Published in final edited form as:

Neuroscience. 2017 February 20; 343: 94–105. doi:10.1016/j.neuroscience.2016.11.040.

Loss of dopamine D1 receptors and diminished D1/5 receptor-mediated ERK phosphorylation in the periaqueductal gray after spinal cord lesion

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Abstract

Neuropathic pain resulting from spinal cord injury is often accompanied by maladaptive plasticity of the central nervous system, including the opioid receptor-rich periaqueductal gray (PAG). Evidence suggests that sensory signaling via the PAG is robustly modulated by dopamine D1- and D2-like receptors, but the effect of damage to the spinal cord on D1 and D2 receptor protein expression and function in the PAG has not been examined. Here we show that 21 days after a T10 or C6 spinothalamic tract lesion, both mice and rats display a remarkable decline in the expression of D1 receptors in the PAG, revealed by western blot analysis. These changes were associated with a significant reduction in hindpaw withdrawal thresholds in lesioned animals compared to sham-operated controls. We investigated the consequences of diminished D1 receptor levels by quantifying D1-like receptor-mediated phosphorylation of ERK1,2 and CREB, events that have been observed in numerous brain structures. In naïve animals, western blot analysis revealed that ERK1,2, but not CREB phosphorylation was significantly increased in the PAG by the D1-like agonist SKF 81297. Using immunohistochemistry, we found that SKF 81297 increased ERK1,2 phosphorylation in the PAG of sham animals. However, in lesioned animals, basal pERK1,2 levels

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Conflict of interest disclosure

The authors declare no financial, personal, or other conflicts of interest. All authors have approved the final version of this article.

were elevated and did not significantly increase after exposure to SKF 81297. Our findings provide support for the hypothesis that molecular adaptations resulting in a decrease in D1 receptor expression and signaling in the PAG are a consequence of SCL.

Keywords

Neuropathic pain; spinal cord injury; behavior; hyperalgesia; SKF 81297; extracellular signal regulated kinase

Neuropathic pain following spinal cord injury ranks as one of the most devastating of human conditions (Masri and Keller, 2012; Keller and Masri, 2013). One theory posits that spinal cord damage causes a loss of inhibitory circuits in the dorsal horn proximal to the injury site, contributing to development of hyperactive nociceptive signaling. This leads to a heightened sensitivity to pain (hyperalgesia) and mechanical hypersensitivity (allodynia) (Drew et al., 2004; Meisner et al., 2010) that appears to involve higher brain centers (Ossipov et al., 2014; Watson and Sandroni, 2016). Treatments deemed safe for relief of chronic neuropathic pain are not always effective for every patient. Unfortunately, development of viable therapeutics continues to be hindered by an incomplete understanding of the circuits and molecules involved in the expression of chronic neuropathic pain.

The rostral ventral medulla (RVM) together with structures in the pons and midbrain constitutes a robust descending antinociception system that suppresses ascending pain signals (Fields et al., 2006; Heinricher et al., 2009). Electrical stimulation of the opioid receptor-rich midbrain periaqueductal gray (PAG) has been shown to relieve pain in animals (Reynolds 1969) and humans (Richardson and Akil 1977; Hamani et al., 2006; Levy et al., 2010; Pereira et al., 2013). Moreover, functional connectivity between the spinal dorsal horn and the PAG is enhanced during painful stimuli (Sprenger et al., 2015). Also of relevance is the observation that deep brain stimulation targeting the PAG has achieved some success in alleviating neuropathic pain in humans (Rasche et al., 2006; Owen et al., 2007; Boccard et al., 2015; Hentall et al. 2016). The link between neuropathic pain and altered function of the PAG has been well-supported by studies employing diverse animal models of chronic neuropathic pain. For example, glutamatergic, serotonergic, purinergic, adrenergic, and cannabinoid receptors in the PAG have recently been identified as molecular targets susceptible to altered function in pain models that include spinal nerve ligation, chronic constriction injury, diabetes-induced neuropathy, and oxaliplatin-induced neuropathic pain (Palazzo et al., 2012; Baptista-de-Souza et al., 2014; Li et al., 2014; Guo et al., 2015; Ho et al., 2015). Opiate receptors within the ventrolateral aspect of the PAG provide powerful regulation of the output neurons that project to the RVM and spinal cord (Cheng et al., 1986; Bandler and Shipley 1994), but opiate analgesics alone do not effectively reduce spinal cord injury pain (Attal 2012; D'Angelo et al., 2013), suggesting that other receptors and signal transduction pathways may be involved in neuropathic pain.

While damage to the spinal cord will cause hundreds of changes in the nervous system that could contribute to hyperalgesia, the dopaminergic system regulates anhedonia and pain perception, and may hold promise for pain suppression therapies (Tracey and Mantyh, 2007). Extrinsic dopaminergic inputs from the ventral tegmental area and substantia nigra

pars compacta project to the PAG (Beckstead 1979; Simon et al., 1979). The A10dc dopaminergic neurons intrinsic to the ventral half of PAG project locally within the PAG, to the ventral striatum, and to the central nucleus of the amygdala (Hökfelt et al., 1976; Ottersen 1981; Hökfelt et al., 1984; Hasue and Shammah-Lagnado 2002; Dong and Swanson 2006). These A10dc neurons exhibit altered activity in rats after sciatic nerve constriction injury (Mor et al., 2015). In rodents, lesion of the large dopaminergic neurons in the ventral PAG attenuates opioid-mediated antinociception (Flores et al., 2004). In humans, decreased dopaminergic transmission has been associated with fibromyalgia (Wood et al., 2007) and neuropathic pain in Parkinson patients (Hanagasi et al., 2011).

Previous studies have implicated PAG D1- and D2-like receptors in the modulation of sensory responses (Flores et al., 2004; Meyer et al., 2009; Chiou et al., 2013). Elevated tyrosine hydroxylase in the PAG of rodents subjected to chronic constriction nerve injury implies an increase in dopamine production (Mor et al., 2015), yet injury-induced changes in dopamine receptor protein levels within the PAG has not been assessed. Furthermore, the effect of spinal cord injury on dopamine receptor expression and signal transduction in the PAG has not been investigated. To test the hypothesis that spinal cord injury-induced maladaptive plasticity involves a compensatory decrease in dopamine receptor expression in the PAG consequent to increased dopamine synthesis in the PAG, we used a rodent model of neuropathic pain (Wang and Thompson 2008; Masri et al., 2009) to examine the effect of localized electrolytic lesion of the spinal cord on dopamine receptor expression and signal transduction in the PAG. We found that dopamine D1 receptor protein levels and D1-mediated signal transduction are both significantly reduced after spinal cord lesion.

METHODS

Animals

Male Sprague-Dawley rats (n=23) weighing 250 – 300g (Harlan Laboratories, Frederick, MD) were used to determine the time course of protein expression by western blot, and to quantify D1 receptor mRNA levels. Male Long-Evans Fos-GFP rats (n=4) weighing 250 - 300g (a gift from Bruce Hope, National Institute of Drug Abuse) were used for immunohistochemical analysis. Male and female GAD65-GFP mice (n=6) weighing 25 – 30 g were used for D1 receptor protein expression analysis (obtained from Adam Puche, University of Maryland School of Medicine). Male and female D1 receptor-Cre mice (D1-Cre, n=8) were used for in vivo signal transduction experiments (obtained from Mary Kay Lobo, University of Maryland School of Medicine). The animals selected for use in this study were chosen not only for the basic foundational information we strove to obtain in this analysis, but also because we anticipated that the experimental design of studies to follow would benefit from the specific transgenic mouse and rat strains that we employed here. All animals were housed in climate-controlled facilities with a 12 h light/dark cycle and free access to food and water. Protocols for the care and use of the experimental animals were approved by the Institutional Animal Care and Use Committee, and conformed to the guidelines of the International Association for the Study of Pain and the principles set forth in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Surgery

SCL was performed in rats and mice according to previously published procedures, with minor changes (Wang and Thompson 2008; Cha 2013). Briefly, animals were anesthetized with either ketamine/xylazine (80/12 mg/kg i.p.) or isoflurane 1-3%. Respiratory rate, corneal reflex, and tail pinch responses were monitored to ensure sufficient anesthesia levels. Lidocaine (2%) was applied to incision sites prior to initiation of surgery. A midline, longitudinal incision exposed the vertebrae from C2 to T2 to create a lesion at C6 (Sprague Dawley rats), and an incision spanning T6 to L2 exposed the vertebrae to create a T10 lesion (Long-Evans rats, GAD-GFP mice, and D1-Cre mice). Lesions were made at the level of C6, as in our previous study (Jiang et al., 2014), with additional groups lesioned at T10 to determine whether molecular adaptations in PAG were dependent on level of the lesion. A laminectomy exposed the spinal cord, the dura mater was removed, and a platinum electrode (5 μ m tip) was inserted into the anterolateral quadrant of the right side of the spinal cord (2.1 mm lateral to the midline) to deliver DC current (10 μ A for 10 s, repeated 4 times, 0.8mm lateral from midline; depth, 1.7 mm from the surface of the spinal cord) to produce a lesion. In mice, the lesion was made by delivering 10 μ A electrical current, repeated 2 times. Following SCL surgery, the muscles were sutured and skin was closed using either sutures or wound clips. Animals were allowed to recover, with the analgesic Rimadyl (5 mg/kg) administered postoperatively every 24 h for 3 d.

Behavioral testing

We have previously shown that SCL produces a decrease in the mechanical withdrawal threshold in rats (Masri et al., 2009); in the current study we sought to confirm the behavioral response to SCL in mice. Animals were habituated for two weeks before behavioral testing was started. Mechanical sensitivity was evaluated by placing animals in Plexiglas boxes on an elevated wire mesh grid and stimulating the hindpaws with calibrated von Frey filaments (Stoelting, Wood Dale, IL). We used the up-down method to determine withdrawal thresholds (Dixon, 1965; Chaplan et al., 1994).

Baseline behavioral tests were conducted on three consecutive days before surgery. Sham and SCL animals underwent further behavioral testing 3, 7, 14, and 21 days after surgery.

Tissue collection and preparation of protein extracts

For D1 receptor protein quantitation, animals were euthanized and brains were rapidly dissected (< 1 min) and frozen in isopentane on dry ice at -50°C for 1 min. For in vivo analysis of pERK1,2, mice were administered either saline or 5 mg/kg (i.p.) of the D1/5 receptor agonist SKF 81297 (Sigma-Aldrich), and euthanized 15 min after injection. Mouse brains were then rapidly frozen in isopentane, as described above. Frozen brains were sectioned coronally (1 mm for rats and 0.5 mm for mice) using a stainless steel coronal brain matrix (Harvard Apparatus, Holliston, MA) and PAG was visually identified using an anatomical atlas as a reference (rat, Paxinos and Watson 2007; mouse, Paxinos and Franklin 2012). A 2 mm diameter tissue sample corer (AgnTho's, Lidings, Sweden) was used to punch and collect tissues from the PAG area. The punched tissues were then stored at -80°C pending isolation of protein.

The approach for tissue processing was based on a previously published method (Voulalas et al., 2011). PAG tissue samples were homogenized in ice-cold buffer comprised of 10 mM HEPES pH7.4, 0.32 M sucrose, 2 mM EDTA, Complete Mini protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN), and phosphatase inhibitors (5 mM NaF, 1 mM NaVO₄) using 18-20 strokes in a Potter-Elvehjem homogenizer. The homogenate was first centrifuged at 1000 × g for 10 min to pellet nuclei and cellular debris, and the supernatant was then centrifuged at 100,000 × g for 45 min at 4 °C to sediment cellular membranes. The supernatant was collected and reserved as a crude cytosolic fraction. The crude membrane pellets were resuspended in a buffer comprised of 25 mM HEPES pH 7.4, 2 mM EDTA, Complete Mini protease inhibitors, and phosphatase inhibitors (see above). Since D1 receptors are highly enriched in detergent-resistant membranes (DRM; Voulalas et al., 2011), crude membranes were further separated into detergent resistant and detergent soluble membrane (DSM) fractions by addition of Triton X-100 (final concentration 1% v/v) overnight at 4 °C, followed by centrifugation at 100,000 × g at 4 °C for 45 minutes. Protein concentration was determined using the Pierce BCA protein assay kit (Thermo Fisher, Waltham, MA).

Western blot analysis

Equal amounts of protein from either the cytosolic (CREB and ERK analysis) or detergent-resistant membrane (D1 receptor) fraction were loaded and separated on NuPage® 4 – 12% Bis-Tris gels with MES running buffer (Invitrogen, Carlsbad, CA). Gels were transferred to nitrocellulose membranes at 30 V for 16 h at 4 °C. Membranes were stained post-transfer with 2% Ponceau S to confirm even lane loading, then blocked with 10% dry milk/10 mM Tris, 150 mM NaCl, 1% Tween 20 (TBST) and probed with primary antibody in 5% milk/TBST. Primary antibodies used in this study included: D1 dopamine receptor (rabbit polyclonal sc-14001, Santa Cruz Biotechnology, Dallas, TX), Thr²⁰²/Tyr²⁰⁴ p44/42 pERK1,2 (mouse monoclonal 9106S, Cell Signaling Technology, Danvers, MA), ERK (rabbit polyclonal sc-93, Santa Cruz Biotechnology), β-actin (mouse monoclonal A-5316, Sigma-Aldrich, St. Louis, MO), β3-tubulin (T-8660, Sigma-Aldrich). Three different Ser¹³³ pCREB antibodies were screened for use in western analysis: rabbit monoclonal (9198S, Cell Signaling Technology), rabbit polyclonal (06-519, EMD Millipore lot from 2016 and Upstate Biotechnology lot from 1999); the Upstate antibody was selected for quantification. Total CREB was quantified with rabbit monoclonal 4820S (Cell Signaling Technology). Secondary antibodies used were horseradish peroxidase (HRP)-conjugated goat anti-rabbit (7074, Cell Signaling Technology) and goat anti-mouse (04-18-15, KPL, Gaithersburg, MD). Pierce West Pico and West Femto Chemiluminescent substrates were used to visualize immunoreactivity. Chemiluminescent images were obtained with Fuji Super RX-N film, and subsequently scanned and quantified by densitometry using Image Studio Lite (v. 3.1, LI-COR Biosciences, Lincoln, NE).

Acute slice stimulation for immunohistochemical analysis

For this portion of the study, we used Long-Evans Fos-GFP rats. A Leica VT1000S was used to prepare coronal rat brain slices from sham and SCL animals 21 days after surgery. The brain was blocked and mounted with the rostral end glued to the stage, submerged in an iced-cold slurry of artificial cerebrospinal fluid (ACSF). By blocking the brain prior to

cutting slices, followed by additional trimming of the tissue surrounding the PAG after mounting for slicing, we were able to prepare slices consisting primarily of the PAG. Consequently, exposure to agonist would be limited to this region. Slices (300 μ m) were collected beginning at approximately -8.52 mm from bregma (Paxinos and Watson, 5th ed.). The low-calcium ACSF for slice preparation contained (in mM) 124 NaCl, 1.75 KCl, 0.63 CaCl₂, 1.3 MgSO₄, 1.25 NaH₂PO₄, 26 NaHCO₃, 10 glucose, and osmolarity was adjusted to 300 – 310 mOsm with sucrose. ACSF was aerated with 95% O₂/5% CO₂ to achieve a pH of 7.2 – 7.3. Freshly cut slices were transferred immediately to a modified interface chamber where they were perfused at 3 mL/min with 95% O₂/5% CO₂-balanced ACSF containing 2.4 mM CaCl₂. This chamber had two separate stages, with two separate perfusion inputs, which enabled simultaneous incubation and stimulation of slices from sham and SCL animals. Slices were allowed to equilibrate for 2 h at 34 °C. The rostral-caudal location of each slice was noted, and sequential, alternating slices were designated for either no treatment (control) or stimulation with the D1/5 receptor agonist SKF 81297. Following the 2 h period of equilibration, control slices were removed, and initiation of perfusion with SKF 81297 commenced. Slices were exposed to agonist for 15 min. All slices were then rinsed with PBS, fixed in 4% paraformaldehyde at 4 °C overnight and transferred to 30% sucrose/PBS overnight, where they remained until thin sections were cut.

Immunohistochemistry

Slices were organized into rostral and caudal PAG areas based on the distance from bregma (-6.00 to 7.20 and 7.56 to 8.52 , respectively) (Paxinos and Watson, 2005). Slices were mounted in OCT and thin sections (40 μ m) containing PAG were cut using a Leica Cryostat. Sections were blocked for 2 h with 10% normal donkey serum (NDS) in PBS with 0.1% Triton X-100, followed by incubation with a pERK1,2 antibody (mouse monoclonal, 1:30,000) in 3% NDS/PBS/TX for 48-72 h at 4 °C. Following extensive washing, sections were incubated with secondary antibody (biotinylated goat anti-mouse, 1:500, Jackson ImmunoResearch, West Grove, PA) for 1 h. Immunoreactivity was visualized using the Elite ABC kit (PK-6100, Vector Laboratories, Burlingame, CA) and 3,3'-diaminobenzidine-peroxidase substrate with nickel acetate to create a black reaction product. Sections were mounted on slides and cover slipped.

We used unbiased methods and the NeuroLucida software to count total number of neurons expressing pERK. Slides were coded, with the code revealed only after all data was blindly collected and analyzed. Because the ventrolateral PAG plays a predominant role in mediating descending inhibition to suppress painful sensations, we created a counting region where the caudal aspect of the ventrolateral regions was enriched (see Introduction); only those sections coming from slices derived from the caudal half of the PAG were included in the analysis. The entire PAG was encircled, and divided into 4 segments using a cross hair that passed through the center of the aqueduct. All cells with a soma > 10 μ m in diameter that were located within the lower half of the encircled caudal PAG were counted. According to Mantyh (1982), this characteristic describes the five major non-glial cell types in the PAG. Quantitative analysis involved calculating the percent change in cells positive for pERK1,2 in unstimulated versus SKF-stimulated slices. The control (sham surgery) and experimental (SCL) groups were analyzed independently.

Statistical analysis

In experiments where D1 receptor protein levels in Sham rats were compared to injured rats 7 and 21 days post-SCL, western blot data satisfied the assumptions of parametric tests and were analyzed using ANOVA followed by the Tukey-Kramer test for post-hoc analysis. In experiments investigating D1 receptor expression in mice at 21 days post-lesion and pERK1,2 levels following in vivo administration of SKF 81297, data also satisfied the assumptions of parametric tests; in these cases, the two-tailed t-test was used for analysis. Immunohistochemical data demonstrated considerable variability; therefore, the Mann-Whitney U test was selected as the test of choice for analysis to examine differences in the medians between groups. Changes in mechanical withdrawal thresholds did not satisfy the assumptions of parametric tests and therefore analysis was conducted using one-way ANOVA on ranks followed by Dunnett's test. Data were reported as mean $\pm$ SEM. Outlier analysis for all data was performed using the single Grubbs' test. The significance level for all tests was set at $p < 0.05$ (GraphPad Prism, 5.0).

Results

D1 receptor protein levels are reduced within the PAG after SCL

To gain insight into dopamine-mediated mechanisms underlying maladaptive signaling in the PAG-brainstem descending pain inhibitory system, we used western blot analysis to examine dopamine receptor expression in the PAG following electrolytic lesion of the spinal cord at C6. We commenced this analysis by first examining basal expression levels of D1, D2, and a target of D1 receptor activation, ERK1,2, in the PAG. We also determined the cellular fraction where these proteins were most abundant. Just as was seen in the striatum and cortex, D1 receptors were highly enriched in DRM (detergent-resistant membranes, Voulalas et al., 2011). ERK1,2 protein was enriched in the cytoplasmic fraction (Fig. 1A). In contrast to cortex and striatum, D2 receptor was undetectable in any PAG fraction, using the same antibody used to detect this protein in other brain regions (Voulalas et al., 2011). Actin was most abundant in the cytoplasmic fraction, less so in DRM, and present in limited amounts in the DSM (detergent-soluble membranes, Fig. 1A). With this information in hand, we focused on the D1 receptor population in the DRM, and ERK1,2 in the cytoplasm.

D1 receptor protein expression was significantly higher in the PAG of sham rats compared to lesioned rats 7 and 21 days after surgery, as seen in the representative example shown in Fig. 1B. Semi-quantitative analysis revealed that the decrease in D1 receptor expression was significant seven days after the lesion, remaining persistently diminished 21 days after lesion, compared to sham-operated rats (Fig. 1C). The striking reduction in D1 receptor protein expression at 21 days ($32.7 \pm 3.7\%$ of Sham control) was associated with significant changes in behavioral indicators of hypersensitivity in rats observed after SCL, measured at the same time points as we have shown previously (Masri et al., 2009; Quiton et al., 2010; Seminowicz et al., 2012; Jiang et al., 2016).

At this point, we hypothesized that the dramatic diminution of D1 receptor protein reflected a response that would transcend species and strain. This was important to demonstrate, since future extensions of this study would involve the use of transgenic mice and rats. To test this

idea, we first examined whether a T10 SCL would also lead to a reduction in mechanical withdrawal threshold and D1 receptor protein levels in mice. SCL in mice caused a decrease in withdrawal threshold that was significant at 14 days and persisted beyond 21 days (Fig. 2A). In rats, these behavioral indicators of hypersensitivity have been observed for at least 42 days after SCL (Masri et al., 2009).

Similar to our results in rats, we found that mice with a SCL at T10 exhibited a decrease in D1 receptor protein in the PAG 21 days after surgery, compared to sham mice (representative example shown in Fig. 2B). Semi-quantitative analysis revealed a significant reduction in D1 receptor levels in the injured mice compared to the shams 21 days after lesion (Fig 2C). Thus, regardless of the species tested or the level of the lesion, SCL animals showed a significantly lower level of D1 receptor protein in the PAG compared to sham animals. The reduction of D1 protein was associated with the development of hypersensitivity 21 days after SCL (Masri et al., 2009).

One feature of D1 receptor protein expression that we noted was the highly variable levels in sham animals. Many confounding factors were considered to explain this variability, including sex of the animals, time of day of sacrifice, and day of sacrifice (14 $\pm$ 7 days) post surgery. However, we could not reliably identify a contributing factor to explain the variability in D1 protein expression. We did not detect a significant decrease in D1 receptor mRNA expression in SCL rats compared to sham animals, despite the significant decreases in D1 protein levels following SCL (Sham, 1.45 ± 0.06 vs. SCL, 1.18 ± 0.38 , $p = 0.53$, Student's t-test).

Lesion-induced reduction of D1/5 dopamine receptor-mediated signal transduction in the PAG

The significant decrease in D1 receptor raised the possibility that cellular functions in the PAG mediated by dopamine would also be diminished after SCL. Since coupling of ERK and CREB phosphorylation to D1 receptor activation has been well established in other brain regions (Brami-Cherrier et al., 2002; Acquas 2007; Papadeas et al., 2008; Pascoli et al., 2012), and as ERK1,2 is phosphorylated in the PAG in response to diverse stimuli (Gioia et al., 2005; Macey et al., 2009; Nasser and McNally, 2013; Sanna et al., 2014; Macey et al., 2015), we selected pERK1,2 and pCREB as markers to assess D1 receptor-mediated signal transduction. As a first step toward addressing this point, we used an in vivo approach to investigate the effect of administration of SKF 81297, a D1/5 receptor agonist, on ERK1,2 and CREB phosphorylation in the PAG of naïve mice. We found that pERK1,2 levels in tissue punches from the PAG of SKF 81297-treated animals were elevated 15 min after exposure to agonist, compared to pERK1,2 levels in saline-treated mice (Fig. 3A). Quantification of this change confirmed that pERK1,2 levels significantly increased 15 min after exposure to agonist, to nearly twice the basal level in unstimulated animals (Fig. 3B); pERK levels 30 min after exposure to agonist were not different from saline-treated animals (Fig. 3C, D). pCREB levels were unchanged 15 and 30 min after stimulation, compared to saline-treated animals (Sal 15, 0.57 ± 0.05 vs. SKF 15, 0.49 ± 0.07 : $t(12) = |4.54|$, $p = 0.47$; Sal 30, 0.56 ± 0.07 vs. SKF 30, 0.59 ± 0.09 : $t(12) = |2.13|$, $p = 0.84$). While this approach did not limit receptor activation to the PAG, it is the first demonstration that in vivo exposure

of an animal to a dopamine agonist can increase pERK1,2 levels in the PAG. More importantly, it validated our use of pERK1,2 as a reporter for D1/5 receptor activity in the PAG. This provided us with an essential tool with which we could advance our study of D1 receptor in PAG after SCL.

Based on these findings, we hypothesized that direct application of SKF 81297 to PAG slices would increase pERK1,2 levels in the ventral PAG. Furthermore, due to the significant diminution of D1 receptor after SCL (Fig. 1), we predicted that the response to agonist would be blunted in lesioned animals compared to shams. For these experiments, we used Fos-GFP transgenic rats because future studies were planned to include a time course analysis and characterization of cells within the PAG that were activated following SCL. In support of our hypothesis and the results from in vivo exposure, immunohistochemical analysis revealed that SKF 81297 could significantly elevate pERK1,2 levels in the ventral PAG of sham-operated animals to more than 1.5 fold over the basal level in slices derived from unstimulated shams (Fig. 4 and Table 1). The basal level of pERK1,2 in slices from SCL mice appeared higher than basal levels in sham mice (Table 1). Strikingly, the number of pERK1,2 immunoreactive cells in slices from SCL mice was not significantly increased by SKF 81297. Considered together, our results suggest that activation of D1/5 receptors in the PAG results in an increase in ERK1,2 phosphorylation, and that this cellular signaling pathway may be uncoupled in animals with reduced D1 receptor in the PAG following SCL.

Discussion

In this study, we used a rodent model of neuropathic pain in which lesion of the anterolateral quadrant of the spinal cord produces behavioral indicators of hyperalgesia, allodynia, and ongoing pain (Davoody et al., 2011). Using standard methods of protein analysis we have shown that SCL is associated with a reduction of D1 receptor protein in the ventral PAG. Furthermore, using both in vivo and in vitro approaches, we provide evidence for the consequences of D1 receptor reduction by showing that diminished D1 receptor is accompanied by diminished D1/5 receptor-mediated ERK phosphorylation in the PAG.

Dopamine receptors in the PAG

A recent study in support of dopamine (and norepinephrine) contributing to the behavioral changes observed in a rodent model of neuropathic pain comes from the work of Mor et al. (2015). This group found significant increases in tyrosine hydroxylase synthesis in the PAG A10dc dopaminergic cell group less than one week after chronic nerve constriction injury. Importantly, the long-term effects of persistently elevated synaptic dopamine on dopamine receptor expression and function is not easily predicted. Furthermore, pharmacological and physiological studies have indicated a role for D1 and D2 receptors in sensory processing in the PAG, yet there has been no consensus as to whether the D1-like (D1/5) or D2-like (D2/3/4) receptors predominate (Flores et al., 2004; Meyer et al., 2009). In terms of basal levels, D2 receptor expression in the PAG has been demonstrated using in situ hybridization and autoradiographic D2 receptor antagonist binding (Wamsley et al., 1989; Martin-Ruiz et al., 2001), but expression of D1 receptors within the PAG has primarily been inferred by the pattern of Cre and EGFP expression in transgenic mouse lines (The GENSAT Project: D1-

Cre mouse lines FK150, EY217, EY262, EY266, and EGFP). In our initial approach, we intended to quantify expression of both D1 and D2 receptors, but since we could not reliably detect D2 receptors via western blot, despite employing the same methodological approach used to detect D2 receptors in the cortex and striatum (Voulalas et al., 2011), we focused initially on D1 expression. Our results from two species and multiple strains of animals indicate that D1 receptors are highly expressed in the PAG relative to D2 receptors. This suggests that elevated dopamine in the PAG may preferentially promote D1-mediated cellular signaling events (Beaulieu and Gainetdinov, 2011).

The decrease in D1 receptor protein expression 7 and 21 days after SCL was associated with a reduction in mechanical sensitivity, reflected in hindlimb mechanical withdrawal thresholds using von Frey filaments in both rats (Quiton et al., 2010; Seminowicz et al., 2012; Jiang et al., 2016) and mice (Fig. 2A). The decrease in D1 receptor at 21d also correlated with an increase in conditioned place aversion 21d after spinal cord lesion in rats (Davoody et al., 2011). The observation that D1 receptors in sham animals showed high animal-to-animal variability was a notable feature of D1 protein expression. Nevertheless, the dramatic decrease in D1 receptor protein levels was not anticipated, and necessitated further examination in order to determine whether such a significant reduction would translate into changes in D1 signaling, or whether alternative cellular mechanisms would compensate for this decrease.

One of the limitations of our analysis was that we did not characterize the cell-type expression pattern of D1 receptors in the PAG, neither did we map changes in the rostral-caudal pattern of D1 expression throughout the PAG following SCL. The neuronal population within the periaqueductal gray is very diverse, and the types of neurons expressing the D1 receptors that are involved in modulation of sensory responses will be characterized in future studies.

Dopamine receptor function/signal transduction in the PAG

Signal transduction driven by D1 receptors has not yet been examined in the PAG. Studies in numerous brain regions and cell types have established that D1 receptor activation is coupled to increased phosphorylation of a number of targets, including ERK1,2 and CREB (Gerfen et al., 2002; Voulalas et al., 2005; Beaulieu and Gainetdinov 2011; Xue et al., 2015), which can occur either directly or indirectly via D1 receptor-mediated activation of PKA (Rangel-Barajas, 2015). Our first approach was to determine whether in vivo exposure to the D1/5 agonist SKF 81297 (Gainetdinov et al., 2003; Lapointe and Guertin 2008; Ott et al., 2014; Lindenbach et al., 2015) would cause an increase in ERK or CREB phosphorylation in the PAG. This methodology has been employed in many studies examining the effect of various agonists and antagonists on cell function in discrete brain regions (Gangarossa and Valient, 2012; Brehm et al., 2015). Using tissue punches taken from the PAG, we detected a rapid and significant increase in pERK1,2, but not pCREB, in response to systemic administration of a D1/5 receptor agonist. Examples of previous studies relevant to our study have shown that (1) activation of D1 receptors by SKF 81297 can increase pERK (Gangarossa et al., 2012; Gangarossa et al., 2013), (2) that the increase in pERK by SKF 81297 can be blocked by the D1 receptor antagonist SCH23390 (Gangarossa et al., 2011), and (3) that the SKF

81297-mediated increase in pERK is PKA-dependent (Florentini et al., 2011; Nagai et al., 2016). To be clear, ERK1,2 is believed to be activated indirectly by PKA through other mechanisms, including Ras/Raf/Rap or the cAMP-dependent, PKA-independent exchange protein Epac (Kawasaki et al., 1998; Gelinis et al., 2008). Taken together, our results suggest that in vivo, ERK is functionally coupled to D1 receptor activation. Future studies will examine PKA-dependence of this event or if other mechanisms are involved.

While CREB is often used as a reliable marker of activity of the D1 receptor/adenylyl cyclase/cAMP/PKA signaling cascade, CREB is not the only molecule activated by this pathway (reviewed in Beaulieu and Gainetdinov 2011; Nishi et al. 2011). Still, our negative finding (no significant increase in pCREB following exposure to D1 receptor agonist) was surprising, but could be explained in a few ways. First, it may reflect the fact that our experimental design was optimal for detecting changes in pERK. For example, Liu and Graybiel (1998) used SKF 81297 to activate pCREB in rat brain, and found that the time of peak pCREB levels varied between brain regions. Second, our experimental paradigm could have led to a preferential increase in ERK phosphorylation; a similar, differential response of CREB and ERK to stimulation of the PAG has been previously reported (Hao et al., 2011). Finally, signal transduction via G protein-coupled receptors is very complex. In recent years, functional receptor heterodimerization has been established for a number of receptors, including the D1 receptor function. Multimerization of D1 receptors with other G protein-coupled receptors can alter D1 signaling properties, specifically with regard to ERK1,2 activation (Ferrada et al. 2009; Moreno et al. 2011; Guitart et al. 2014). Thus, a thorough analysis of CREB activation in the PAG in response to D1 agonist, in intact and SCL animals, would be of great interest for future studies.

An obvious limitation to in vivo, systemic administration of a D1/5 agonist is that D1 receptors residing outside of the PAG could be driving activity within the PAG. Therefore, the use of acutely prepared slices containing the PAG helped us to address this particular technical limitation. With the slice preparation, we intentionally focused our attention on the caudal aspect of the PAG, a region that constitutes the ventral, ventrolateral, and a fraction of the lateral PAG. The rationale for selecting this area for our analysis was that the ventrolateral PAG has been well established as the locus for opioid-mediated analgesia (Moreau and Fields, 1986; Helmstetter and Landeira-Fernandez, 1990; Williams et al., 1995). In contrast, the physiological functions attributed to the rostral PAG (dorsal and lateral) involve cardiovascular regulation and non-opioid analgesia (Linnman et al., 2012). In support of our findings in vivo, we observed an increase in ERK1,2 phosphorylation within 15 minutes of agonist exposure in vitro. Additional experiments are required to determine whether the increase in pERK1,2 represents a direct coupling of the D1 receptor signaling cascade to this kinase or an indirect effect of D1 activation. Nevertheless, the fact that in acute slices the same agonist significantly elevated pERK1,2 suggests that the circuits regulating D1/5 receptor-mediated ERK phosphorylation in the PAG are intact in this reduced preparation. It also lends support to the idea that D1 receptors located proximally to the PAG are involved in activation of ERK1,2.

We observed that the decrease in D1 receptors in the PAG following SCL was associated with a significant blunting of D1/5-mediated signaling in this region. While this could

involve a functional uncoupling of D1 receptor from its signaling cascades, it may simply reflect the fact that fewer D1 receptors are available for activation by agonist. Also of interest was the apparent increase in basal pERK1,2 in the PAG of SCL animals. This could also explain the lack of a response to D1 receptor agonist after SCL, due to a reduced pool of ERK1,2 available for phosphorylation, leading to a “ceiling effect.” These questions also warrant additional investigation in future studies.

Role of dopamine receptors in the modulation of sensory responses

Based on the current literature, we hypothesize that the consequence of reduced D1 receptor activity in the PAG is a significant impact on opioid-mediated nociception. Early reports demonstrated that 6-OHDA-mediated depletion of dopaminergic neurons in the PAG reduced morphine's analgesic effects (Deakin and Dostrovsky, 1978). More recently, Flores et al. (2004) demonstrated that not only 6-OHDA lesion but also infusion of a D1 receptor antagonist into the PAG significantly decreased opioid-mediated analgesia. It should be noted that there have been no studies to date where administration of D1 antagonist directly into the PAG causes hypersensitivity in naïve animals. Since the SCL-induced changes in D1 receptor levels is certainly not occurring without concurrent changes in other signaling systems, we would not predict that application of D1 receptor antagonist alone to the PAG would result in hypersensitivity. In other words, a decrease in D1 receptor protein expression in the PAG contributes to neuropathic pain mechanisms after injury, but cannot alone be responsible for causing this debilitating condition. The study by Meyer et al. (2009) further illuminated the contribution of dopaminergic signaling to antinociception when they found that systemic exposure to the dopamine receptor antagonist α -flupenthixol attenuated the analgesic effects of intra-PAG injection of morphine. In the same study, the intra-PAG injection of the non-selective dopamine receptor agonist apomorphine elicited a strong antinociceptive response that was blocked by a D2, but not D1 receptor antagonist. In the in vitro arm of this study, they revealed that the action of dopamine in the PAG mirrored that of met-enkephalin, namely inhibition of evoked inhibitory post-synaptic currents (IPSCs). Moreover, they established that α -flupenthixol blocked the reduction of IPSCs by dopamine, but not met-enkephalin, which supported the notion that the modulatory effect of dopamine occurred either in parallel with, or subsequent to inhibition of GABA release by opioids. Finally, the frequent occurrence of pain in Parkinson's patients (Hanagasi et al., 2011; Ha and Jankovic, 2012; Sophie and Ford, 2012), and the ability of some antidepressants that inhibit dopamine reuptake to provide relief from chronic pain (Semenchuk et al., 2001; Katz et al., 2005; Sindrup et al., 2005; Shah and Moradimehr, 2010; Hache et al., 2011; Hache et al., 2015) lend additional credence to our hypothesis that deficits of dopaminergic signaling underlie neuropathic pain following spinal cord injury. For example, the serotonin and norepinephrine reuptake inhibitor amitriptyline is one of the few pharmacological agents that have shown promise for reducing spinal cord injury pain and post-stroke neuropathic pain. Of relevance to our findings is that amitriptyline upregulates D1 receptor expression (Lammers et al., 2000; Huzarska et al., 2006), dopamine receptor signaling pathways (Bohm et al., 2006; Lauterbach 2012), spares dopaminergic neurons (Paumier et al., 2015), and increases dopamine levels (Dimatteo et al., 2000; Vlainich et al., 2011).

Conclusion

In summary, we have found that electrolytic lesion of the anterior lateral quadrant of the spinal cord is accompanied by the development of hypersensitivity, a robust reduction in D1 receptor protein levels, and abnormal D1/5 signaling in the PAG. Considering the precedent for functional interactions between dopaminergic signaling and opioid signaling in the PAG, our data suggest that the decrease in D1 receptors in the PAG may contribute to the pathology that follows damage to the spinal cord.

Acknowledgements

This project was supported by the National Institute of Neurological Disorders and Stroke Research Grant R01-NS069568 to R.M. We thank Bruce Hope, Mary Kay Lobo, and Adam Puche for transgenic animals. We are grateful to the members of the laboratory of Asaf Keller for insightful discussions.

Abbreviations used

DRM	Detergent-resistant membranes
DSM	Detergent-soluble membranes
ERK1,2	Extracellular signal-regulated protein kinases 1 and 2
pERK1,2	Phosphorylated ERK 1 and 2
i.p.	Intraperitoneal
IPSC	Inhibitory post-synaptic potential
PAG	Periaqueductal grey
PKA	Protein kinase A
SCL	Spinal cord lesion

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Figure 1.

D1 dopamine receptor protein levels are reduced in the PAG of rats following SCL. A, Basal expression and cellular distribution of D1 and D2 receptors, ERK1,2 and actin in the PAG. Cyto = cytoplasm, DSM = detergent soluble membranes, and DRM = detergent resistant membranes. B, Expression of D1 receptors in the PAG of sham and SCL animals 7 and 21 days after surgery, detected with a D1-specific antibody. C, Quantitation of D1 receptor protein indicates that, compared to sham animals, D1 levels are significantly reduced 7 days after SCL, and remain at low levels through 21 days after injury ($F(2, 20) = 11.99$, $p = 0.0004$, ANOVA with Tukey-Kramer post hoc test). (*) indicates significant difference relative to sham surgery. The number of experimental animals per group is indicated in the graph.

Figure 2.

The SCL-induced decreases in withdrawal threshold and D1 receptor protein are species-independent. A, Mechanical withdrawal thresholds progressively decline over time following SCL in mice. B, Western blot analysis showing D1 receptor protein levels in three Sham and three SCL animals, compared to β -actin levels. C, Quantification of D1 receptor protein levels in Sham and SCL animals as a function of β -actin levels reveals a significant reduction in D1 receptors after SCL ($t(4) = |3.02|$, $p = 0.039$, two-tailed t-test). (*) indicates

significant difference relative to sham surgery. The number of animals per condition is indicated in the graph.

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Figure 3.

Elevation of ERK phosphorylation in the PAG following D1/5 receptor stimulation. A, Systemic exposure of mice to SKF 81297 elicits an increase in pERK1,2 levels in the PAG 15 min after i.p. injection. B, Quantitation of pERK1,2 reveals a significant enhancement of ERK activation in response to agonist ($t(13) = |2.44|$, $p = 0.03$). C, ERK phosphorylation is transient; D, pERK levels were not significantly different 30 min after SKF 81297 administration ($t(12) = |2.29|$, $p = 0.93$). Data were analyzed using the two-tailed t-test with

(*) indicating significant difference relative to saline-injected controls. The number of experimental animals per condition is indicated in the graphs.

Figure 4.

Coupling of ERK phosphorylation to D1/5 receptor stimulation in the PAG is impaired in animals with SCL. A, In PAG slices derived from sham animals, the number of pERK1,2-positive cells is low compared to slices from sham animals perfused with SKF 81297 (B), where the number of pERK1,2 immunoreactive cells increases significantly 15 min after stimulation. By contrast, the number of pERK1,2-positive cells in unstimulated PAG slices derived from SCL animals (C) is not significantly different from pERK1,2 levels in PAG

slices from SCL that were exposed to SKF 81297 for 15 min. Scale bar for A-D, 400 μm ; A1-D1, 100 μm ; A2-D2, 50 μm . Statistical information is presented in Table 1.

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Table 1

Quantification of pERK1,2 immunoreactive cells in PAG slices from Sham and spinal cord lesioned (SCL) rats, in the absence and presence of SKF 81297.

	n	Median	U	U'	P
Sham Control	4	33			
Sham SKF 81297	6	54	2.5	21.5	0.043
SCL Control	6	51			
SCL SKF 81297	6	53	17	19	0.872

* indicates significant difference relative to unstimulated controls; Mann-Whitney U test