Painful facet joint injury induces neuronal stress activation in the DRG: Implications for cellular mechanisms of pain

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A R T I C L E  I N F O

Article history:
Received 30 May 2008
Received in revised form 16 July 2008
Accepted 16 July 2008

Keywords:
Facet joint
Strain
Pain
Stress response
BiP
Western blot

A B S T R A C T

The cervical facet joint is implicated as one of the most common sources of chronic neck pain, owing to its rich nociceptive innervation and susceptibility to injurious mechanical loading. Injuries to the facet joint and its ligament can induce inflammation in the joint and spinal cord. Inflammatory molecules which are known to have a role in pain can also stimulate the integrated stress response (ISR). Therefore, we hypothesize that ISR is activated by facet joint injury in a rodent model of pain. To address this hypothesis, we assessed the expression of binding protein (BiP) (also known as growth-related protein 78 (GRP78)), a marker of endoplasmic reticulum stress response, in the dorsal root ganglion (DRG) after painful facet joint injury. In a rodent model of facet joint injury, dynamic distraction of the C6/C7 joint (injury, n = 12) was imposed; sham procedures were performed separately (sham, n = 8). Forepaw mechanical allodynia was assessed postoperatively for 7 days as a quantitative measure of pain symptoms. The C6 DRG was harvested and assessed for BiP expression using triple label immunofluorescent confocal microscopy and immunoblot analyses. BiP was significantly higher (p < 0.001) in the DRG after injury than sham and was expressed predominantly in neurons. Similarily, quantification of BiP by immunoblot demonstrated a significant 2.1-fold increase (p = 0.03) in injury compared to sham at day 7. Findings suggest neuronal stress activation is associated with painful facet joint injury, and that joint loading may directly mediate the behavior of DRG neurons in this class of injury.

Chronic pain from whiplash and neck injuries is among the most common acquired musculoskeletal disorders in the United States, with approximately 30% of Americans suffering chronic pain and an annual cost of $61 billion. The cervical facet joint is implicated as one of the most common sources of chronic neck pain, owing to its rich nociceptive innervation and susceptibility to injurious mechanical loading. Injuries to the facet joint and its ligament can induce inflammation in the joint and spinal cord. Inflammatory molecules which are known to have a role in pain can also stimulate the integrated stress response (ISR). Therefore, we hypothesize that ISR is activated by facet joint injury in a rodent model of pain. To address this hypothesis, we assessed the expression of binding protein (BiP) (also known as growth-related protein 78 (GRP78)), a marker of endoplasmic reticulum stress response, in the dorsal root ganglion (DRG) after painful facet joint injury. In a rodent model of facet joint injury, dynamic distraction of the C6/C7 joint (injury, n = 12) was imposed; sham procedures were performed separately (sham, n = 8). Forepaw mechanical allodynia was assessed postoperatively for 7 days as a quantitative measure of pain symptoms. The C6 DRG was harvested and assessed for BiP expression using triple label immunofluorescent confocal microscopy and immunoblot analyses. BiP was significantly higher (p < 0.001) in the DRG after injury than sham and was expressed predominantly in neurons. Similarly, quantification of BiP by immunoblot demonstrated a significant 2.1-fold increase (p = 0.03) in injury compared to sham at day 7. Findings suggest neuronal stress activation is associated with painful facet joint injury, and that joint loading may directly mediate the behavior of DRG neurons in this class of injury.

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response and pain. While facet joint injury is common, the cellular mechanisms driving pain remain poorly understood. An in vivo model of painful mechanical injury to the cervical facet joint is used to probe whether painful facet joint loading can induce BiP expression to identify the role of joint loading in modulating BiP responses in afferent neurons of spinal joints.

All procedures were approved by the Institutional Animal Care and Use Committee. Male Holtzman rats (350–430 g) were housed under USDA- and AAALAC-compliant conditions with free access to food and water.

Surgical procedures were performed under inhalation anesthesia and were modified from previously reported methods [14,15]. Following a skin incision in the back of the neck, muscle and soft tissue were cleared from C5 to T1. The laminae, facet capsules, and spinous processes at the C6/C7 facet joints were exposed bilaterally. The interlaminar ligament was transected at C5–T1 to enable attachment of each of the C7 and C6 vertebral to a loading device via microforceps. The loading device imposed controlled distraction of the C6/C7 facet joint using a stepper motor (Danaher Precision Systems) by displacing the C6 microforceps rostrally. Joint distraction was applied at a rate of 15 mm/s to simulate the injury loading profile of the C6/C7 facet joint and its capsule during whiplash [22,28,31]. The C6/C7 facet joints underwent either: (1) injury distraction (0.3 mm; n = 12) sufficient to produce sustained mechanical allodynia [6, or (2) sham procedures without any distraction (0 mm; n = 8).

Imaging and biomechanical measurements were acquired during joint distraction to quantify the applied loading and to validate that the same injury severity was applied in all cases. Particles (diameter = 0.17 mm) were affixed to the laminae surrounding the C5/C6 joint on the right side and used to define the overall joint distraction. Additional particles were also placed in a grid covering the right C6/C7 facet capsule, to similarly track the full-field capsule displacements during loading. All particles were tracked using high-speed imaging (Phantom v4.3 CCD camera; Vision Research, Inc.). In order to quantify the severity of loading to the capsule, maximum principal strain was computed using LS-DYNA software (LSTC) [6,15,21].

Bilateral mechanical allodynia was assessed in the forepaws on days 1, 3, 5, and 7, using 2 strengths of von Frey filaments (2, 4 g; Stoelting Co.) [32]. Procedures for assessing forepaw allodynia were previously reported and validated [11,15]. Briefly, on each day, rats were acclimated to the tester and the environment. Each filament was applied to the plantar surface of each forepaw in three rounds of testing. Each round applied 10 stimulations and was separated by 10 min between rounds. The total number of paw withdrawals was counted as the number of positive responses out of the total 30 stimulations for each forepaw. The responses for the right and left paws were averaged for each group on all testing days. Prior to surgery, baseline measurements were taken as each rat’s control.

The C5 left DRG was harvested on day 7 to analyze and quantify BiP expression. In separate groups of rats, immunohistochemistry identified the cell types expressing BiP, and immunoblot analysis was performed to quantify BiP levels. As such, half of the rats that received injury (n = 6) and half from the sham group (n = 4) were randomly selected and processed for each type of tissue analysis.

For immunofluorescent histochemistry, rats were deeply anesthetized followed by transcardiac perfusion with phosphate-buffered saline (PBS) and paraformaldehyde. Samples were post-fixed in 4% paraformaldehyde and paraffin-embedded. Transverse sections (10 µm) were mounted on APES-coated slides, incubated at 55 °C, deparaffinized and rehydrated as previously described [3]. Sections were blocked with 10% normal goat serum (Chemicon International). Mouse monoclonal antibodies to BiP (clone 40, 1:500,000; BD BioSciences), and the neuronal marker, microtubule associated protein (MAP2; SMI-52, 1:100; Covance Research Products) were used. Tyramide Signal Amplification Technology (New England Biolabs) was used to amplify the BiP signal. The MAP2 primary antibody was visualized by a Cy-3-conjugated, goat anti-mouse antibody (1:200). Confocal microscopy was performed on a Bio-Rad Radiance 2100 equipped with Argon Green He/Ne, Red Diode and Blue Diode lasers to excite the Cy-3 as described previously [30]. Metamorph 6.0 image analysis software (Universal Imaging Inc.) was used to quantify BiP. Total BiP was measured by the integrated pixel intensity for BiP per image and was the total pixel intensity per image multiplied by the area of pixel positive for the signal. The integrated pixel intensity for BiP overlapping positive pixels for MAP2 was used to determine the colocalization of BiP with neurons. Integrated BiP pixel intensity and the colocalization of BiP with neurons were both separately quantified.

For immunoblotting, rats were anesthetized and perfused with PBS. The DRG was removed and homogenized in lysis buffer and cocktails of protease and phosphatase inhibitors. Homogenized tissue was centrifuged at 25,000 rpm for 30 min and the supernatants were collected from the lysates. Total protein concentration of the lysates was determined using a BCA protein assay reagent (Pierce Biotechnology). Protein (10 µg) was loaded into each lane of a 4–12% Bis–Tris gradient gel (Invitrogen Corp.) for separation. A broad-range molecular weight ladder was run on each gel. Subsequent to separation, proteins were transferred onto a PVDF membrane and blocked in TBS with 1% Tween-20 (TBS-T) and 5% non-fat milk for 1 h at room temperature. The membrane was incubated at 4 °C overnight with mouse monoclonal antibody to BiP (1:1000; BD Biosciences) in TBS-T with 5% bovine serum albumin. The membrane was washed and incubated with HRP-conjugated secondary antibody (1:1000, Pierce Biotechnology) in 0.1% Tween-20 containing 1% non-fat milk.
cytoplasmic in neurons (Fig. 2A), a localization consistent with
day 7. Regardless of treatment, BiP staining was predominantly
responses for all time points. The mechanical allodynia produced by a facet joint
distraction injury was significantly (*p < 0.05) elevated over sham
for both types of tissue assays. There was no significant difference in the mechanical allodynia measured
in either joint distraction or maximum principal strain.

Significant differences in allodynia were determined between
groups over time using a repeated measures ANOVA with Bonferroni
post hoc test. Significance was defined at p < 0.05 for all statistical analyses.

The mean applied joint distraction for the injury group
was 0.23 ± 0.19 mm, with a corresponding mean maximum principal strain of 21.5 ± 12.3%. There was no significant differ-
ence in either joint distraction or maximum principal strain in the capsule between the groups of rats with tissues
prepared for immunofluorescence (distraction = 0.30 ± 0.21 mm; strain = 18.1 ± 4.9%) and those for immunoblotting (distraction = 0.16 ± 0.15 mm; strain = 24.9 ± 16.8%). Similarly, there was no significant difference in the mechanical allodynia measured
between either of the groups used for the two types of tissue assays.

Mechanical allodynia was immediately elevated above baseline
unoperated responses after a facet joint distraction injury, for both
filaments (Fig. 1) and remained significantly (p < 0.001) elevated on
each day. In contrast, sham procedures did not produce allodynia at
any time point. The mechanical allodynia produced by a facet joint
distraction injury was significantly (p < 0.001) elevated over sham
responses for all time points.

Facet joint injury also increased BiP expression in the DRG at
day 7. Regardless of treatment, BiP staining was predominantly
cytoplasmic in neurons (Fig. 2A), a localization consistent with
its role as a chaperone protein in the ER. However, the overall
level of BiP immunostaining in the DRG was significantly greater
(p < 0.05) after injury than sham (Fig. 2B). A significant increase in
neuronal BiP immunostaining on day 7 was observed after painful
injury relative to sham (p < 0.001) (Fig. 2C). Similar increases in BiP
protein in the DRG were also confirmed by immunoblot analysis.
Quantification of BiP by normalizing to actin expression showed a
2.1-fold significant (p = 0.03) increase in injury compared to sham
(Fig. 3).

This study is the first to demonstrate that facet joint loading suf-
icient to produce behavioral sensitivity also increases a marker of
integrated stress response activation (Figs. 2 and 3). The increased
BiP-immunoreactivity observed predominantly in neurons of the
DRG after painful injury supports activation of the integrated stress
response in association with facet-mediated pain. These findings
suggest that the neurons innervating the facet joint and/or its cap-
sule may act as ‘sensors’ of the capsule’s injury. This finding is
consistent with electrophysiological reports of altered signaling
and persistent after-discharges in afferents of the facet joint during
its injurious loading [4]. Taken with the literature, our data further
implicate cellular responses of neuronal afferents in the facet cap-
sule as having an essential role in chronic pain [4,14]. This study
does not directly define relationships between the magnitude of
capsule loading and BiP responses or allodynia; studies are needed
to define BiP in specific neuron populations and evaluate if such
responses are sensitive to mechanical inputs and/or relate directly
to resulting allodynia.

The current study demonstrates that ISR activation, indicated by
increased BiP, occurs in neurons of the DRG after painful facet dis-
traction. Given the presence of gial activation in the spinal cord at
the associated injury level [14,35], activation of the ISR in the DRG
is not surprising. Activation of the ISR alters translational initiation
by directly phosphorylating, and subsequently inactivating, the ini-
tiating factor required for charging the initiator methionine tRNA

Fig. 2. BiP immunostaining increases in DRG neurons after injury (A). Using triple label immunofluorescent confocal microscopy, BiP staining (green) is more prominent in injured DRG neurons (MAP2; red). Nuclei are shown in blue. Red and green co-localize to yellow. Scale is 30 μm for both images. Quantification of total BiP-integrated pixel intensity (B), and integrated pixel intensity of BiP colocalizing with MAP2, normalized to MAP2 area (C) show increased BiP expression. Data are presented as average ± standard deviation, with significant increases (*) over sham.
This study demonstrates activation of the ISR in a pain model. BiP expression in DRG neurons increases after painful facet joint distraction, which implies those neurons may be injured. While this study focused on neuronal behavior, glial cells are also involved in pain, and also in this injury model [5,14,35]. These results suggest an association between the neuronal stress response and pain, and the potential for mechanics to directly mediate behavior of affected neurons. Continued investigations will advance the understanding of the cellular mechanisms by which mechanical trauma-induced neuroinflammation contributes to pain, and shed light on the development of effective therapeutic interventions.

Acknowledgements

This work was funded in part by grants from the NHTSA/SCIB (DTNH-22-04-H-01423), the CDC/NCIPC (R49-CCE00689), the NIH/NINDS (NS056885), and support from a departmental GAANN fellowship.

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Fig. 3. BiP protein increases at day 7 after an injury. A single band of protein was detected by immunoblot analysis for either BiP or actin. Quantification of BiP nor-
[23] malized by actin shows a 2.1-fold increase in injury over sham (*p = 0.03). Data are presented as average ± standard deviation.


