Neuroscience 193 (2011) 377–386

ACTIVATING TRANSCRIPTION FACTOR 4, A MEDIATOR OF THE INTEGRATED STRESS RESPONSE, IS INCREASED IN THE DORSAL ROOT GANGLIA FOLLOWING PAINFUL FACET JOINT DISTRACTION

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Abstract—Chronic neck pain is one of the most common musculoskeletal disorders in the US. Although biomechanical and clinical studies have implicated the facet joint as a primary source of neck pain, specific cellular mechanisms still remain speculative. The purpose of this study was to investigate whether a mediator (activating transcription factor; 4ATF4) of the integrated stress response (ISR) is involved in facet-mediated pain. Holtzman rats underwent C6/C7 facet joint loading that produces either painful (n=16) or nonpainful (n=8) responses. A sham group (n=9) was also included as surgical controls. Behavioral sensitivity was measured and the C6 dorsal root ganglia (DRGs) were harvested on day 7 to evaluate the total and neuronal ATF4 expression. In separate groups, an intra-articular ketorolac injection was administered either immediately (D0 ketorolac) or 1 day (D1 ketorolac) after painful facet joint loading. Allodynia was measured at days 1 and 7 after injury to assess the effects on behavioral responses. ATF4 and BiP (an indicator of ISR activation) were separately quantified at day 7. Facet joint loading sufficient to elicit behavioral hypersensitivity produced a threefold increase in total and neuronal ATF4 expression in the DRG. After ketorolac treatment at the time of injury, ATF4 expression was significantly (P<0.01) reduced despite not producing any attenuation of behavioral responses. Interestingly, ketorolac treatment at day 1 significantly (P<0.001) alleviated behavioral sensitivity at day 7, but did not modify ATF4 expression. BiP expression was unchanged after either intervention time. Results suggest that ATF4-dependent activation of the ISR does not directly contribute to persistent pain, but it may sensitize neurons responsible for pain initiation. These behavioral and immunohistochemical findings imply that facet-mediated pain may be sustained through other pathways of the ISR.

Key words: ISR, ATF4, facet joint, pain, ketorolac, BiP.

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Abbreviations: ATF4, activating transcription factor 4; DRG, dorsal root ganglion; ISR, integrated stress response; MAP, microtubule-associated protein; NSAID, nonsteroidal anti-inflammatory drug; PERK, PKR-like endoplasmic reticulum stress kinase; PKR, double-stranded RNA-activated protein kinase.

Neck pain affects up to 70% of individuals in their life span, and it is one of the most commonly reported origins of musculoskeletal pain in the general population (Côté et al., 1998; Natvig et al., 2010). In particular, the facet joint is one of the most common sources of pain in the cervical spine (Barnsley et al., 1995; Lord et al., 1996; Manchikanti, 1999). The facet joint capsule contains not only mechanoreceptors for proprioception but also nociceptive fibers that provide a means for transmitting pain signals (Cavanaugh et al., 1996; Inami et al., 2001; McLain, 1994). Mechanical loading of the facet joint, in particular stretch of its capsule, has been demonstrated as a primary mechanism of pain generation in biomechanical and *in vivo* studies (Cavanaugh et al., 1996; Cusick et al., 2001; Deng et al., 2000; Gore et al., 1987; Grauer et al., 1997; Ito et al., 2004; Lee et al., 2004a; Ono et al., 1997; Panjabi et al., 2004; Pearson et al., 2004; Siegmund et al., 2001). Indeed, local anesthetic blocks to the nerves of the facet joint can alleviate, or even abolish, pain in up to 62% of chronic pain cases from mechanical neck injury (Aprill and Bogduk, 1992; Barnsley et al., 1993; Yoganandan et al., 1998). Despite the strong biomechanical and clinical data implicating the facet joint and its capsule's involvement in pain, the cellular mechanisms related to pain from injury to this joint still remain speculative.

Inflammatory processes contribute to persistent pain through a variety of mediators (Kawakami and Weinstein, 1986; McMahon et al., 2005; Millan, 1999). Several different animal models of painful joint inflammation have reported cytokine upregulation and glial activation in the dorsal root ganglion (DRG) and spinal cord (Fenzi et al., 2001; Lee et al., 2008; Miyagi et al., 2006). Glial activation can alter neuronal signaling and can also cause excessive glutamate release (Kawakami and Weinstein, 1986). A number of *in vitro* and *in situ* studies have shown that the release of glutamate and cytokines can directly induce the integrated stress response (ISR) in neurons and other cells, which is critical for cell development and function (Cardozo et al., 2005; Kharroubi et al., 2004; Oyadomari et al., 2001; Shim et al., 2004). Despite mounting evidence linking inflammatory responses to activation of the ISR and the known role of inflammation in pain (Hartwig et al., 2003; Inglis et al., 2005; Lee et al., 2008; Markowitz et al., 2007), there is still very limited information on the role of the ISR in facet- or joint-mediated pain.

The integrated stress response, also known as the endoplasmic reticulum (ER) stress response, is a common cellular response to disruption of homeostasis in

0306-4522/11 $ - see front matter
doi:10.1016/j.neuroscience.2011.07.059

injury or disease states (Dong et al., 2008; Harding and Ron, 2002; Katayama et al., 2004; Rao and Bredesen, 2004). The ISR is a tripartite pathway initiated by three ER-localized proteins, double-stranded RNA-activated protein kinase (PKR)-like endoplasmic reticulum stress kinase (PERK), inositol-requiring enzyme (IRE1a), and activating transcription factor 6. Activation of the ISR culminates in increased expression of the ISR binding protein (BiP), which plays a major role in the repair of unfolded and misfolded proteins (Schröder and Kaufman, 2005). In addition to BiP, each pathway activates proteins that enhance protein folding, establish homeostasis, and attenuate translation. The latter is a direct consequence of activation of the PERK pathway by phosphorylation and subsequent attenuation of eukaryotic initiation factor 2α (eIF2α), which promotes translation initiation. Interestingly, phosphorylation of eIF2α favors translation of activating transcription factor 4 (ATF4), which has been shown to promote apoptotic cell death via transactivation of C/EBP-homologous protein (CHOP) (Cherasse et al., 2007; Ohoka et al., 2005; Yamauchi et al., 2007). In neurons, ATF4 can contribute to long-term synaptic plasticity in mice (Chen et al., 2003). Sustained modification of spinal neurons has also been observed in our rat model of facet-mediated pain (Quinn et al., 2010). That model also exhibits spinal neuroinflammation and disrupted homeostasis in the injured afferents, as well as increased glutamate activity in the spinal cord and ISR activation in the DRG after facet injury in association with sustained behavioral sensitivity (Dong et al., 2008; Dong and Winkelstein, 2010; Lee et al., 2008; Quinn et al., 2010). Although we have previously observed increases in neuronal BiP in the DRG after painful facet joint injury, no studies have investigated the extent and pathway of ISR activation in facet-mediated pain.

The objectives of this study were to investigate whether ATF4 in injured afferents is involved in behavioral hypersensitivity that develops after painful facet joint injury. As such, facet capsule stretch was applied in our rat model at magnitudes that do and do not produce sustained behavioral hypersensitivity in the forepaw (Dong et al., 2008; Dong and Winkelstein, 2010; Lee and Winkelstein, 2009) to characterize ATF4 expression in the affected DRG after joint injury and to determine if painful joint loading is associated with the upregulation of ATF4 expression. In addition, to determine whether any changes in ATF4 are related to behavioral sensitivity, additional studies were performed assessing ATF4 and BiP expression after painful loading conditions with a nonsteroidal anti-inflammatory drug (NSAID) treatment. Ketorolac is an NSAID that reduces pain by nonselectively inhibiting cyclooxygenase (COX) activity, which leads to diminished production of prostaglandins (Cassinelli et al., 2008; Dogan et al., 2004; Turner et al., 2011). Specifically, ketorolac injection reduces joint inflammation and postoperative pain in clinical and animal models (Convery et al., 1998; Ng et al., 2006; Swift et al., 1998). Therefore, in this study, ketorolac was administered to the injured joint either immediately after injury at day 0 or at 1 day after painful loading, in separate groups. In those studies, BiP expression was evaluated along with ATF4 to assess the effects of treatment on ISR activation.

EXPERIMENTAL PROCEDURES

Animal care and surgical procedures

All procedures were approved by the University of Pennsylvania Institutional Animal Care and Use Committee and adhered to the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain (Zimmermann, 1983). Male Holtzman rats (Harlan Sprague–Dawley, Indianapolis, IN, USA) weighing 350–425 g were housed under USDA- and AAALAC-compliant conditions with free access to food and water and a 12/12 h light–dark cycle.

All surgical procedures were performed under inhalation anesthesia (4% isoflurane for induction, 2.5% for maintenance) and have been previously described (Dong et al., 2008; Dong and Winkelstein, 2010; Lee et al., 2004b). Briefly, an incision was made from the base of the skull to the second thoracic vertebra and the bilateral C6/C7 facet joints were exposed by removing the surrounding soft tissue and musculature. The C6 and C7 laminae were rigidly attached to microforceps of a customized loading device that imposed a controlled joint injury by displacing the C6 vertebra rostrally, holding it for 30 s and returning to its initial unloading position, while the C7 vertebra remained stationary. Two different C6/C7 facet joint distractions were applied separately to either induce (0.5 mm; painful $n=16$) or not induce (0.2 mm; nonpainful $n=8$) behavioral hypersensitivity, based on previous studies (Dong et al., 2008; Dong and Winkelstein, 2010). Sham procedures were also performed as a surgical control with no applied joint distraction (0 mm; sham $n=9$) but all other surgical procedures. The magnitude of the injury severity was measured by quantifying vertebral and joint capsule distractions during joint loading. Polystyrene particles were affixed to the C6 and C7 laminae and joint capsule for motion tracking. Joint distraction was defined as the maximum displacement of C6 laminae relative to C7, and the capsule distraction was defined as the average resultant displacement of the rostral edge of its capsule relative to the caudal edge. As an additional biomechanical measure of injury severity, maximum principal strain for the capsule was calculated using engineering software (LS-DYNA) (Dong and Winkelstein, 2010; Lee et al., 2004b; Weisshaar et al., 2010; Winkelstein et al., 2000). Joint displacements and capsule strains were compared between painful and nonpainful groups using an unpaired *t*-test.

A subset of rats from the painful group was randomly selected to receive intra-articular administration of ketorolac (12 μg in 10 μl saline) either immediately (D0 ketorolac; $n=4$) or at 1 day (D1 ketorolac; $n=5$) after painful joint loading. Joint injection procedures were performed under inhalation anesthesia (2.5% isoflurane), and ketorolac (Sigma-Aldrich; St. Louis, MO, USA) was administered in the bilateral C6/C7 facet joints using a 10-μl syringe with a 33G beveled needle (Hamilton; Reno, NA, USA). The needle was gently inserted into the facet joint by piercing through its capsule in the dorsal medial region with the injection bolus delivered slowly.

Behavioral assessment

Behavioral sensitivity was assessed by measuring bilateral mechanical hyperalgesia in the forepaws on days 1, 3, 5, and 7 after painful and nonpainful distraction injury or sham procedures. Prior to surgery, rats were also assessed for hyperalgesia to provide a baseline measurement to serve as an unoperated control response for each rat. Methods to measure hyperalgesia were adopted from Chaplan's up/down method and have been previously reported and validated (Chaplan et al., 1994; Decosterd and

Woolf, 2000; Hubbard and Winkelstein, 2005; Lee et al., 2008). The response threshold was measured using increasing strengths of von Frey filaments (Stoelting, Wood Dale, IL, USA), ranging from 0.6 to 26 g, to stimulate the forepaw. The lowest-strength filament to provoke a positive withdrawal response was taken as the response threshold if a withdrawal response was also confirmed for application of the next higher filament. Each testing session consisted of three rounds of five stimulations to each forepaw, with at least a 10-min rest period separating each round. The left and right forepaw responses for each rat were averaged for each group on each testing day. A repeated measures ANOVA with Bonferroni correction compared temporal hyperalgesia between painful, nonpainful, and sham groups. Additionally, hyperalgesia for each postoperative day was also compared across groups using a one-way ANOVA.

For the rats receiving ketorolac treatment, forepaw mechanical allodynia was measured at baseline, day 1, and day 7 to assess and determine if the onset of behavioral sensitivity was consistent with previous reports using the same distraction magnitude (Dong et al., 2008; Dong and Winkelstein, 2010; Lee et al., 2004a), and to define the behavioral sensitivity on the day when DRG tissue was assayed (day 7). For each test session, forepaw mechanical allodynia was performed in three rounds for each paw. Each round consisted of 10 tactile stimulations to the plantar surface of each forepaw using a 4-g von Frey filament (Stoelting) that has been shown to robustly detect allodynia after injury in the cervical spine (Dong and Winkelstein, 2010; Hubbard and Winkelstein, 2008; Lee et al., 2004a; Rothman et al., 2005). For each session, the total number of paw withdrawals was counted for each paw of each rat and responses for the left and right paws were averaged for each group. Mechanical allodynia for D0 ketorolac and D1 ketorolac were compared using an ANOVA with repeated measures for the two time points.

DRG harvest and immunohistochemistry

The C6 DRGs on the left side were assayed on day 7 from all groups to assess ATF4 expression and its co-localization with neurons, using fluorescent confocal microscopy. Rats were deeply anesthetized and underwent transcardiac perfusion with 250 ml of phosphate-buffered saline (pH 7.4), followed by 300 ml of 4% paraformaldehyde. DRGs were removed and postfixed in 4% paraformaldehyde for 2–4 h before being transferred to 50% ethanol for overnight incubation. DRG samples were dehydrated in a series of graded ethanol solutions and then paraffin embedded. Thin (10 μm) axial tissue sections were mounted on APES-coated slides and incubated at 55 °C overnight before deparaffinization and rehydration, as previously described (Dong et al., 2008). Antigen retrieval was performed by incubating slides at 95 °C in a water bath for 1 h using target retrieval solution (Dako, Carpinteria, CA, USA). Sections were washed three times in PBS for 10 min each and blocked using 10% normal goat serum (Chemicon International; Billerica, MA, USA) with 0.3% Triton X-100 for 2 h at room temperature. Sections were incubated overnight in rabbit polyclonal ATF4/CREB2 (1:200; Santa Cruz Biotechnology; Santa Cruz, CA, USA) and mouse microtubule-associated protein (MAP2; 1:200; Covance Research Products; Princeton, NJ, USA); MAP2 is a neuronal marker that stains the soma and dendrites of neuronal cells (Jalava et al., 2007; Matus et al., 1981). Sections were then washed with PBS and incubated in Alexa 488 goat anti-rabbit and Alexa 546 goat anti-mouse (1:1000; Invitrogen; Carlsbad, CA, USA) for 2 h in the dark. After three rounds of 5 min of washing, slides were mounted using anti-fade gel (Biomeda; Foster City, CA, USA). Negative controls with no primary antibody as well as tissue sections from naïve unoperated rats were also included to verify staining methods.

Total and neuronal ATF4 expression in the DRG were separately analyzed using quantitative densitometry and compared between groups. Four tissue sections from each DRG were imaged at 40× magnification using a Zeiss LSM 510 confocal microscope (Carl Zeiss; Thornwood, NY, USA), equipped with Argon, HeNe, and Coherent Chameleon fs-pulsed NIR lasers. Total ATF4 expression in the DRG was measured as the percentage of pixels above a defined threshold, which was chosen based on expression in normal tissue (Lee et al., 2004a; Rothman and Winkelstein, 2010). The co-localization of ATF4 with MAP2 was defined as areas in the DRG that were positive for both ATF4 and MAP2 staining above the defined threshold, chosen based on normal tissue; it was calculated as the percentage of pixels that were positive for both markers relative to the total number of pixels positive for ATF4 in each DRG sample. Each of the total and neuronal expression of ATF4 was averaged for all rats in each injury group and normalized to sham levels. A one-way ANOVA with post hoc Bonferroni correction was used to compare ATF4 immunoreactivity between groups (painful, nonpainful, D0 ketorolac, D1 ketorolac).

Both ATF4 and BiP immunoreactivity were evaluated at day 7 after injury using samples from the matched C6 DRGs from each rat in the D0 ketorolac and D1 ketorolac groups receiving ketorolac treatment. Tissue from the sham group was also included in the BiP assay as a control. Mouse monoclonal antibodies to BiP (clone 40, 1:10,000; BD BioSciences; San Diego, CA, USA) and MAP2 (1:100; Covance Research Products) were used, followed by sequential secondary incubations of Alexa 488 F'ab goat anti-mouse and Alexa 568 F'ab goat anti-mouse (1:1000; Invitrogen). Using the same quantification methods as described above for ATF4, both total and neuronal ATF4 and BiP expression in the DRG for each group were quantified, averaged, and compared between D0 ketorolac, D1 ketorolac, and sham using one-way ANOVA, for each marker separately.

RESULTS

There was no visual or biomechanical evidence of rupture of the facet capsule for any rat in this study. The mean applied vertebral distraction was 0.49±0.09 mm in the painful group and 0.19±0.03 mm in the nonpainful group, with corresponding average capsule distractions of 0.34±0.08 mm and 0.15±0.03 mm. The painful and nonpainful groups underwent significantly different vertebral ($P<0.001$) and capsule ($P<0.001$) distractions. Similarly, the average maximum principal strain in the capsule for painful loading (31.2±14.0%) was significantly higher ($P=0.047$) than that for nonpainful loading (18.5±9.7%) of the joint. Although these biomechanical metrics indicate that the painful and nonpainful groups underwent significantly different injuries, there was no difference in any of biomechanical parameters defining the severity of joint loading for either of the injury groups receiving ketorolac treatment, supporting that both of the D0 ketorolac and D1 ketorolac treatment groups received similar injuries.

Tactile hypersensitivity in the forepaw was induced by facet joint distraction in the painful group only, with a significant ($P<0.01$) reduction in the threshold for paw withdrawal in that group compared to both nonpainful and sham groups as early as day 1 after injury (Fig. 1). Although hyperalgesia was immediate (day 1) after painful joint loading and the reduction in withdrawal threshold was sustained through the entire testing period until day 7, the threshold responses induced by sham and nonpainful joint distraction remained at baseline levels for all postoperative days (Fig. 1). The withdrawal threshold for nonpainful and sham was not significantly different from each other. Yet,

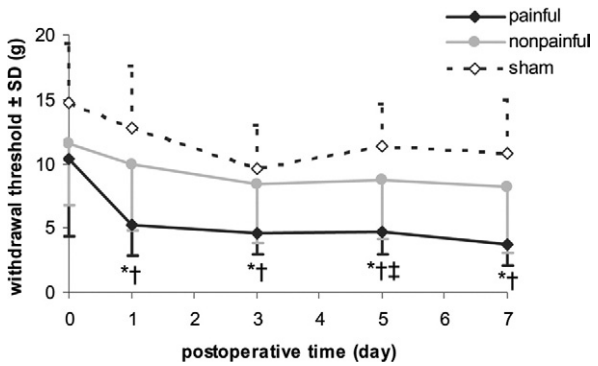

Fig. 1. Mechanical hyperalgesia as measured by the average response threshold to von Frey filament stimulation in the forepaw. Increase sensitivity corresponds to a decreased response threshold. Painful distraction significantly reduced thresholds below nonpainful distraction (* $P<0.002$) and sham controls († $P<0.001$) for all testing days. Nonpainful and sham responses were only significantly (‡ $P=0.02$) different from each other on postoperative day 5.

the threshold response for both groups was significantly ($P<0.03$) higher than the painful group on all postoperative days (Fig. 1).

For both groups of rats that underwent painful joint distraction and also received ketorolac treatment, mechanical allodynia was developed at day 1 regardless of the timing of treatment (Fig. 2). Both groups exhibited a greater than four-fold increase in allodynia over responses of the sham controls at day 1, and there was no difference in allodynia between the D0 ketorolac and D1 ketorolac groups at that time point (Fig. 2). However, at day 7 after treatment, mechanical allodynia was significantly ($P<0.001$) reduced in the D1 ketorolac group only, while remaining unchanged in the group receiving treatment at the time of injury (D0 ketorolac) (Fig. 2).

Mirroring the behavioral outcomes, ATF4 expression at day 7 in the DRG resulting from the nonpainful distraction was not different from sham, but ATF expression for painful joint distraction was significantly higher than both the nonpainful ($P<0.05$) and sham ($P<0.01$) responses (Fig. 3). Both total and neuronal ATF4 for painful group was more than twice that in the nonpainful group (Fig. 3P, Q). Unlike the behavioral outcomes, ketorolac treatment at the time of injury (D0 ketorolac) reduced total ATF4 expression, but this was not the case for the later treatment time (D1 ketorolac). Total ATF4 expression in the D0 ketorolac group was significantly ($P<0.001$) lower than the painful distraction without any treatment and reduced to levels that were comparable to those after the nonpainful distraction (Fig. 3). However, total ATF4 immunoreactivity evident after D1 ketorolac treatment was comparable to the ATF4 levels in the painful group and was significantly ($P=0.006$) elevated above nonpainful responses (Fig. 3). Neuronal ATF4 expression in the DRG demonstrated similar responses as did the total ATF4 expression, with D0 ketorolac showing no difference from the nonpainful distraction but the D1 ketorolac group exhibiting a significant ($P<0.001$) three-fold increase over nonpainful levels (Fig. 3).

Despite differences in the behavioral responses and ATF4 expression at day 7, the amount of total and neuronal BiP expressed in the DRGs at day 7 was not different between the two treatment groups (D0 ketorolac, D1 ketorolac) (Fig. 4). Total BiP expression for D0 ketorolac was significantly greater ($P=0.006$) than the levels in sham but was not different from those for D1 ketorolac (Fig. 4). However, neuronal BiP expression was significantly greater ($P<0.05$) for both D0 ketorolac and D1 ketorolac than sham (Fig. 4).

DISCUSSION

This study demonstrates that the differences in forepaw sensitivity produced by painful and nonpainful mechanical loading of the facet joint are also associated with similar graded changes in ATF4 expression in the DRG (Figs. 1 and 3). Previous studies with the same pain model have shown that the behavioral sensitivity measured in the forepaw is consistent with that which is also produced in the back of the neck (Lee et al., 2008, 2009). While whiplash and facet joint pain are most commonly reported to occur in the back of the neck along the “coat hanger” distribution, more than 60% of patients also report widespread sensitivity radiating to other parts of the body, including the trunk and upper extremity (Curatolo et al., 2001; Hincapié et al., 2010; Koelbaek Johansen et al., 1999; Mayou and Radanov, 1996; Scott et al., 2005). Further, the behavioral and ATF4 responses were also modulated differentially by intra-articular ketorolac injection (Fig. 3). Intra-articular ketorolac administration was selected based on clinical evidence of its improved effectiveness in offering postoperative pain relief in knee arthroscopy surgery (Convery et al., 1998; Ng et al., 2006); yet, oral administration of NSAIDs is more common and should be the focus of future studies with this painful joint model.

The robust increase in total and neuronal ATF4 expression observed after painful loading compared to the nonpainful group (Fig. 3) suggests that behavioral hypersensitivity may be sustained, in part, through ATF4-medi-

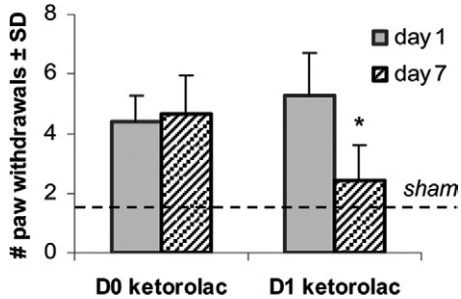

Fig. 2. Forepaw mechanical allodynia (MA) for D0 ketorolac and D1 ketorolac groups at days 1 and 7 after injury. Increase sensitivity corresponds to a higher number of paw withdrawals. The dashed line indicates the average sham response. There was no significant difference between day 1 and day 7 MA for D0 ketorolac. However, following D1 ketorolac treatment, mechanical allodynia was significantly (* $P=0.006$) reduced to almost sham levels by day 7. The MA response for both treatment groups at day 1 was not significantly different from each other.

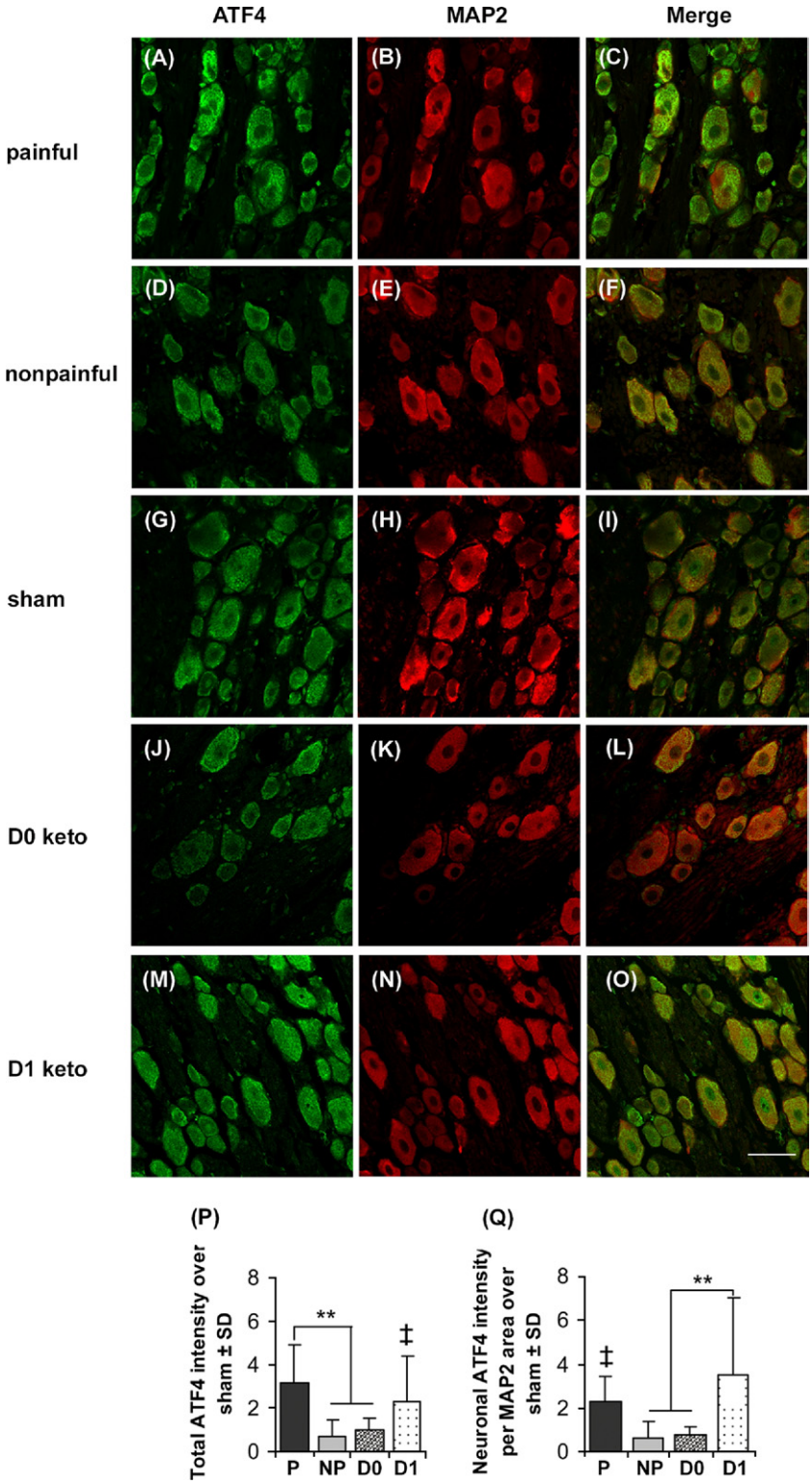

Fig. 3. ATF4 expression (green) in neurons (red; MAP2) of the DRG. ATF4 immunostaining was increased after painful distraction (A–C) compared to the nonpainful (D–F) and sham (G–I). Although D0 ketorolac (J–L) reversed such elevation, D1 ketorolac (M–O) showed no change in the ATF4 expression. Quantification of total ATF4 pixel intensity (P) showed a significantly higher amount of ATF4-immunoreactivity after painful distraction (** $P<0.001$); total ATF4 for D1 ketorolac was significantly higher than the nonpainful (‡ $P=0.006$). Similarly, neuronal ATF4 intensity normalized to MAP2 area (Q) also quantified a significantly increased expression in painful compared to nonpainful (‡ $P=0.04$) distractions. Neuronal ATF4 expression remained significantly elevated after D1 ketorolac (** $P<0.001$) but not after the D0 ketorolac treatment. Data are presented as fold increase over sham; the scale bar (50 μm) applies to all panels. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

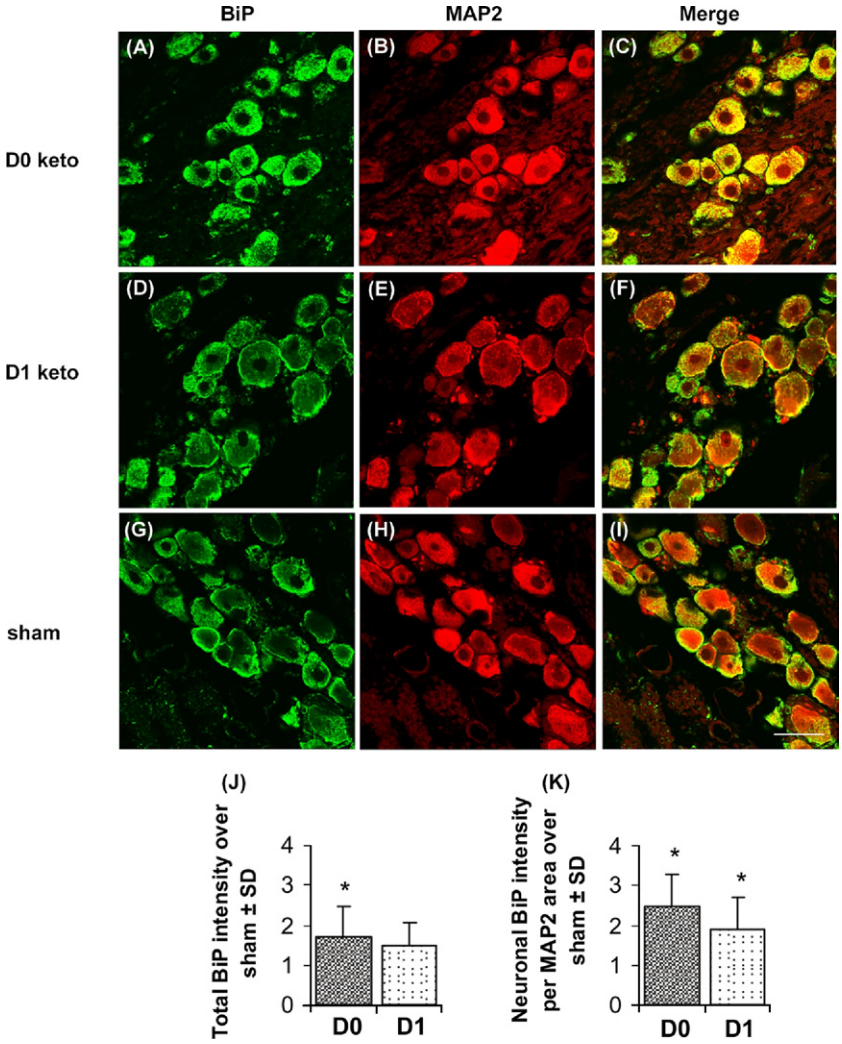

Fig. 4. BiP expression (green) in neurons (red; MAP2) of the DRG. BiP immunoreactivity was more prominently expressed in D0 ketorolac (A–C) compared to D1 ketorolac (D–F) and sham controls (G–I). Quantification of total BiP intensity (J) and neuronal BiP intensity normalized to MAP2 area (K) showed no significant difference between the two treatment groups. Asterisk (* $P<0.05$) sign indicates significant elevation over sham; data are presented as fold increase over sham. The scale bar (50 μm) applies to all panels. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

ated pathways of the ISR in the affected neurons of the C6/C7 facet joint. Induction of ATF4 in spinal neurons has been shown to play an important role in programmed cell death in a spinal cord ischemia model in the rabbit, and a number of *in vitro* studies found a direct relationship between upregulation of ATF4 and decreased neuronal and liver cell survival (Jousse et al., 2007; Magne et al., 2011; Ohoka et al., 2005; Yamauchi et al., 2007). Collectively, those studies point to the apoptotic characteristics of ATF4 activation. As such, findings from our study suggest that cell death in the DRG may be induced by prolonged activation of the ATF4 pathway of the integrated stress response after painful facet joint loading. However, ATF4 also activates several target genes of the ISR that promote cell survival (Harding et al., 2000, 2003; Lu et al., 2004), which highlights the need for future investigations to examine the specific cell fate for ATF4-positive neurons in this model. Previous studies with similar joint injury scenarios have shown that excessive stretch to this joint's ligament can cause microstructural damage and axonal dysfunction in the capsule, activate and injure the innervating nociceptors, and also induces neuronal hyperexcitability in the spinal cord (Chen et al., 2006; Kallakuri et al., 2008; Markowitz et al., 2007; Quinn et al., 2007, 2010). Given that the cell bodies of the afferents of the facet joint reside in the DRG, it is not surprising to find that noxious facet joint loading sufficient to produce sustained behavioral hypersensitivity is associated with cellular responses in the DRG that could potentially alter their normal functioning via protein misfolding or even cell death.

Significant upregulation of BiP expression was observed in the DRG previously in the same model after painful joint injury (Dong et al., 2008), suggesting that the ISR is activated in the injured afferents of the facet joint. Findings from the current study confirm such activation, with a significant increase in both total and neuronal BiP

expression after the D0 ketorolac treatment that failed to attenuate behavioral hypersensitivity (Figs. 2 and 4). Although treatment with ketorolac at day 1 (D1 ketorolac) did attenuate behavioral sensitivity and reduce it to nonpainful and sham levels (Fig. 2), the BiP expression at day 7 in that group was not different from that observed after D0 ketorolac treatment (Fig. 4). In contrast, ATF4 immunoreactivity was significantly reduced in that group (Fig. 3), suggesting that although ATF4 activation may have been blocked by the D0 ketorolac treatment, the cellular integrated stress response was still activated. Taken together with the observed maintenance of behavioral sensitivity in that group (Fig. 2), these data imply that a different arm of the ISR, one which is independent of ATF4, may be contributing to the maintenance of behavioral hypersensitivity. In an *in vitro* model mimicking endoplasmic reticulum stress in human chondrocytes, it was suggested that ATF4 is involved in joint diseases, such as osteoarthritis (Hamamura et al., 2009). However, results from current study indicate that ATF4 might not be directly responsible for the persistence of pain, despite being activated after painful joint loading. Since the downstream effectors of the ISR affect the broad aspects of cell fate (Lin et al., 2007; Pidoux and Armstrong, 1993), in addition to the ATF4 response, additional work is needed to also probe the expression of the other pathways of the ISR, the IRE1α and ATF6 pathways, in order to understand which of them may be responsible for the maintenance of pain. An *in vitro* study using human embryonic kidney cells found that the stress response mediated by the PERK pathway persisted even after 30 h after stress onset in culture, whereas IRE1 was quickly attenuated within 8 h with ATF6 attenuation being slightly delayed (Lin et al., 2007). Since each of these signaling pathways varies remarkably with time, more studies are necessary to delineate the time profile of all three pathways to identify the differential contributions of various aspects of ISR to facet-mediated pain.

Our observations support activation of the ISR in facet mediated injury; however, dampening of the ISR pathways specifically following insult or injury has not been investigated. Interestingly, ATF4 can be activated and repressed by mechanisms independent of the ISR. Enhanced translation of ATF4 may also occur when eIF2α is phosphorylated by PKR and general control nondepressible-2 (GCN2). In addition to double-stranded RNA, PKR can be activated by interferon (Wek et al., 2006), which could be relevant in this model since interferon has been shown to sensitize nociceptive neurons and contribute to the generation of joint pain (Cuellar et al., 2009; Li et al., in press; van Baarsen et al., 2010). GCN2 is activated under conditions of nutrient deprivation and is thought to induce apoptosis more readily than activation by PERK or PKR (Muaddi et al., 2010). Taking those findings in the literature together with the results of the current study suggests it would be useful to determine the activity of these kinases in facet-mediated pain following both painful and nonpainful distractions. Finally, the ISR may be active while ATF4 is downregulated. One can envision that global translation inhibition may occur in the absence of enhanced ATF4 translation when amino acids are scarce. In addition, there is evidence that ATF4 is downregulated at the mRNA level by TRIF-mediated signaling through toll-like receptors 3 and 4 (Woo et al., 2009). This latter hypothesis may explain our observation of BiP remaining elevated suggestive of maintained ISR activation, whereas ATF4 levels are no longer elevated after D0 ketorolac treatment.

Biomechanical and behavioral outcomes from this study not only support that transient whiplash-like loading to the capsular ligament can elicit persistent pain behaviors, but also further indicate that the biomechanical threshold for activation of cellular responses might actually be between 20 and 30% strain. Consistent with the biomechanical data reported here, studies using human cadavers to simulate whiplash loading reported strains ranging from 19 to 40% at accelerations of 3.5–8 g at C6/C7 capsule, while the physiologic range was less than 11% (Kaneoka et al., 1999; Panjabi et al., 1998; Pearson et al., 2004; Yoganandan et al., 2002). Similarly, nociceptors innervating the facet capsule were reported to be activated at strains above 11% (Lu et al., 2005; Yoganandan et al., 1998). An excessive stretching of facet capsule beyond its physiologic range can result in altered axonal morphology in the nerve fibers in the facet capsule (Kallakuri et al., 2008). With an imposed strain magnitude more than twice the physiologic tolerance, it can be inferred that axonal damage might also be occurring in this study. A number of *in vivo* and *in vitro* studies reported that axonal injury can trigger the protective mechanisms of ISR and lead to changes in membrane potential and transient depolarization (Cardozo et al., 2005; Galbraith et al., 1993; Kharroubi et al., 2004; Penas et al., in press). Taken together with the literature, findings from this study suggest that although ATF4 may not be directly responsible for the maintenance of pain, it may be a regulator for, or a potential contributor to, the enhanced neuronal excitability observed in the spinal cord (Quinn et al., 2010; Steiger et al., 2004). Certainly, additional work is necessary to investigate the relationship between ATF4 activation and neuronal excitation in the spinal cord.

CONCLUSIONS

The data presented here demonstrate that facet joint loading sufficient to induce heightened behavioral sensitivity can also trigger ATF4 activation in the DRG, but the persistence of pain may not be directly sustained by ATF4-dependent pathways of ISR. Although ketorolac treatment given at the time of joint injury did not attenuate behavioral sensitivity, it did reverse the ATF4 response in the DRG despite a lack of change in BiP expression. These findings suggest that pain may be maintained through other pathways of ISR, which mandates further investigation to fully outline the timing and extent of ISR activation that may contribute to the initiation and maintenance of pain. Nevertheless, results from this study suggest a role for cellular stress response in the persistence of facet-mediated pain.

Acknowledgments—This work was funded in part by grants from the National Institutes of Health/National Institute of Arthritis, Mus-

culoskeletal and Skin Diseases (AR056288) and the Catharine D. Sharpe Foundation.

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(Accepted 24 July 2011)
(Available online 28 July 2011)