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GABA-A RECEPTOR ACTIVITY IN THE NORADRENERGIC LOCUS COERULEUS DRIVES TRIGEMINAL NEUROPATHIC PAIN IN THE RAT; CONTRIBUTION OF NA α 1 RECEPTORS IN THE MEDIAL PREFRONTAL CORTEX

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Abstract

Trigeminal neuropathic pain is described as constant excruciating facial pain. The study goal was to investigate the role of nucleus locus coeruleus (LC) in a model of chronic orofacial neuropathic pain (CCI-ION). The study examines LC's relationship to both the medullary dorsal horn receiving trigeminal nerve sensory innervation and the medial prefrontal cortex (mPFC). LC is a major source of CNS noradrenaline (NA) and a primary nucleus involved in pain modulation. Although descending inhibition of acute pain by LC is well established, contribution of the LC to facilitation of chronic neuropathic pain is also reported. In the present study, a rat orofacial pain model of trigeminal neuropathy was induced by chronic constrictive injury of the infraorbital nerve (CCI-ION). Orofacial neuropathic pain was indicated by development of whisker pad mechanical hypersensitivity. Hypersensitivity was alleviated by selective elimination of NA neurons, including LC (A6 cell group), with the neurotoxin anti-dopamine- β -hydroxylase saporin (anti-D β H-saporin) microinjected either intracerebroventricularly (i.c.v.) or into trigeminal spinal nucleus caudalis (spVc). The GABA_A receptor antagonist, bicuculline, administered directly into LC (week 8) inhibited hypersensitivity. This indicates a valence shift in which increased GABA_A signaling ongoing in LC after trigeminal nerve injury paradoxically produces excitatory facilitation of the chronic pain state. Microinjection of NA α 1 receptor antagonist, benoxathian, into mPFC attenuated whisker pad hypersensitivity, while NA α 2 receptor antagonist, idazoxan, was ineffective. Thus, GABA_A-mediated activation of NA neurons during CCI-ION can facilitate hypersensitivity through NA α 1 receptors in the mPFC. These data indicate LC is a chronic pain generator. Published by Elsevier Ltd on behalf of IBRO.

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CONFLICTS OF INTEREST

The Authors declare no conflicts of interest.

Keywords

dopamine-beta-hydroxylase; anti-D β H-saporin; mechanical allodynia; spinal trigeminal caudalis; CCI-ION; chronic orofacial neuropathic pain

INTRODUCTION

The pontine locus coeruleus (LC) nucleus is a major source of norepinephrine/noradrenaline (NA) in the central nervous system and a well-known mediator of descending inhibition of pain. The highly divergent efferent axonal projections of the LC innervate all levels of the neuraxis with an extensive network of ascending and descending projections to accentuate specific responses (Grzanna and Molliver, 1980; Westlund and Coulter, 1980; Westlund et al., 1981, 1982, 1983; Mantz et al., 1988; Aston-Jones et al., 2004; Gompf et al., 2010; Chandler and Waterhouse, 2012; Eschenko et al., 2012). A major NA efferent pathway from the LC innervates the medial prefrontal cortex (mPFC). This circuit optimizes behaviorally relevant, cognitive functions (Aston-Jones and Cohen, 2005; Marzo et al., 2014). For example, salient internal or external events can alter function or “reset” large-scale neural populations. This can be mediated by the targeted release of NA in the mPFC and can then shift the excitatory/inhibitory balance of the mPFC to a more excitable state. Therefore, we hypothesized that continuous activation within the NA LC-mPFC circuit provided by a chronic nerve injury model could shift pain modulation from inhibition to facilitation. To test this, we evaluated neuropathic pain behavior after either: (1) destruction of NA neurons in the LC; or (2) administration of α -adrenergic antagonists into the mPFC. Elimination of ascending and descending NA input was tested, as was the effect of NA α 1 and NA α 2 receptor activation.

Modulation of nociceptive transmission and pain perception are influenced by direct NA projections to trigeminal and spinal cord dorsal horn neurons. Several studies have shown that neurons of both the LC and the rostral ventromedial nucleus raphe magnus can either inhibit or facilitate spinal pain transmission in different physiological states (Grzanna and Molliver, 1980; Westlund and Coulter, 1980; Nuseir and Proudfit, 2000; Millan, 2002). Although the descending feedback inhibition of pain by LC is well understood during acute pain conditions (Jones and Gebhart, 1987), the circuitry and molecular changes associated with central NA neurons that lead to chronic pain facilitation after peripheral nerve injury are still unclear (Martin et al., 1999; Taylor et al., 2000; Viisanen and Pertovaara, 2007; Brightwell and Taylor, 2009). Unique to the present study in comparison to many previous studies is the duration of the behavioral study. The present studies were initiated to understand pain facilitation by the LC through 8 weeks post injury in a chronic orofacial neuropathic pain (CCI-ION) model in contrast to most previous studies that have used pain models persisting 1–3 weeks. In the present study, a model of trigeminal neuropathic facial pain was induced by chronic constrictive injury of the infraorbital nerve, the second branch of the trigeminal nerve coursing across the maxillary bone (Vos et al., 1994). Trigeminal neuropathic pain is described as excruciating, constant burning pain and its treatment is a significant challenge. The nerve injury was confirmed by persistent mechanical hypersensitivity through an 8-week time course and by examining the expression profile of

the injury biomarker activating transcription factor 3 (ATF3) in trigeminal ganglion (TG) neurons at the end of the study. The role gamma amino butyric acid (GABA) receptors play in the interneuronal modulation of LC and the effects of NA receptor activation in the mPFC were also investigated.

An initial study determined whether the selective elimination of NA neurons using anti-dopamine- β -hydroxylase saporin (anti-D β H-saporin) alleviates or facilitates the chronic facial neuropathic pain. The immunotoxic anti-D β H-saporin is taken up specifically by NA nerve endings and destroys NA neurons after retrograde transport to the cell bodies. Since the nerve injury increased expression of biomarkers for both NA and GABA (GAD65), the physiological effect of GABA was determined in a second experiment by administering the GABA_A receptor antagonist bicuculline methiodide directly into the LC to block GABA_A activation. Ongoing nerve injury would be expected to increase GABAergic inhibitory tone in the LC. The present study tests an alternative hypothesis that after long standing nerve injury, LC activation can be potentiated by GABA_A receptor mediated neuronal activation (De Koninck, 2007; Doyon et al., 2013; Wei et al., 2013). Then blocking GABA_A signaling with bicuculline would decrease LC activity causing decreased hypersensitivity.

Finally, to test whether LC is providing anti-nociceptive or pro-nociceptive effects on the mPFC in the ongoing pain state, NA α 1 and NA α 2 receptor antagonists (benoxathian and idazoxan hydrochloride, respectively) were microinjected directly into the mPFC to block the effects of NA input. The mPFC is a key neural region activated by sustained nociceptive input during the transition from acute nociceptive processing to central generation of pain based on numerous fMRI studies (Baliki et al., 2006, 2012; Apkarian et al., 2013). It was hypothesized that if this major ascending NA input from LC to mPFC inhibits chronic neuropathic pain, then injecting a NA α 2 receptor antagonist would increase the tactile hypersensitivity that develops after nerve injury. If the ascending LC NA input to mPFC was facilitating chronic facial neuropathic pain, then injecting the NA α 1 receptor antagonist would decrease the tactile hypersensitivity that developed after nerve injury.

EXPERIMENTAL PROCEDURES

All experimental procedures were approved by the Institutional Animal Care and Use Committees at the University of Kentucky and VA Medical Center, Lexington, Kentucky and were carried out following the Guidelines of the National Institutes of Health and the American Pain Association regarding the care and use of animals for experimental procedure. All measures were taken to minimize the number of animals used and their discomfort in these studies. Fig. 1 illustrates the experimental timeline for behavioral testing and drug injections.

Chronic constriction injury of the infraorbital nerve

Male Sprague–Dawley rats ($n = 53$) weighing 250–300 g (Harlan, Indianapolis, IN, USA) were housed under a 12-h light–dark cycle (7 AM–7 PM) with food and water *ad libitum*. Rats were anesthetized with a mixture of ketamine and xylazine (80 mg/kg, i.p. + 10 mg/kg, i.p.) prior to the surgery as previously described (Vos et al., 1994; Ma et al., 2012). The heads of the rats were shaved and stabilized in a stereotaxic frame. After the skin was

cleansed with betadine followed by 70% ethanol, the left intraorbital nerve was exposed using a sterile surgical blunt dissection. Two chromic gut (5-0) ligatures were loosely tied around the left intraorbital nerve (2 mm apart). In order to determine the appropriate desired degree of constrictive force, the nerve was observed microscopically while the ligature was tightened so that the circulation through the superficial vasculature was retarded but not occluded. For the sham operation, the intraorbital nerve was exposed but not ligated. Following the nerve injury or sham surgery, the skin incision was closed with wound auto clips.

Assessment of whisker pad mechanical threshold

Bilateral assessment of the whisker pad mechanical withdrawal threshold was performed weekly throughout the 9-week experimental time course. To minimize observer subjectivity, the person performing the behavioral test was blinded to treatment groups. Prior to von Frey testing, each rat was acclimatized by holding them for 15 min in large cloth gloves to minimize stress-induced effects (Aloisi et al., 1994). Baseline behavioral testing was done for 2 weeks. Eight von Frey fibers (0.4, 0.6, 1.0, 2.0, 4.0, 6.0, 8.0, 15.0 g; Stoelting, Wood Dale, IL, USA) were used to determine the mechanical sensitivity of the vibrissal whisker pad. A modified up-and-down method was used with a cut off maximal threshold of 18.72 g (Ma et al., 2012). Initially, an intermediate von Frey monofilament (2.0 g) was applied perpendicularly to the vibrissal whisker pad with a slight bending force. In the event of a positive response (head withdrawal three times out of five), a weaker filament was next applied. In the event of a negative response, a stronger filament was applied. Each filament was applied five times at intervals of a few seconds. Care was taken to avoid direct vibrissal stimulation during von Frey fiber applications. Testing proceeded in this manner until four fibers applied after the first one resulted in a withdrawal response, allowing the estimation of the mechanical withdrawal threshold (in grams force) using a curve fitting algorithm. A withdrawal threshold decrease was an indication of mechanical allodynia. The mechanical threshold testing continued once a week for 8 weeks after nerve injury.

Selective elimination of LC noradrenergic A6 neurons with anti-D β H-saporin

Twenty-one days after the nerve injury when hypersensitivity was maximal, rats were anesthetized under a mixture of ketamine and xylazine (80 mg/kg, i. p. + 10 mg/kg, i.p.). Stereotaxic surgery was performed to intracerebroventricularly (i.c.v.) inject the NA-specific neurotoxin anti-D β H-saporin to selectively eliminate D β H-containing neurons as described previously (Taylor et al., 2000). Injections of 5- μ g anti-D β H-saporin (CCI-ION $n = 6$, sham surgery $n = 4$) or 5- μ g mouse IgG saporin as the control ($n = 3$) were made into the left lateral ventricle to eliminate NA neurons with rostral projections (Advanced Targeting Systems, San Diego, CA, USA). The saporin was dissolved in 10- μ l sterile saline and infused using a 10- μ l Hamilton syringe over 5 min at a rate of 2 μ l/min. The needle (30-gauge beveled) remained in place for 10 min at the end of the injection. Stereotaxic coordinates (mm) with reference to bregma for left lateral ventricle were A–P (anterior–posterior) = -0.8 , Lat (lateral) = $+1.5$ at two depths, D–V (dorsal–ventral) = -4.3 and -3.3 (Paxinos and Watson, 1986).

For bilateral brainstem injections to eliminate NA neurons with input to the trigeminal nucleus caudalis, the stereotaxic coordinates were A–P = –14.5, Lat = ± 2 –3, and D–V = –8.5 (in mm) with the injection needle angled 20°. The saporin injection (CCI-ION $n = 6$, sham $n = 4$) or control IgG (CCI-ION $n = 3$) was made slowly over 10 min (4 $\mu\text{g}/4 \mu\text{l}/\text{side}$). After saporin injections, sufficient time (2 weeks) was allowed for the neurotoxic effects to take place (Wrenn et al., 1996; Wiley and Kline, 2000). Effects on the mechanical allodynia were assessed with the weekly testing. LC cell loss was assessed with Cresyl Violet staining.

Microinjection of bicuculline methiodide, benoxathian & idazoxan hydrochloride (IDA)

Three weeks after the CCI-ION surgery and the presence of mechanical allodynia confirmed with behavioral testing, a cohort of rats was deeply anesthetized with a mixture of ketamine and xylazine (80 mg/kg, i.p. + 10 mg/kg, i.p.) and placed in a stereotaxic frame. After a 2 cm skin incision was made on the midline of the head to expose the cranium, a 26-gauge dual microinjection guide cannula (Plastics One, Roanoke, VA, USA) was inserted for bilateral injections into either the LC or mPFC. Final coordinates with reference to bregma were: LC (A–P = –10.0, Lat = ± 1.3 , D–V = –7.0) and mPFC (A–P = ± 2.6 , Lat = ± 0.8 , D–V = –4.1). The bilateral cannula was secured to the skull with three machine screws and dental cement (Lang Dental, Wheeling, IL, USA). The skin was closed with 5-0 nylon sutures (Ethicon, Somerville, NJ, USA), and the rats were allowed a recovery period of at least 14 days.

The mechanical withdrawal threshold was again evaluated in weeks 5–6 before drug application. A 30-gauge stainless steel microinjection cannula (microinjector) was lowered through the guide cannula into the LC to microinject GABA_A receptor antagonist bicuculline methiodide bilaterally (freshly prepared in 0.9% saline, $n = 5$, 0.2 $\mu\text{g}/1.0 \mu\text{l}/\text{side}$, at 0.5 $\mu\text{l}/\text{min}$, Sigma Chemical Co., St Louis, MO, UAS) or vehicle ($n = 4$).

Animals with bilateral mPFC implanted microcannulae received a microinjection of $\alpha 1$ -adrenergic receptor antagonist, benoxathian hydrochloride (0.8 $\mu\text{g}/0.5 \mu\text{l}/\text{side}$, 0.25 $\mu\text{l}/\text{min}$, Sigma) (CCI-ION $n = 6$, Sham $n = 3$), or NA $\alpha 2$ receptor antagonist, IDA (9 $\mu\text{g}/0.5 \mu\text{l}/\text{side}$, 0.25 $\mu\text{l}/\text{min}$, Sigma) (CCI-ION $n = 6$, Sham $n = 3$). The microinjector was left in place for 2 min after drug injection to allow for drug diffusion. Head withdrawal threshold was then measured at 10-, 20-, 40- and 90-min time points after the microinjections. Using a Latin square cross-over design, half of the animals received drug and half received the 0.9% saline vehicle in alternate weeks.

Immunohistochemistry

At the end of the study the rats were deeply anesthetized with sodium pentobarbital (150 mg/kg) and perfused transcardially first with 0.1 M phosphate-buffered saline containing 0.1% heparin followed by ice cold 4% paraformaldehyde. Brains and TG were dissected, post-fixed in 4% paraformaldehyde at 4 °C overnight, transferred to a 30% sucrose solution in 0.1 M PB for 24 h, and then embedded into O.C.T. compound (Tissue-Tek, Sakura, Torrance, CA, USA). Using a cryostat, 20 μm sections for pons and 12 μm sections for TG were cut serially and mounted onto treated glass slides (Superfrost Plus, VWR Micro slides, Radnor, PA, USA). For all immunohistochemistry, comparison groups were batched stained with the same immunochemical solutions. The trigeminal ganglia and pons sections were

washed with 0.1 M phosphate-buffered saline (pH 7.4) and blocked with 5% powdered milk in phosphate-buffered saline (30 min, room temperature). Sections were incubated overnight at room temperature with their respective primary antibodies in 3% milk/phosphate-buffered saline. After removal of the primary antibody, the sections were washed 3×10 min in 0.1 M phosphate-buffered saline and subsequently incubated with their respective secondary antibodies in 3% milk/phosphate-buffered saline for 1 h. Sections were rinsed, and coverslipped with anti-fade glycerol-based mounting media with/without DAPI (Vector Laboratories, Burlingame, CA, USA).

LC lesions after D β H-saporin injections were verified using D β H immunohistochemistry. Sections were incubated in rabbit anti-D β H (1:1000; #22806, Immunostar, Inc., Hudson, WI, USA) followed by incubation with fluorescent AlexaFluor 594 anti-rabbit IgG 2° antibody (1:1000; Molecular Probes, Invitrogen, Eugene, OR, USA). To confirm the neuronal injury, TG sections were incubated in rabbit anti-ATF3 antibody (1:500; SC-188, Santa Cruz, CA, USA) followed by incubation with fluorescent AlexaFluor 594 anti-rabbit IgG 2° antibody (1:1000; Molecular Probes, Invitrogen). ATF3 is a member of the ATF/CREB family of transcription factors. Its presence is indicative of neuronal injury in a variety of stressed tissues (Hai et al., 1999). Consistent with this previous finding is the increase in ATF3 expression maintained in affected trigeminal ganglia neurons in our studies at 9–12 weeks after nerve injury (Ma et al., 2012). Immunohistochemistry for GAD-65 was performed with mouse anti-glutamate decarboxylase (GAD65) monoclonal antibody (1:50; Developmental Studies Hybridoma Bank, Iowa City, IA, USA) followed by incubation with fluorescent AlexaFluor 488 anti-mouse IgG 2° antibody (1:1000; Molecular Probes, Invitrogen).

Fluorescence images of tissue sections were photographed with a Nikon E1000 microscope (Nikon Instruments, Inc., Melville, NY, USA) equipped with CoolSNAP ES CCD Camera (Photometrics, Tucson, AZ, USA) and the MetaVue 6.1 Image Analysis System program (Molecular Devices, Downingtown, PA, USA). Five images per animal were taken with 20 $\times$ magnification objective from all comparison groups consistently with the same digital imaging settings. Staining intensity of the biomarkers was quantified with the MetaMorph off-line program (Molecular Devices, Downingtown, PA, USA). Due to the intense immunoreactivity of GAD65 after nerve injury, the photomicroscopic images in Fig. 3 are shown with a reduced exposure time to allow optimal visualization of the individual GABA terminals as well as emphasize the bilateral increase in D β H and GAD65 staining in rats with nerve injury compared to the sham controls. The image quantitative analysis of ATF3-positive neurons in the trigeminal ganglia was done by an observer blinded to treatment group. The ATF3 immunopositive neurons were counted and averaged $\pm$ SEM (four animals/group) from five trigeminal ganglia sections per animal spaced at 120 μ m intervals through the entire ganglia.

Statistical analysis

Statistical analyses of the behavioral studies were performed with SPSS software using non-parametric tests as detailed in the text. The two-tailed Student's *t*-test was used to compare cell counts and intensity of immunostaining between two groups with and without

trigeminal nerve injury. All data were expressed as mean $\pm$ SEM (standard error of the mean). A $p < 0.05$ was considered significant.

RESULTS

Anti-D β H saporin eliminates noradrenergic neurons innervation

To evaluate the impact of the LC on development of chronic facial pain, we selectively eliminated noradrenergic LC neurons with either lateral ventricle (i. c.v.) or trigeminal nucleus injections of anti-dopamine- β -hydroxylase saporin (anti-D β H-saporin, 5 μ g/10 μ l). The LC was identified as a dense cluster of D β H immunoreactive NA cells in the lateral pons at the lateral edge of the fourth ventricle (Fig. 2A, B). Anti-D β H-saporin i.c.v. injection eliminated most of the pontine NA neurons bilaterally in the LC (A6) (Fig. 2C, D) and the subcoeruleus, as well as the medullary NA neurons (A1/A2). Semi-quantitative neuronal counts in LC after CCI-ION + anti-D β H-saporin (15.2 ± 4.9 , $n = 4$) were significantly decreased compared to naïve controls (174.5 ± 27.6 cells, $n = 3$) ($p < 0.001$, Student's t -test). Injection of the neutral control IgG-saporin into the lateral ventricle did not change D β H immunoreactivity in the LC (Fig. 2A, B) or other NA cell regions. The results were similar with anti-D β H-saporin and control IgG-saporin injected into spinal trigeminal nucleus caudalis (spVc) bilaterally (data not shown). Significant loss of NA terminal staining in the periaqueductal gray, parabrachial area, amygdala and hypothalamus was also noted (data not shown).

Effect of traumatic nerve injury on TG neurons and mechanical hyperalgesia

Fig. 3A, B illustrates the mechanical hypersensitivity that develops and persists after induction of the CCI-ION orofacial pain model. CCI-ION reduced the mechanical withdrawal threshold from baseline of 18.72 ± 0 g to 4.54 ± 1.7 g. By contrast, mechanical threshold did not change in naïve rats or rats with sham surgery (18.72 ± 0 g). We confirmed the neuronal injury in our CCI-ION model at week 8 with immunohistochemical quantification of transcription factor ATF3, a neuronal injury biomarker (Obata et al., 2003). Sham surgery was associated with low basal expression of ATF3 in TG neurons (Fig. 3C, E). In contrast, CCI-ION dramatically increased ATF3 immunoreactivity in TG neurons on the side ipsilateral to the nerve injury (Fig. 3D, E) (CCI-ION 98.75 ± 4.99 cells vs. sham 4.75 ± 2.84 ; $n = 4$ /group, $p < 0.001$, Student's t -test), but not on the contralateral side (data not shown).

Noradrenergic LC lesions alleviate development of mechanical allodynia

As illustrated in Fig. 3A, the mechanical threshold was increased within 2 weeks after i.c.v. injection of anti-D β H-saporin from 4.58 ± 1.34 g to 13.23 ± 0.74 g and was maintained at the same level through weeks 6–9. Compared to rats with nerve injury receiving control IgG-saporin (1.22 ± 0.16 g), there was a statistically significant difference in the mechanical threshold ($*p < 0.05$, non-parametric Games-Howell test). The saporin conjugated antibodies did not produce overt non-specific effects. Like the naïve rats, the observed head withdrawal thresholds in sham animals remained at baseline level (18.72 ± 0 g) through the entire experimental time course. The i.c.v. injection of anti-D β H-saporin eliminated NA neurons throughout the CNS indicated by absence of immunoreactive D β H in tissue sections

processed simultaneously with tissues from control animals. Fig. 3B compares the averaged mechanical threshold of the ipsilateral vibrissal whisker pads showing the statistically significant differences between the experimental groups: naïve, nerve injury (CCI-ICV) + anti-D β H-saporin, and CCI-ICV + IgG saporin ($^{**}p < 0.01$, $^{***}p < 0.001$, non-parametric Dunnett's T3 test).

Targeting coeruleotrigeminal NA neurons as well, we injected anti-D β H-saporin into the trigeminal brainstem nuclei bilaterally. In these animals, whisker pad mechanical threshold significantly increased from 0.26 ± 0.87 g to 10.37 ± 3.12 g at 8 weeks (Fig. 3A) compared to the nerve injury group injected with control IgG-saporin (0.45 ± 0.06 g, $^{*}p < 0.05$, non-parametric Games-Howell test).

Microinjection of GABA_A receptor antagonist bicuculline methiodide into LC alleviated mechanical hypersensitivity

To test the effect of GABA_A receptor activation of the LC on trigeminal neuropathic pain, the GABA_A receptor antagonist bicuculline was microinjected into the LC 5 weeks after the nerve injury (Fig. 4A). This study was done because expression of immunoreactivity to GABA-synthesizing enzyme glutamic acid decarboxylase (GAD65) was significantly increased in the LC evaluated 8 weeks after nerve injury (Fig. 4B, C). Nerve injury induced up-regulation of GABA_A receptors in the LC suggested GABA might be tonically activating GABA_A receptors, leading to inhibition of LC descending inhibitory influence on trigeminal neuropathic pain. To test this, the GABA_A receptor antagonist, bicuculline methiodide, was microinjected bilaterally into the LC (0.2 μ g/side in 1.0- μ l saline) in one cohort. As shown in Fig. 4A, when delivered 5 weeks after nerve injury, bicuculline alleviated mechanical hypersensitivity tested at 10 min (18.72 ± 0.00 g vs. 2.36 ± 1.11 g) and 20 min (14.17 ± 1.89 g vs. 2.36 ± 1.11 g) post infusion as compared to saline-injected animals ($^{**}p < 0.005$, non-parametric Dunnett's T3 test). The increase in mechanical threshold peaked at 10 min and returned to pre-drug injection values by 90 min.

Injection of NA α 1 antagonist, but not NA α 2 antagonist, into medial prefrontal cortex alleviates mechanical hypersensitivity

A major NA efferent pathway from the LC includes axonal projections to the mPFC, an important site of activation in chronic pain patients. To test the hypothesis that nerve injury tonically activates this pathway, we bilaterally injected either the NA α 1-receptor-selective antagonist benoxathian hydrochloride (0.8 μ g/0.5 μ l/side) or the NA α 2-receptor-selective antagonist IDA (9 μ g/0.5 μ l/side) into the mPFC. As shown in Fig. 5, benoxathian alleviated mechanical hypersensitivity versus vehicle injection at post-infusion time points 10 min (14.42 ± 1.98 g vs 1.60 ± 0.38 g), 20 min (9.84 ± 2.30 g vs 2.30 ± 0.53 g), and 40 min (7.76 ± 2.26 g vs 1.48 ± 0.25 g) ($^{*}p < 0.05$, $^{**}p < 0.005$, non-parametric Games-Howell test). The effect peaked at 10 min and returned to pre-drug injection values by 90 min. Benoxathian did not produce overt non-specific effects. By contrast, idazoxan did not change mechanical allodynia induced by nerve injury (Fig. 5) ($p > 0.05$, not significant, non-parametric Dunnett's T3 test).

DISCUSSION

In the present study, the role of LC was examined using the CCI-ION trigeminal neuropathic pain nerve injury model in rats through a chronic 5–8 week post injury time period. It was determined that elimination of NA neurons with anti-D β H-saporin, including LC neurons, resulted in reduced mechanical hypersensitivity in rats with nerve injury. Reduced hypersensitivity was observed after elimination of NA input in week 4 with injections into the trigeminal dorsal horn or i.c.v. injections to eliminate LC neurons with ascending projections to higher brain sites. The pharmacological studies in week 6 indicated that antagonism of GABA_A receptors in the LC had a paradoxical inhibitory effect on nerve injury induced hypersensitivity. A facilitative effect of NA signaling in the mPFC in rats with nerve injury induced by CCI-ION was mediated by NA α 1 receptors. Paradoxical data also indicated a diminished influence of inhibitory NA α 2 receptor signaling in rats with well-established nerve injury induced pain-related behaviors. These studies focused on alterations observed at the level of the LC and the mPFC suggest NA facilitation increases vulnerability for a chronic pain state as discussed by others previously (Denk et al., 2014). Discussed below are mechanisms and circuitry generating the predominance of NA facilitation rather than inhibition demonstrated in this CCI-ION model.

The ascending projections of noradrenergic LC neurons to medial prefrontal cortex contribute to development of CCI-ION-induced mechanical hypersensitivity

Subdivisions of NA LC neurons project to specific regions of the CNS, including cortex, hippocampus, hypothalamus, amygdala, spinal cord and cerebellum (Loughlin et al., 1986a, b). The source of NA innervation of the mPFC is the LC (Aston-Jones et al., 1984; Aston-Jones and Cohen, 2005). The present study demonstrated that D β H-saporin microinjected either i.c.v. or into the spinal trigeminal nucleus decreased mechanical hypersensitivity after the infraorbital nerve injury. The i. c.v. administration of anti-D β H-saporin eliminated NA A6 neurons of the LC and decreased the intensity of tactile allodynia. In support of these findings, another study showed that intrathecal anti-D β H-saporin decreased the intensity of allodynia and hyperalgesia after sural nerve injury (Brightwell and Taylor, 2009). Both of these studies indicate targeted destruction of the LC NA system reduces tactile allodynia in chronic neuropathic pain persisting more than three weeks. On the other hand, several studies (Martin et al., 1999; Li et al., 2002; Jasmin et al., 2003) which used anti-D β H-saporin via an intrathecal (i.t.) spinal route of administration found that the mechanical reflexive von Frey threshold either did not change or it increased pain-related behaviors. Several explanations could account for this discrepancy. Firstly, the results of the i.c.v. route of administration here indicate significant loss of LC A6 neurons and reduced NA terminal staining in the periaqueductal gray, parabrachial area, amygdala, and hypothalamus. It has been shown previously that i. c.v. administered anti-D β H-saporin depletes both pontine and medullary NA neurons (Jasmin et al., 2003). The spVc route of administration eliminated pontine LC, sub-coeruleus, and parabrachial neurons which have descending spinal projections to the spinal trigeminal nucleus and spinal cord (Westlund and Coulter, 1980; Westlund et al., 1981, 1982, 1983). With the medullary injection, there is less potential involvement of LC neurons with ascending projections to the forebrain sites that modulate focused attention and amplify nociceptive responses. Removal of any contribution of

ascending projections of the A1/A2 NA cell groups has not been considered here and would require additional investigation.

LC neuronal activation indicated by increased expression of c-fos and pCREB after nerve ligation has been shown previously (Brightwell and Taylor, 2009; Martins et al., 2015). In further support of a facilitative function for ascending LC NA pathways during *chronic* neuropathic pain rather than the inhibitory function described in other *acute* pain models, we specifically performed pharmacological studies with NA α 1 or NA α 2 receptor antagonists to block NA signaling in the forebrain mPFC region. The restricted nature of NA LC axonal projections to discrete sub-regions of prefrontal cortex, such as the mPFC and the anterior cingulate cortex (ACC) produces focused arousal (Chandler and Waterhouse, 2012; Chandler et al., 2013). Physiological levels of NA released under normal conditions strengthen prefrontal cortical functions such as enhancing working memory and focusing attention via actions at post-synaptic NA α 2 receptors. Effects of high levels of NA released during chronic stress conditions mediate anxiety, and stress induced affective disorders eventually impairing mPFC executive function (Nakane et al., 1994; Ramos and Arnsten, 2007). Despite the higher affinity of NA for NA α 2 receptors (56 nM) than for NA α 1 receptors (330 nM) (Mohell et al., 1983; O'Rourke et al., 1994), it is well established that higher NA levels stimulate NA α 1 receptors and impair mPFC functions (Birnbaum et al., 1999). Continuous stress induced NA facilitation impairs the functional integrity of termination sites such as the mPFC and create an allostatic overload (McEwen, 2004; Jarcho et al., 2012; Jett and Morilak, 2013). Allostatic overload is the inability to maintain homeostasis when the individual is exposed to repeated or chronic stress (McEwen, 2000). Dysregulation within the NA system has been implicated in the pathogenesis of anxiety and depressive disorders, as well as disruption of memory, learning, and cognitive flexibility. Disruption of the executive/motivational function of the prefrontal cortex and its interactions with the limbic system can create failure of the mesolimbic reward circuit and vulnerability for chronification of pain with alteration of brain activity, structure and circuitry (Birnbaum et al., 1999; Baliki et al., 2006, 2010, 2012; Wood et al., 2007; Hashmi et al., 2013; Navratilova and Porreca, 2014). Demonstrated experimentally, unilateral electrical stimulation of the LC elicits bilateral LC activation and sustained activation of the mPFC (Marzo et al., 2014). Chronification of the aversiveness of clinical pain has been demonstrated longitudinally with fMRI as greater functional connectivity between the nucleus accumbens and the mPFC shifting connectivity strength from pain-related to emotion-related brain activation (Baliki et al., 2012; Hashmi et al., 2013).

Facilitatory role of NA LC neurons in chronic pain conditions

It is postulated that NA circuitry facilitates chronic stress-induced anxious depression when the dysregulation becomes the new set point (Finlay et al., 1995; Goddard et al., 2010). An extension of this hypothesis would be that pain, as a motivationally relevant stimulus, is chronified by the underlying NA circuitry dysregulation. Significant concentrations of facilitative postsynaptic NA α 1 and inhibitory NA α 2 receptors are associated with ascending NA projections to the mPFC (Pieribone et al., 1994; Nicholas et al., 1996; Arnsten et al., 1998; Ramos and Arnsten, 2007). The results of the present studies confirmed that by microinjecting benoxathian hydrochloride to block NA α 1 receptors in the mPFC, tactile

allodynia after nerve injury was alleviated. However, microinjection of IDA, a selective NA α 2 receptor antagonist had no effect on mechanical hypersensitivity that developed after long term nerve injury. Thus, we propose that during conditions of CCI-ION and ongoing stress created in weeks 5–8 by the trigeminal nerve injury model, NA α 1 receptor facilitation in the mPFC becomes engaged, and overrides the effects of NA α 2 receptor inhibition. The net result of excitation of medial prefrontal neurons would be chronic facilitation of nociceptive hypersensitivity (Fig. 6).

Thus, under conditions of persisting or intense noxious input or tissue injury, the modulatory effect attributable to the LC is a switch from inhibition to facilitation. Several studies concur that NA LC lesions can also significantly reduce the tonic behavioral responses to the intense stimulation induced by intraplantar formalin (Martin et al., 1999; Taylor et al., 2000) and prevent autotomy in a nerve denervation model (Al-Adawi et al., 2002). On the other hand, most studies reported in the literature use acute pain measures to demonstrate the endogenous feedback inhibition provided by the NA system as reviewed previously (Westlund and Coulter, 1980; Tsuruoka and Willis, 1996; Millan, 1999, 2002; Tsuruoka et al., 2003).

The present study provides results indicating that the NA α 1 driven facilitative role of NA LC neurons becomes dominant over pain inhibition, suggesting this mechanism contributes to the transition to chronic pain. This implies the LC is a chronic pain generator. The results also concur with studies implying that with persisting trigeminal nociceptive input, the LC contributes to facilitation of mechanical hypersensitivity through both descending and ascending circuitry (Martins, et al., 2013; Martins et al., 2015).

Increased GAD expression in the LC of Rats with CCI-ION

Another mechanism investigated here that might account for the switch to a facilitatory role for LC is related to the bilateral increases in expression of GAD65 in the LC observed 8 weeks after induction of the nerve injury. Initial expectation was that these neuroplastic changes in the LC would result in reduced activity of NA neurons. However, intra-LC administration of bicuculline methiodide, a selective GABA_A receptor antagonist alleviated tactile mechanical allodynia. Thus, dysregulated GABA_A signaling is contributing to the facilitation by NA LC neurons at the 5–8-week time points studied post nerve injury.

As shown in other studies, GAD65 staining within the LC and its surrounding dendritic field supports previous findings that innervation by GABAergic interneurons provides functional modulation of LC neurons (Iijima and Ohtomo, 1988; Osmanovic and Shefner, 1990; Aston-Jones et al., 2004). In addition to innervation by the peri-coerulear GABAergic neurons, speculation about the source of the GABA input should also include the GABAergic projections of central amygdalar and posterior lateral hypothalamic neurons that innervate the LC (Dimitrov et al., 2013).

GABA has been shown to inhibit LC activity under many conditions. However, in the present study, the finding that GAD65 immunoreactivity is greatly increased at prolonged time points and that GABA_A receptor block reduces hypersensitivity suggests ongoing, strong GABAergic synaptic activation long-term. The long term pathological conditions in

the present study could produce compensatory increase in extracellular K^+ and significantly increasing $[Cl^-]_i$ with upregulation of KCC2. In spinal cord and trigeminal dorsal horn, KCC2 modulation of GABAergic function has been implicated in the loss of inhibition or the disinhibition that develops over time (Moore et al., 2002; Coull et al., 2003; Price et al., 2005; De Koninck, 2007; Wei et al., 2013). A valence shift from inhibitory to excitatory GABAergic $[K^+]_o$ responses occurs with disruption of KCC2 homeostasis and diminished Cl^- homeostatic maintenance under intense activation of GABA_A receptors. Imbalance and dysfunction of K^+-Cl^- cotransporters is emerging as a key regulator of pain conditions implicated in the induction, transition, and maintenance of acute to chronic somatic neuropathic pain (Price et al., 2005; De Koninck, 2007; Doyon et al., 2013). It is proposed that restoring the balance would alleviate chronic pain. Future study is necessary to understand the implication of the increased GABA_A facilitation in LC.

The present studies find increased GAD65 expression in the LC after duration in the orofacial neuropathic pain model for many weeks. We hypothesize that increased dysregulated GABAergic signaling in the LC after peripheral nerve injury causes intense activation of GABA_A receptors in the LC. Effectiveness of gabapentin in reducing hypersensitivity is speculated to reduce the increased expression and release of GABA in the LC after peripheral nerve injury (Yoshizumi et al., 2012). In another study, persistent pain after acute hindpaw inflammation induced by complete Freund's adjuvant reportedly resulted in decreased GABAergic inhibition by activating a selected group of medullary raphe neurons that signal increase in descending pain facilitation (Zhang et al., 2013). While nerve injury may initially increase inhibitory tone in the LC, the effect of reducing GABAergic tone with bicuculline in the present study was to alleviate mechanical hypersensitivity. Based on the present findings, long-term intense activation of GABA_A receptors after the nerve injury become a participant in the valence shift causing net excitation of LC neurons contributing to LC facilitation of chronic orofacial pain. Thus, we speculate LC serves as a chronic pain generator. Clearly, additional study is necessary to unravel the paradoxical findings presented by the present studies of hypersensitivity persisting at longer time points.

In summary, the data reported here for the CCI-ION trigeminal nerve injury model are supportive of LC facilitation of mechanical hypersensitivity in chronic pain states as demonstrated by an increase in mechanical threshold after NA specific neurotoxic lesions. Contributory to the role of LC as a chronic pain generator is dysregulated GABA_A signaling indicated by the significant increase in GAD65 staining and the decrease in mechanical hypersensitivity after bicuculline. Also contributory to the valence shift from inhibition to excitation of hypersensitivity by NA locus LC (A6) neurons is the balance shift from NA α 2 to NA α 1 signaling in the mPFC. This study extends the existing literature for the role of the mPFC within the pain connectome as an essential contributor to central sensitization in addition to its role in anxiety and depression that accompany chronic pain. The present findings provide strong support for predominantly facilitative rather than inhibitory actions of LC NA neurons that become dysregulated during chronic pain. Speculation arises as to whether the underlying NA circuitry valence shift contributes to or generates the chronic pain that becomes the new normal for many patients.

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Abbreviations

ATF3	activating transcription factor 3
BEN	benoxathian hydrochloride
BIC	bicuculline methiodide
CCI-ION	chronic constriction injury of trigeminal nerve maxillary V2 infraorbital branch
DβH	dopamine-β-hydroxylase
GABA_A	gamma amino butyric acid receptor A
GAD65	glutamate decarboxylase
i.c.v	intracerebroventricularly
IDA	idazoxan hydrochloride
LC	nucleus locus coeruleus
mPFC	medial prefrontal cortex
NA	noradrenaline
NAα1 or NAα2	noradrenergic receptor alpha 1 or noradrenergic receptor alpha 2
spVc	spinal trigeminal nucleus caudalis
TG	trigeminal ganglion
VEH	vehicle
Vth	trigeminal nerve

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Fig. 1.

Time line for the studies. Surgical induction of the CCI-ION trigeminal neuropathic pain model, behavioral testing, saporin and drug injections are indicated with arrows.

Fig. 2.

The intracerebroventricular injection of anti-D β H-saporin depletes D β H immunoreactivity in the LC. The upper panel illustrates the LC in coronal sections of the pons from Paxinos G and Watson C, The Rat Brain Atlas 2nd Ed. 1986, plate 57, utilized for the stereotaxic injections. (A, B) Representative photomicrographs illustrating D β H immunoreactivity in the LC of control IgG saporin-injected animal in the ipsi- and contra-lateral pons. (C, D) Anti-D β H-saporin injection essentially eliminated D β H immunoreactivity bilaterally from the LC.

Fig. 3.

The anti-DβH-saporin but not control IgG saporin alleviated mechanical hypersensitivity induced by trigeminal nerve injury. (A) The time course of mechanical allodynia on the ipsilateral vibrissal whisker pad in rats with nerve injury, with and without anti-DβH-saporin is shown as the withdrawal threshold (g) at baseline through week 8 after nerve injury * $p < 0.05$, non-parametric Games-Howell test. (B) The histogram shows the averaged head withdrawal threshold of the ipsilateral vibrissal whisker pads. Comparisons were made among the three experimental groups: naïve, nerve injury (CCI-ICV) + anti-DβH-saporin and CCI-ICV + IgG saporin. ** $p < 0.01$, *** $p < 0.001$, non-parametric Dunnett's T3 test. (C, D) Increased expression of ATF3 in trigeminal ganglia of rats with trigeminal nerve injury. Representative images were taken from the trigeminal ganglion of sham (C) and injured animal (D) ipsilateral to the nerve injury with immunostaining for the neuronal cell injury marker ATF3. (E) Injury biomarker ATF3 increase in trigeminal ganglia in week 8 post-CCI-ION. Nerve injury induces a significant increase in ATF3 immunopositive cells (e.g. arrows) in the trigeminal ganglia on the injured side. The bar graph shows group comparisons of the average number of cells positively stained for ATF3 in the trigeminal ganglia of CCI-ION vs. sham group. Values represent mean $\pm$ SEM. *** $p < 0.001$, Student's t -test.

Fig. 4.

(A) Mechanical allodynia induced by trigeminal nerve injury is alleviated after LC microinjection of bicuculline methiodide, a selective GABA_A receptor antagonist. When delivered in 5 week after nerve injury, bilateral microinjection of bicuculline methiodide but not saline, reduced mechanical hypersensitivity of the ipsilateral vibrissal whisker pad of rats with trigeminal nerve injury. Values represent mean $\pm$ SEM ($n = 4-5$), ** $p < 0.005$, non-parametric Dunnett's T3 test. (B) CCI-ION injury increased GAD65 immunoreactivity bilaterally in the LC. Photomicrographs illustrate dual immunostaining in the LC for DβH (red) and GAD65 (green) with DAPI counterstain (blue) for control rats and rats with nerve injury (week 8). (C) Quantification of GAD65 immunoreactivity in the LC. The bar graph indicates grouped data representing mean $\pm$ SEM for GAD65 immunostaining. * $p < 0.05$,

Student's t -test. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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Fig. 5.

(A) $\text{N}\alpha_1$ receptor antagonist microinjection into mPFC reduced mechanical hypersensitivity. When delivered 6 weeks after nerve injury, bilateral microinjection of benoxathian hydrochloride but not saline, reduced mechanical hypersensitivity indicated by an increase in the withdrawal threshold for von Frey fibers applied to the vibrissal whisker pad ipsilateral to the nerve injury. The $\text{N}\alpha_1$ antagonist had no effect on the sham animals. * $p < 0.05$, ** $p < 0.005$, non-parametric Games-Howell test. (B) $\text{N}\alpha_2$ receptor antagonist microinjection into mPFC had no effect on mechanical hypersensitivity. Idazoxan hydrochloride, a selective $\text{N}\alpha_1$ receptor antagonist injected directly into the mPFC bilaterally had no effect ($p > 0.05$, not significant, non-parametric Dunnett's T3 test) on the mechanical hypersensitivity six weeks after nerve injury.

Fig. 6.

Hypothetical model of the shift in function of LC noradrenergic neurons from pain inhibition to pain facilitation associated with persisting trigeminothalamic activation. While noradrenergic system $\text{NA}\alpha_2$ receptors mediate inhibition of nociception in many acute pain models, a valence switch by LC to facilitation occurs with conditions of persisting and intense nociceptive activation. The present study demonstrates reduced hypersensitivity after GABA_A antagonism in the LC or after elimination of NA input with anti-D β H-saporin microinjected into either the lateral ventricle or the spinal trigeminal caudalis dorsal horn. $\text{NA}\alpha_1$ receptor signaling in the mPFC contributes to the chronic pain facilitation by LC that becomes dominant over pain inhibition in our long term chronic CCI-ION orofacial pain model.