages of allodynia development shown in Fig. 1. Data presented are numbers of genes in each indicated functional group that were differentially expressed (up- or down-regulated) in DRG at designated time points post SNL. The number in parenthesis on top of each bar indicates the actual number of differentially expressed genes in that particular group. TF – transcription factors.

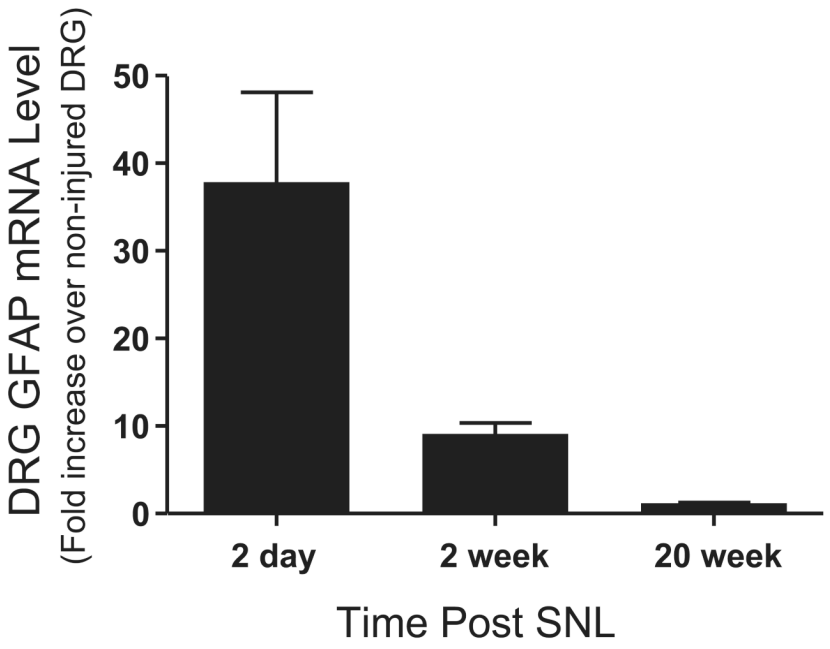

Fig. 3. Spinal nerve ligation injury in rats induced GFAP mRNA upregulation in injured DRG

Gene expression profiling with Affymetrix rat genome U34A GeneChip indicated that left L5/6 spinal nerve ligation (SNL) injury induced upregulation of GFAP mRNA in injured (ipsilateral) DRG compared with that in non-injured (contralateral) DRG at the early stages of tactile allodynia development. The increases were diminished at 20 weeks post injury when the behavioral hypersensitivity in injured rats returned to the control level. Data presented are the means ± SEM from three gene chips in each group.

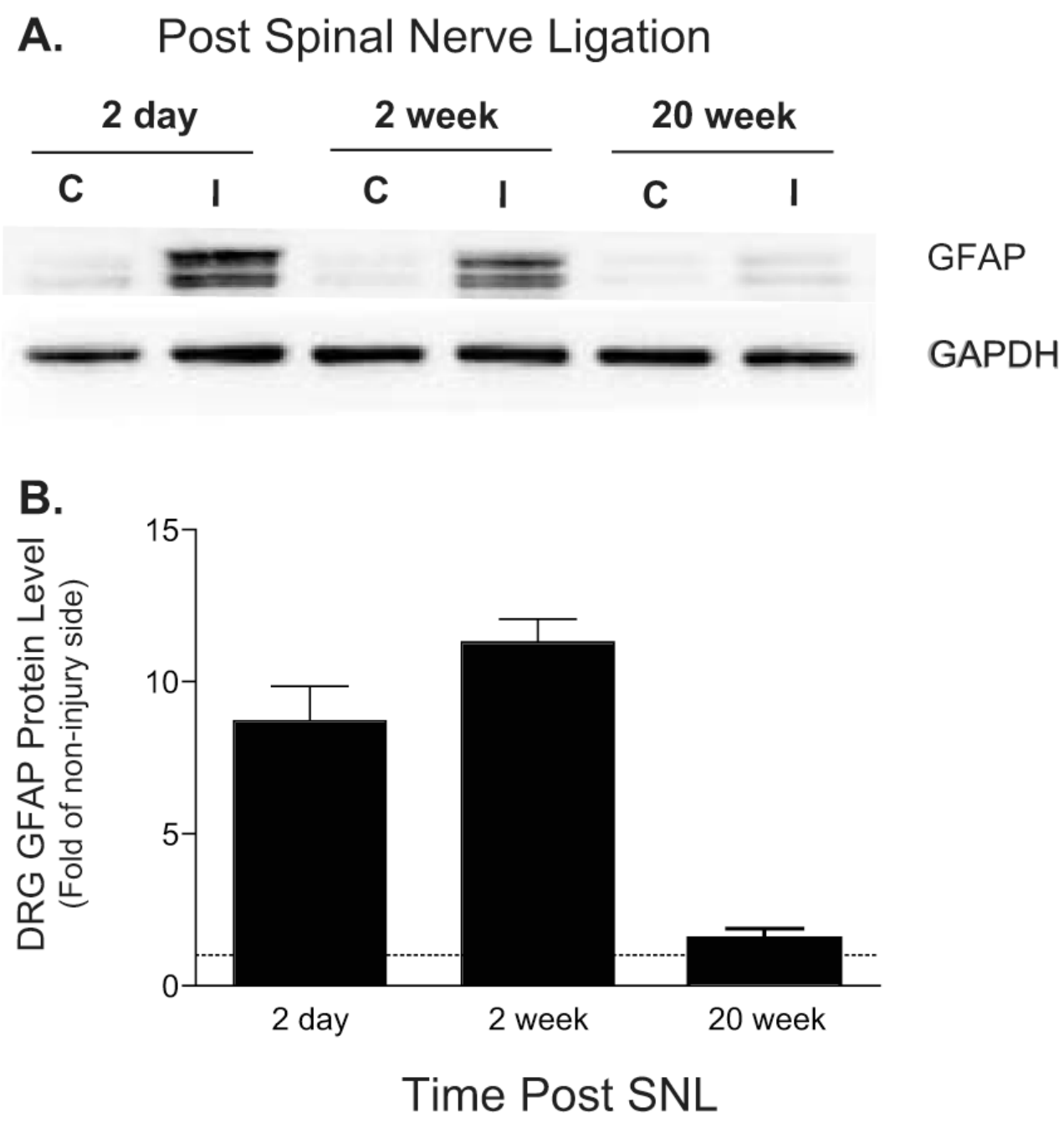

Fig. 4. Spinal nerve ligation injury in rats induced GFAP protein upregulation in injured DRG

L5/6 DRG were collected from both sides of rats at designated time points after left L5/6 spinal nerve ligation (SNL) injury followed by Western blot analysis to examine the protein levels of GFAP as described in the Experimental Procedures. **A.** Representative Western blots showing GFAP protein levels in L5/6 DRG at different time points post SNL. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH): loading control. C-contralateral side. I-injury side. **B.** Summarized Western blot data presented as fold changes in the injury side compared with that in the non-injury side (dotted line) after taken the ratio of GFAP/GAPDH within each sample group. Data are presented as the means ± SEM from at least three independent experiments.

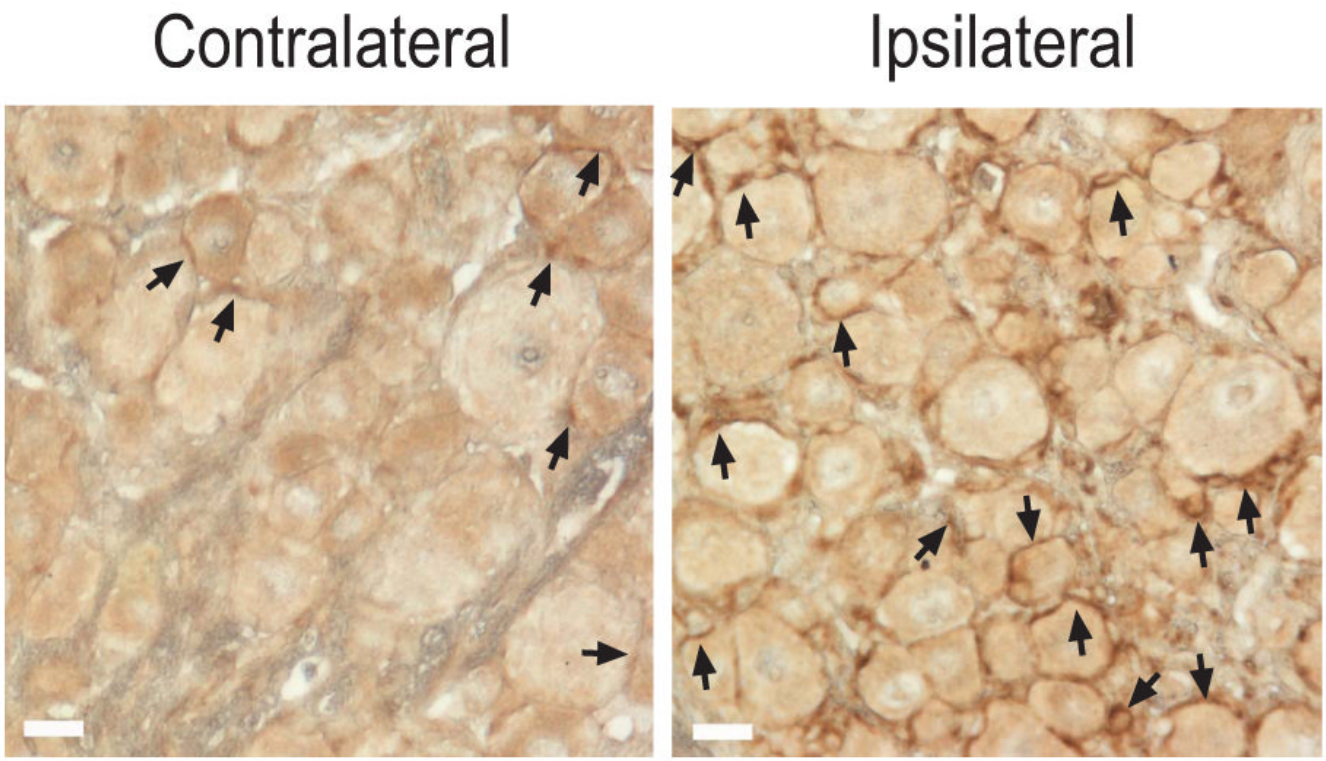

Fig. 5. Immunohistostaining data showing increased GFAP immunoreactivity in non-neuronal cells in injured DRG

L5 DRG were collected from both sides of rats two-weeks after left L5/6 spinal nerve ligation (SNL) injury followed by immunohistostaining to determine the localization of GFAP immunoreactivity as described in the Experimental Procedures. GFAP immunoreactivity (arrows) levels in non-neuronal cells were low in non-injured DRG (contralateral). After nerve injury, GFAP reactivity was increased in non-neuronal cells in injured DRG (ipsilateral). No staining was detected in sections from both sides when GFAP antibodies were omitted. Scale bar = 20 μm.

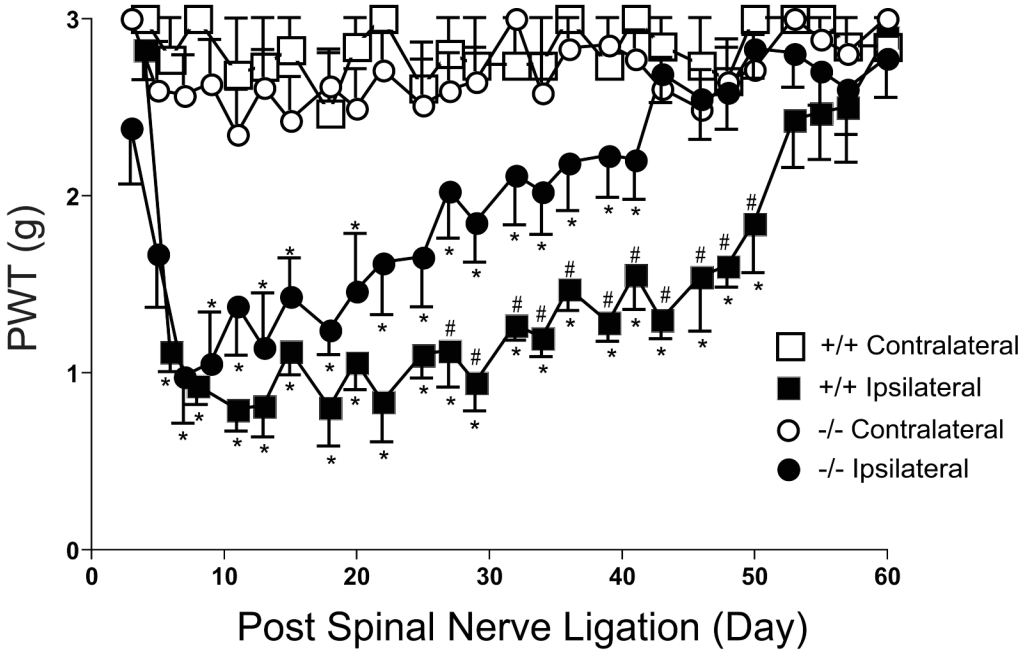

Fig. 6. Spinal nerve ligation injury in GFAP knockout mice resulted in a shortened duration of allodynia

Left L5 spinal nerve ligation (SNL) was performed in GFAP knockout mice (-/-) and their age- and gender-matched wild-type littermates (+/+), and behavioral response to mechanical stimulation was tested in these mice blindly at designated time points after injury as described in the Experimental Procedures. Spinal nerve injured wild-type mice developed tactile allodynia at the injury-side (ipsilateral) compared with the contralateral side. SNL GFAP knock out mice showed a shorter duration of allodynia in the injury (ipsilateral) side than their gender- and age-matched SNL wild-type littermates. Data presented are the means ± SEM from at least nine mice in each group. For clarity, statistical significance was shown only at the $p < 0.05$ level. * $p < 0.05$ compared with data from the contralateral side within the same group of mice. # $p < 0.05$ compared with data from the injury (ipsilateral) side of SNL GFAP knockout (-/-) mice.

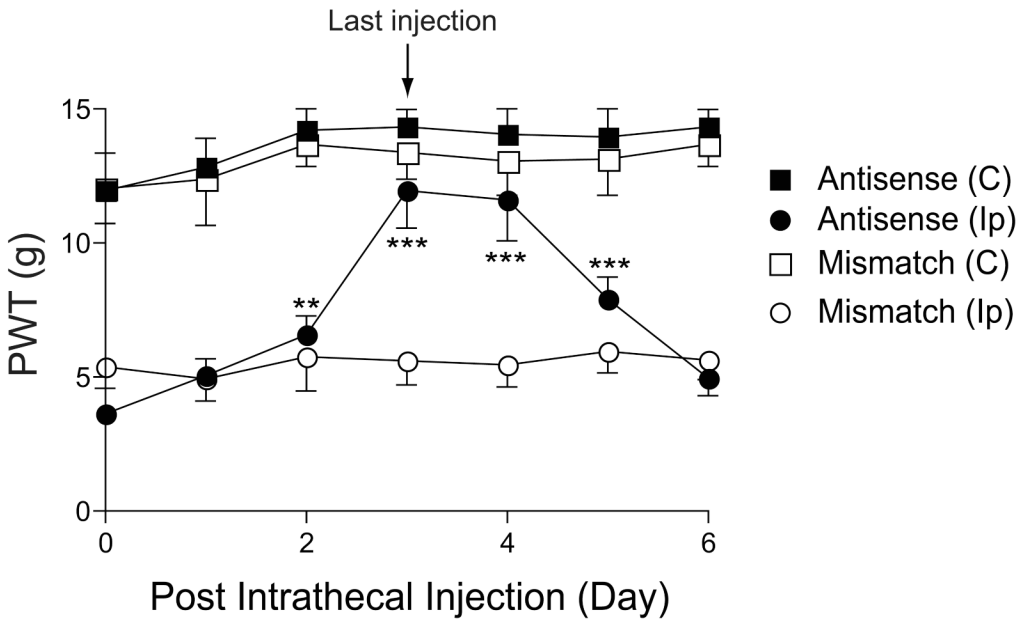

Fig. 7. Intrathecal treatments with GFAP antisense, but not mismatch, oligodeoxynucleotides reversed tactile allodynia in spinal nerve injured rats

Behavioral response to mechanical stimulation was tested in left L5/6 spinal nerve ligated (SNL) rats treated with intrathecal antisense or mismatch oligodeoxynucleotides as described in the Experimental Procedures. The treatment (50 μg/rat/day) started six-weeks post SNL and lasted for four days. Behavioral test was performed blindly before daily i.t. injection and continued for three more days after the last injection. Data presented are the means ± SEM from six rats in each group. C-contralateral, Ip-ipsilateral (injury) side. ** $p < 0.005$, *** $p < 0.0005$ compared with the pretreatment level.

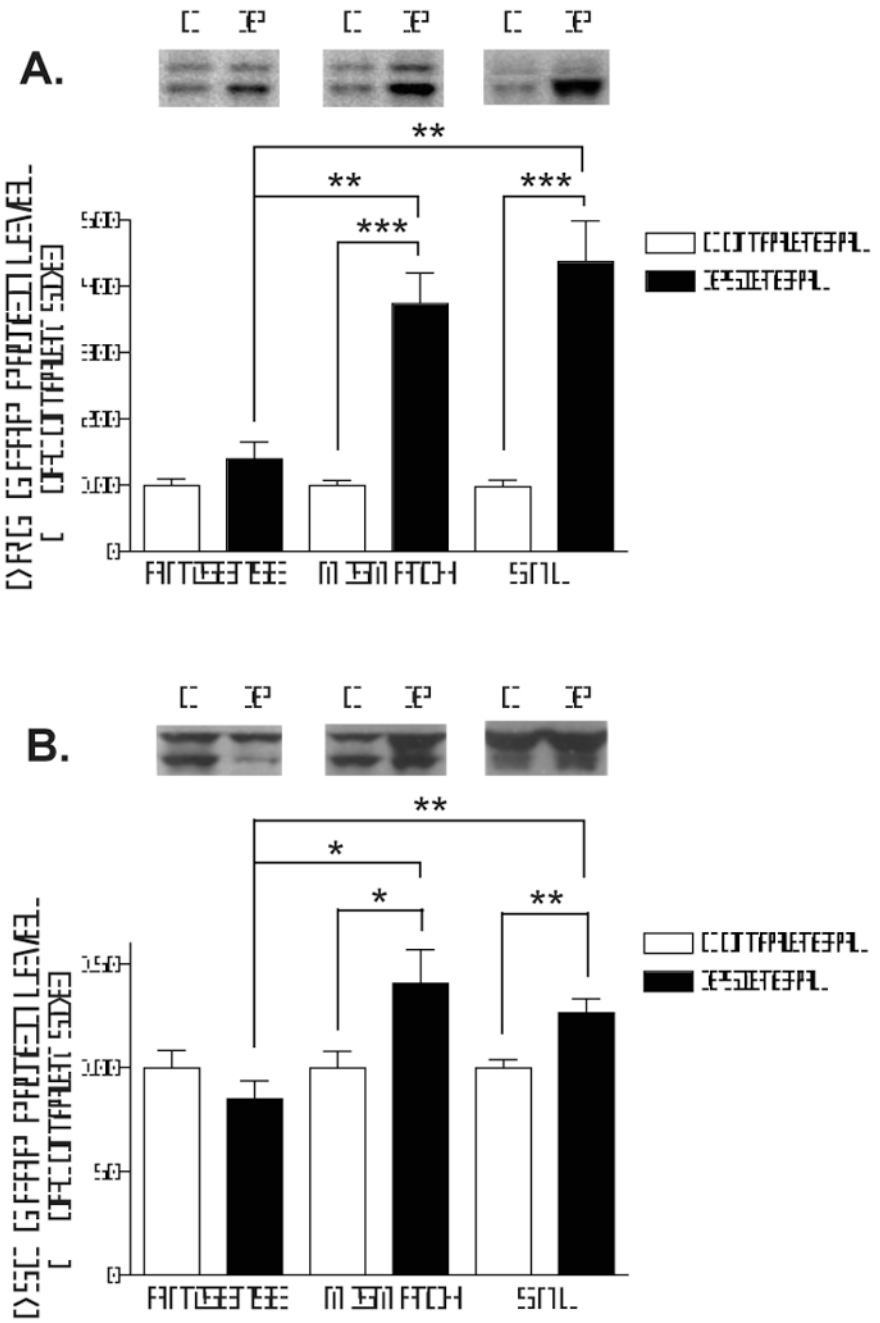

Fig. 8. Intrathecal treatments with GFAP antisense oligodeoxynucleotides diminished injury-induced GFAP expression in L5/6 DRG and dorsal spinal cord of spinal nerve ligated rats

L5/6 DRG and dorsal spinal cord were collected from both sides of left L5/6 spinal nerve ligated rats either one-day after the last injection of the four-day intrathecal antisense or mismatch oligodeoxynucleotide treatments (50 μg/rat/day, starting after six-weeks of SNL), or without intrathecal treatment (SNL). Western blot analysis was used to examine GFAP protein levels in DRG (**A**) and dorsal spinal cord (DSC) (**B**) samples as described in the Experimental Procedures. For loading control, the same plots were stripped and re-blotted with GAPDH or β-actin antibodies. Band density ratios of GFAP over GAPDH or β-actin were taken before signals from the ipsilateral (injury) side were compared with that from the

contralateral (non-injury) side. Representative Western blots are presented at the top of each corresponding treatment group. Summarized Western blot data are shown in the bar graphs as the means ± SEM from at least six groups of rats. Averaged value from each sample set was used for calculating the means if the same samples were examined in more than one Western blots. C-contralateral, Ip-ipsilateral (injury) side. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$.