Impaired Pain-evoked Analgesia after Nerve Injury in Rats Reflects Altered Glutamate Regulation in the Locus Coeruleus

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ABSTRACT

Background: Patients with neuropathic pain show reduced endogenous analgesia induced by a conditioned noxious stimulus. Here, the authors tested whether peripheral nerve injury impairs descending noradrenergic inhibition from the locus coeruleus (LC) after L5–L6 spinal nerve ligation (SNL) in rats.

Methods: A subdermal injection of capsaicin was used to examine noxious stimulation–induced analgesia (NSIA), evoked LC glutamate and spinal noradrenaline release, and evoked LC neuronal activity in normal and SNL rats. The authors also examined the role of presynaptic metabotropic glutamate receptors or the astroglial glutamate transporter-1 (GLT-1).

Results: SNL increased basal extracellular glutamate concentration in the LC (170.1%; 95% CI, 44.7 to 295.5; n = 15) and basal spinal cord noradrenaline release (252.1%; 95% CI, 113.6 to 391.3; n = 15), which was associated with an increased tonic LC neuronal activity and a down-regulation of GLT-1 in the LC. SNL reduced NSIA (–77.6%; 95% CI, –116.4 to –38.8; n = 14) and capsaicin evoked release of glutamate in the LC (–36.2%; 95% CI, –49.3 to –23.2; n = 8) and noradrenaline in the spinal cord (–38.8%; 95% CI, –45.1 to –32.5; n = 8). Capsaicin-evoked LC neuronal activation was masked in SNL rats. Removing autoinhibition of glutamatergic terminals by metabotropic glutamate receptor blockade or increasing GLT-1 expression by histone deacetylase inhibition restored NSIA in SNL rats. SNL-induced impairment of NSIA was mimicked in normal rats by knockdown of GLT-1 in the LC.

Conclusions: These results suggest that increased extracellular glutamate in the LC consequent to down-regulation of GLT-1 contributes to LC dysfunction and impaired pain-evoked endogenous analgesia after nerve injury. (**ANESTHESIOLOGY 2015; 123:899-908**)

PERIPHERAL nerve injury can result in chronic neuropathic pain, which is associated with a plethora of changes in the peripheral and central nervous systems related to pain signaling. The gate control theory of pain posits a modulatory role of descending systems on spinal cord sensory processing, which is important to endogenous analgesia.[1] Patients with established neuropathic pain have a reduced ability to physiologically recruit descending inhibition,[2] and patients with reduced descending inhibition are at greater risk for chronic pain after surgery,[3] suggesting that disruption of endogenous analgesia plays a permissive role in chronic pain. However, the underlying mechanisms for disruption of endogenous analgesia remain unclear.

Although endogenous analgesia is mediated by several peripheral and central mechanisms,[4–6] descending noradrenergic inhibition is essential to endogenous analgesia and recovery from hypersensitivity after nerve injury in rats.[7] As an important endogenous analgesic neurotransmitter, noradrenaline is released in the spinal cord from bulbospinal

What We Already Know About This Topic

- Patients with chronic pain have an impaired noxious stimulation–induced analgesia (NSIA), the ability to reduce painful stimuli through activation of the endogenous analgesia system

What This Article Tells Us That Is New

- The authors investigated the circuitry for impaired noxious stimulation–induced analgesia (NSIA) in rats with neuropathic pain
- The authors demonstrate glutamate dysregulation in the locus coeruleus, and the resulting impairment of descending inhibitory control contributes to the loss of NSIA in neuropathic pain

axons that originate in the locus coeruleus (LC) and adjacent nuclei in the brain stem and suppresses activation of spinal nociceptive neurons by actions on α2-adrenoceptors.[8] Among various neurochemical inputs in the LC, glutamate is the primary excitatory neurotransmitter on noradrenergic neurons, acting through α-amino-3-hydroxy-5-methyl-

4-isoxazolepropionic acid (AMPA) receptors.[9] Glutamate also inhibits its own release *via* group 2 and group 3 metabotropic glutamate receptors (mGluRs) located on glutamatergic terminals.[10] Extracellular glutamate is classically removed by two types of astroglial glutamate transporters, primarily glutamate transporter-1 (GLT-1) and also glutamate–aspartate transporter.[11,12] Knockdown or blockade of GLT-1 increases basal extracellular glutamate concentrations in the LC of normal rats. Although peripheral nerve injury also increases basal extracellular glutamate concentrations in the LC,[13] whether this reflects changes in GLT-1 has not been examined. We previously demonstrated that GLT-1 expression decreases in the spinal cord after L5–L6 spinal nerve ligation (SNL), associated with increased extracellular glutamate concentrations, and that inhibition of histone deacetylase (HDAC) restores both GLT-1 and glutamate to normal.[14] Based on these results, the current study focused on the LC and tested the hypothesis that peripheral nerve injury increases extracellular glutamate by down-regulating GLT-1 to inhibit evoked glutamate release in the LC *via* activation of mGluRs and thereby diminishes evoked LC neuronal activity from noxious input important to pain-induced endogenous analgesia.

To measure endogenous analgesia in rats, we used a validated method, noxious stimulation–induced analgesia (NSIA), which is reduced in animals after neuropathic injury and in humans with neuropathic pain.[2,4] The current study examined whether SNL impairs NSIA and reduces noxious stimuli–evoked release of glutamate in the LC and subsequent LC neuronal activation and spinal noradrenaline release. We reasoned based on the adaptive gain theory of LC function[15] that increasing tonic LC activity after SNL would reduce the phasic response to the spinal cord in response to this noxious stimulation and necessary for NSIA. We tested mechanisms by which these impairments occur by the use of presynaptic mGluRs blockade, HDAC inhibition, and quantification of AMPA receptor subunits, glutamate receptors 1 and 2 (GluR1 and GluR2), in spinally projecting noradrenergic neurons in the LC.

Materials and Methods

Animals

Male and female Sprague-Dawley rats (6 weeks old at arrival) from Harlan Industries (USA), housed under a 12-h light–dark cycle with free access to food and water, were used. All experiments were approved by Animal Care and Use Committee at Wake Forest University School of Medicine (Winston-Salem, North Carolina).

Surgeries and LC Cannula Verification

Unilateral L5 and L6 SNL was performed as described previously.[16] Briefly, animals were anesthetized with 2.0% isoflurane in oxygen, the right L6 transverse process was removed, and the right L5 and L6 spinal nerves were tightly ligated

using 5-0 silk suture. Three to four weeks after SNL surgery, some animals were anesthetized with 2% isoflurane and placed securely in a stereotaxic frame (KOPF, USA). A sterile stainless steel guide cannula (CXG-8 for microdialysis, Eicom Co., Japan, or C315G for drug or small interfering RNA (siRNA) injection, Plastic One, USA) was implanted into the left or right LC as described previously.[17] The coordinates for placement of the tip of the guide cannula were 9.8 mm posterior and 1.4 mm lateral to the bregma, and 6.5 mm ventral from the surface of the dura mater, according to the rat brain atlas.[18] Animals were allowed to recover from the surgery at least 1 week before the experiment. After the experiment, 175 animals with LC guide cannulae except 18 animals for Western blotting received an intra-LC injection of methylene blue (0.5 µl) and were euthanized by an intravenous or intraperitoneal injection of pentobarbital (150 mg/kg). The brain was removed, postfixed with 4% buffered formaldehyde, and sectioned, and the placement of the cannula was verified visually. In the current study, six rats were excluded from the behavioral and microdialysis experiments (three rats in each experiment) due to cannula malplacement.

Drugs and siRNA Treatments

Animals were randomly assigned to drug or siRNA treatment. For intra-LC drug administration, (2S)-2-Amino-2-[(1S,2S)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid (LY341495; Tocris Bioscience, USA), 6-cyano-7-nitroquinoxaline-2,3-dione disodium salt (CNQX; Tocris Bioscience), and vorinostat (Tocris Bioscience) were dissolved in 0.033 M NaOH, double distilled water, and dimethyl sulfoxide, respectively, diluted with saline to the final concentrations, and injected through the LC guide cannula at a rate of 0.1 µl/min. For intrathecal drug administration, idazoxan hydrochloride (Tocris Bioscience) was dissolved in saline and injected in the L5–L6 intervertebral space using a 30-gauge needle. Knockdown of GLT-1 in the LC was performed as we previously described.[13] A mixture of siRNAs for rat GLT-1 (SMARTpool #M-091209-02, Thermo Fisher Scientific Inc., USA) or a nontargeting siRNA pool (#D-001206-14, Thermo Fisher Scientific Inc.) was dissolved in double distilled water, diluted with the transfection reagent (i-Fect; Neuromics, USA) to achieve a final concentration of 8.3 pmol/0.5 µl, and injected through the LC guide cannula for five consecutive days.

Behavior Test: NSIA

Nociceptive mechanical thresholds in the hindpaw were measured with a Randall-Selitto analgesiometer (Ugo Basile, Italy) as previously described.[19] A cutoff pressure of 250 g was used to avoid potential tissue injury. All animals were trained for 3 days with this apparatus before baseline values were measured. NSIA was performed as recently reported.[7] Under brief isoflurane anesthesia (2%), capsaicin (150 µg/50 µl/rat; Sigma-Aldrich, USA) or vehicle (50 µl/rat; 50% ethanol

in saline) was injected into left forepaw, and the withdrawal thresholds in the left hindpaw (contralateral to SNL injury) were measured. In the current study, no animal showed distress or self-mutilating behavior in the forepaw after capsaicin injection. The authors (M.K. and T.S.) who conducted behavior tests were blinded to treatments and groups.

Western Blotting for GLT-1 in the LC
Western blotting for GLT-1 in the LC was performed as previously reported.[13] Under deep isoflurane anesthesia (5%), animals were euthanized by decapitation and the brain stem was quickly removed and placed in ice-cold saline. Brainstem slices (2 mm thickness) containing the LC were obtained using a precision brain slicer (RBM-4000C, ASI Instruments, Inc., USA), and the region of the left LC was carefully dissected under the surgical microscope. The LC and adjacent tissue were homogenized, lysed, and centrifuged for 10 min at 4°C at 1,000g. Protein content in each supernatant was measured using a standard method.[20] Samples (25 μg protein) were placed on 10 to 20% gradient gels (Criterion Tris-HCl Gel; Bio-Rad, USA), run at 100V for 1h, and transferred to polyvinylidene difluoride membrane (Bio-Rad). The membrane was blocked with 1% bovine albumin serum in Tris-buffer saline containing 0.1% Tween 20 and incubated overnight at 4°C with a guinea pig anti-GLT-1 (1:5,000, AB1783; Millipore, USA) or a rabbit α-tubulin (1:5,000, 2125S; Cell Signaling, USA). After washing with Tris-buffer saline containing 0.1% Tween 20, the membrane was incubated for 1h at room temperature with a corresponding horseradish peroxidase–conjugated secondary antibody (1:5,000; anti-guinea pig or 1:5,000; anti-rabbit, Santa Cruz Biotechnology, Inc., USA), treated for 1 min with West Pico hemiluminescence substrate (Thermo Fisher Scientific Inc.), and exposed to x-ray film (Kodak BioMax film, Sigma-Aldrich). The density of each specific band was measured using a computer-assisted imaging analysis system (MCID Elite7.0 image analysis system, Imaging Research Inc., Canada).

Microdialysis for LC Glutamate and Spinal Noradrenaline
Simultaneous microdialysis in the spinal cord and LC was performed according to our previous reports with minor modifications.[13,14] On the day of the experiment, anesthesia was induced with 2% isoflurane and then maintained with 1.5% isoflurane during the study. A heating blanket was used to maintain rectal temperature at 36.5° ± 0.5°C, and the right jugular vein was cannulated for saline infusion (3.2 ml kg^{-1} h^{-1}). For microdialysis in the left lumbar spinal dorsal horn, the L3–L6 level of spinal cord was exposed by the T13–L1 laminectomy. Microdialysis probes (CX-I-8-01, outer diameter = 0.22 mm, inner diameter = 0.20 mm, length = 1 mm; Eicom Co.) were inserted into the left spinal dorsal horn and left LC 1 h before the experiment and perfused with Ringer's solution (1.0 μl/min). Then, after

two 30-min baseline samples, capsaicin, at the same dose used for NSIA, or vehicle was injected into the left forepaw. Glutamate and noradrenaline contents in the microdialysates were measured by separate high-pressure liquid chromatography systems with electrochemical detection (HTEC-500, Eicom Co.), as we previously reported.[14,17]

Immunohistochemistry
Capsaicin-evoked Neuronal Activation in the LC. Under brief isoflurane anesthesia (2%), capsaicin, at the same dose used for NSIA, or vehicle was injected into the left forepaw. Thirty minutes later, animals were euthanized with an intraperitoneal injection of pentobarbital (150 mg/kg) and perfused with 0.01 M phosphate-buffered saline containing 1% sodium nitrite, followed by 4% formalin in 0.1 M phosphate-buffered saline. The brain stems were dissected out, postfixed in the same fixative, cryoprotected with 30% sucrose in 0.1 M phosphate-buffered saline, and sectioned at a 16 μm thickness. Immunostaining for phosphorylated cyclic adenosine monophosphate response element binding protein (pCREB) and dopamine-β-hydroxylase (DβH) in the LC was performed as previously described.[17] After being pretreated with 1% normal donkey serum (Jackson Immuno Research Laboratories, USA), the sections were incubated with a rabbit monoclonal anti-pCREB antibody (1:500, 06-519; Millipore) and a mouse monoclonal anti-DβH antibody (1:500, MAB 308; Millipore) overnight. Subsequently, the sections were incubated with Cy2 conjugated anti-mouse IgG (1:200, Jackson Immuno Research Laboratories) and Cy3 conjugated anti-rabbit IgG (1:600, Jackson Immuno Research Laboratories).

Retrograde Tracing and Immunostaining for GluR1/2 in LC Neurons. T13–L1 laminectomy and intraspinal injection were performed as previously described.[21] Red-fluorescent beads (F8793; Life Technologies, USA) were filled in a glass micropipette (10 to 20 μm tip diameter) and four pairs of bilateral injections (50 nl/site, each 500 μm apart in the rostrocaudal axis) were performed at 500 μm from the midline and 500 μm under the surface of the dorsal spinal cord using a micro injector (Nanoinject, Drummond Scientific Co., USA). Seven days after injection, the brainstem sections were prepared as described in the previous paragraph. The sections were pretreated with 3% normal donkey serum, incubated with a combination of rabbit monoclonal anti-GluR1 antibody (1:500, 04-855; Millipore) with mouse monoclonal anti-DβH antibody (1:500), or mouse monoclonal anti-GluR2 antibody (1:1,000, MAB397; Millipore) with rabbit monoclonal anti-tyrosine hydroxylase (TH) antibody (1:500, P40101-0; Pel-Freez Biologicals, USA) overnight. Subsequently, the sections were incubated with donkey anti-rabbit or anti-mouse biotin (1:500, Jackson Immuno Research Laboratories), followed by Cy2 conjugated streptavidin (1:200, Jackson Immuno Research Laboratories) and AlexaFluor 647 conjugated anti-mouse or anti-rabbit IgG (1:500, Jackson ImmunoResearch Laboratories).

Image Analysis. For quantification, four to five brainstem sections containing the LC were randomly selected from each rat and digital images were captured using a Nikon Eclipse Ni fluorescent microscope system (Nikon, Japan). All images for each experiment were taken at the same time with the same camera settings, and the authors performing the image analysis (M.K. and C.E.M.-U.) were blinded to treatment or group. Positive immunostaining for pCREB, GluR1, or GluR2 was defined using a constant threshold for each protein applied across all sections. DβH- or TH-immunoreactive (IR) cells with or without pCREB, GluR1, or GluR2 immunostaining were counted in the entire LC from four to five sections per rat. Results are reported as % of total DβH- or TH-IR cells per rat. DβH or TH-IR cells containing fluorescent beads were defined as spinally projecting LC neurons.

Statistical Analysis

A limitation of the current study is that power analysis was not conducted before the study to determine appropriate sample sizes. Data are presented as mean ± SD. Behavioral data and microdialysis data were analyzed by two-way repeated-measures ANOVA where time and group (surgery or treatment) were considered as independent variables, and Tukey *post hoc* test was used for multiple comparisons using SigmaPlot software (Systat Software Inc., USA). Other data were analyzed by one-way or two-way ANOVA followed by Tukey *post hoc* test with two-tailed hypothesis. *P* value less than 0.05 adjusted by SigmaPlot software was considered significant.

Results

Behavior Study

Role of LC-spinal Noradrenergic Pathway in NSIA. In intra-LC (left side) and intrathecal saline-treated normal rats, fore-paw injection of capsaicin into the left forepaw significantly increased withdrawal thresholds in the left hindpaw 30 min after injection compared with vehicle (fig. 1), demonstrating NSIA. This NSIA effect was significantly reduced by intra-LC injection of the AMPA antagonist CNQX, indicating importance of glutamatergic signaling in the LC. NSIA was also significantly reduced by intrathecal injection of the α2-adrenoceptor antagonist idazoxan, indicating the importance of spinal noradrenaline release. Neither intra-LC CNQX nor intrathecal idazoxan affected withdrawal thresholds in forepaw vehicle-injected rats. Consistent with our recent observation,[7] these results confirm that activation of LC-spinal noradrenergic pathway after capsaicin injection is essential to NSIA in normal rats.

Impact of SNL on NSIA in Male and Female Rats. In male and female rats, there was a significant main effect of group on withdrawal thresholds in the right hindpaw ipsilateral to SNL (fig. 2A; $F_{5, 48} = 35.47$; $P < 0.001$). *Post hoc* testing revealed that normal female rats showed slightly but
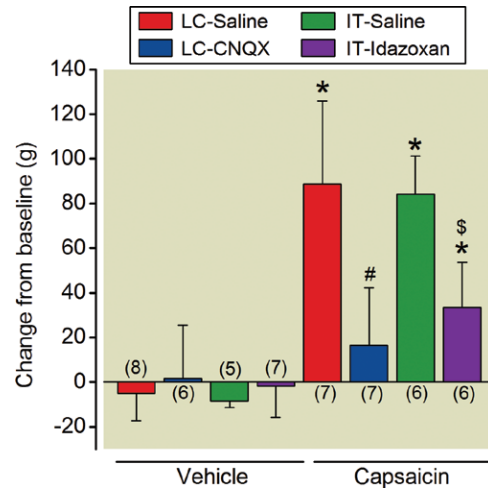

Fig. 1. Role of locus coeruleus (LC)-spinal noradrenergic pathway in noxious stimulation–induced analgesia in normal rats. Normal rats received an injection of saline or CNQX (6-cyano-7-nitroquinoxaline-2,3-dione disodium salt; 0.3 μg/0.5 μl) in the left LC, or an intrathecal (IT) injection of saline or idazoxan (30 μg/10 μl), followed by a subdermal injection of vehicle or capsaicin (150 μg/50 μl) into the left forepaw. Withdrawal thresholds in the left hindpaw were measured before (baseline) and 30 min after capsaicin. Groups were analyzed by two-way ANOVA (intra-LC/intrathecal treatment × forepaw treatment) followed by Tukey *post hoc* comparisons. Data (mean ± SD) are presented as change from baseline. *$P < 0.05$ *versus* vehicle, #$P < 0.05$ *vs.* LC-saline, $$P < 0.05$ *vs.* IT-saline.

significantly lower withdrawal thresholds in the right hindpaw than those of normal male rats, that SNL resulted in mechanical hypersensitivity in the ipsilateral hindpaw 2 to 6 weeks after surgery in male rats and 6 weeks after surgery in female rats, and that withdrawal thresholds in the ipsilateral hindpaw 6 weeks after SNL showed no sex difference ($P = 0.92$).

In male rats treated with forepaw injection of capsaicin (fig. 2B), there was a significant main effect of time ($F_{3, 102} = 39.97$; $P < 0.001$) on the withdrawal thresholds in the contralateral hindpaw and a significant group × time interaction ($F_{9, 102} = 3.59$; $P < 0.001$), but no main effect of group ($F_{3, 102} = 2.13$; $P = 0.12$). *Post hoc* testing revealed that significant NSIA was observed only at 30 min after injection and that the group × time interaction reflected a progressive reduction in NSIA as a function of time since SNL injury. Similarly, in female rats, SNL resulted in a reduction of NSIA compared with normal rats (group: $F_{1, 14} = 123.40$, time: $F_{1, 14} = 116.05$, group × time: $F_{1, 14} = 15.73$; $P < 0.01$ for all F values). There was no sex difference in NSIA in either normal ($P = 0.60$) or SNL conditions ($P = 0.49$). Based on this lack of sex difference in SNL-induced hypersensitivity and impairment of NSIA, we used male rats 6 weeks after SNL for the following studies.

Presynaptic Inhibition on LC Glutamate Release after SNL. An intra-LC injection of the presynaptic mGluR antagonist LY341495 had no effect on withdrawal threshold ipsilateral to injection in normal rats ($P = 0.68$), but
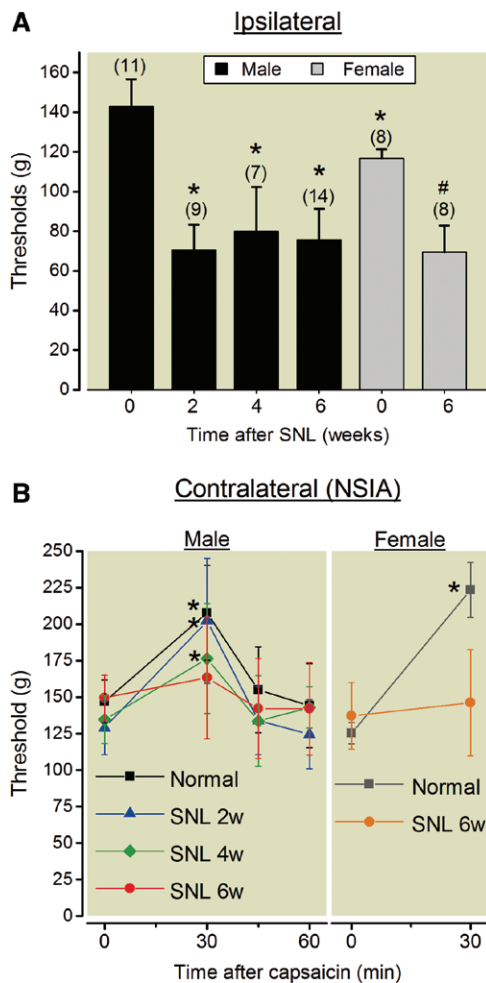
Fig. 2. Effects of spinal nerve ligation (SNL) on hypersensitivity in the ipsilateral hindpaw (*A*) and noxious stimulation–induced analgesia (NSIA) in the contralateral paw (*B*). Withdrawal thresholds in the ipsilateral hindpaw to SNL were measured at 0 (normal), 2 (SNL 2w), 4 (SNL 4w), or 6 weeks (SNL 6w) after surgery. Then, all animals received a forepaw injection of capsaicin (fig. 1) and withdrawal thresholds in the contralateral hindpaw were measured over the time. Data are presented as mean ± SD. Groups were analyzed by two-way ANOVA (Surgery × Sex) or (Surgery × Time) followed by Tukey *post hoc* comparisons. (*A*) *P < 0.05 *versus* normal male. #P < 0.05 *versus* normal female. (*B*) *P < 0.05 *versus* time 0.

Fig. 3. Effects of intra-locus coeruleus (LC) LY341495 on hindpaw withdrawal thresholds in rats after spinal nerve ligation (SNL). LY341495 (0 to 2.0 μg/0.5 μl) was injected into the LC either contralateral (left) or ipsilateral (right) to SNL and withdrawal threshold in the hindpaw ipsilateral to LC-injection was measured 30 min after injection (*A*). Some rats also received a forepaw injection of capsaicin (*B*, CAP) or intrathecal injection of idazoxan (IT-Ida, 30 μg/10 μl) following LC-LY341495. Data are presented as mean ± SD. Groups were analyzed by one-way ANOVA (Treatment) followed by Tukey *post hoc* comparisons. *P < 0.05 *versus* vehicle, #P < 0.05 *versus* LY341495 2.0 μg alone. NSIA = noxious stimulation–induced analgesia.

increased GLT-1 expression ($P = 0.03$) and NSIA ($P = 0.04$) (fig. 4B) compared with saline in SNL rats, while not affecting baseline withdrawal thresholds in the hindpaw contralateral to SNL (vorinostat: 127 ± 10 g *vs.* saline: 129 ± 20 g; $P = 0.77$; n = 9 in each group), consistent with our previous observations in the spinal cord that the reduced GLT-1 expression after SNL was reversed by HDAC inhibitors.[14,22] In normal rats, repeated LC injections of GLT-1 siRNA, at a dose that reduces GLT-1 expression and increases basal extracellular glutamate level in the LC,[13] mimicked the effect of SNL on NSIA, while not affecting baseline withdrawal threshold (GLT-1 siRNA: 139 ± 12 g *vs.* control siRNA: 135 ± 16 g; $P = 0.52$; n = 9 in each group). These results suggest that down-regulation of GLT-1 in the LC plays a critical role for impaired NSIA after SNL.

Increased Tonic and Decreased Evoked LC Activity after SNL

LC Glutamate and Spinal Noradrenaline Release. Basal glutamate concentrations in microdialysates were significantly greater from the left LC of SNL rats (2.7 ± 2.3 ng/30 μl; n = 15) than those of normal rats (1.0 ± 0.8 ng/30 μl; $P = 0.01$; n = 14), consistent with current behavioral results that demonstrate autoinhibition of glutamatergic terminals in the LC after SNL *via* mGluRs. Similarly, basal noradrenaline concentrations in microdialysates from the left lumbar spinal dorsal horn of SNL rats (4.0 ± 2.5 pg/30 μl) were significantly greater than those of normal rats (1.5 ± 0.7 pg/30 μl; $P < 0.005$). These results suggest

increased it bilaterally and dose dependently in SNL rats, an effect reversed by intrathecal idazoxan (fig. 3A). Also in SNL animals, intra-LC injection (left) of a low dose (0.4 μg) of LY341495, which did not alter withdrawal threshold alone ($P = 0.55$), restored NSIA (fig. 3B). These results demonstrate SNL-induced autoinhibition of glutamatergic terminals in the LC, consistent with tonically elevated extracellular glutamate concentrations in the LC after SNL.

Down-regulation of GLT-1 in the LC after SNL. Expression of GLT-1 protein in the LC from saline-treated SNL rats was significantly lower than that of normal rats (fig. 4A; $P < 0.01$). Intra-LC vorinostat treatment significantly
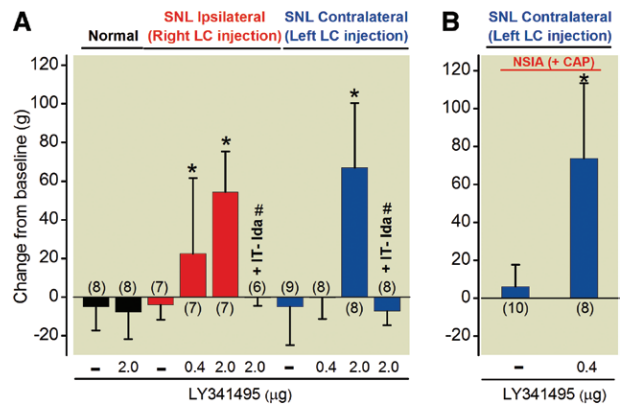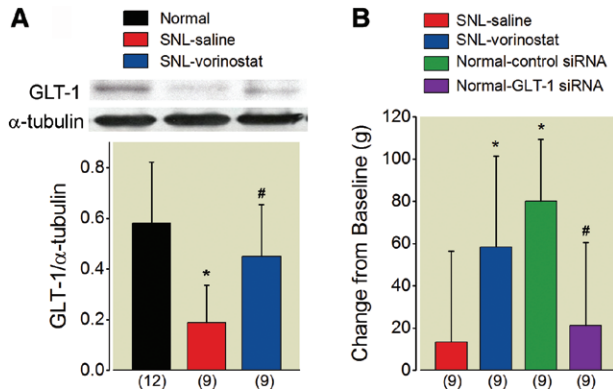
Fig. 4. Noxious stimulation–induced analgesia (NSIA) and glutamate transporter-1 (GLT-1) expression in the locus coeruleus (LC). Data are presented as mean ± SD. Groups were analyzed by one-way ANOVA (Condition/Treatment) followed by Tukey *post hoc* comparisons. (*A*) Representative Western blotting images and quantification of GLT-1 and α-tubulin in the left LC (contralateral to injury) from normal and spinal nerve ligated (SNL) rats. In rats after spinal nerve ligation (SNL), saline (0.5 μl) or the histone deacetylase inhibitor vorinostat (0.25 μg/0.5 μl) was injected into the left LC once a day for 2 weeks starting from 4 weeks after injury, and their brain stem were collected immediately after testing NSIA. *P < 0.05 versus normal. #P < 0.05 versus saline. (*B*) NSIA by capsaicin (150 μg/50 μl) in SNL rats treated with intra-LC saline or vorinostat and in normal rats treated with repeated injections of control or GLT-1 selective small interfering RNA (siRNA; 8.3 pmol/0.5 μl for 5 days) into the left LC. *P < 0.05 versus saline. #P < 0.05 versus control siRNA.

that SNL increases tonic activity of spinally projecting LC neurons.

For capsaicin-evoked glutamate release in the LC, there were significant main effects of group ($F_{3, 50} = 11.64$; $P < 0.001$), time ($F_{2, 50} = 11.08$; $P < 0.001$), and the group × time interaction ($F_{6, 50} = 3.01$; $P = 0.014$) (fig. 5A). For capsaicin-evoked noradrenaline release in the spinal cord, there was a significant main effect of group ($F_{3, 50} = 19.69$; $P < 0.001$), but no main effect of time ($F_{2, 50} = 0.55$; $P = 0.58$) or group × time interaction ($F_{6, 50} = 1.56$; $P = 0.18$) (fig. 5B). Capsaicin-evoked release of glutamate in the LC and noradrenaline in the spinal cord were reduced in SNL rats compared with normal rats at peak time points (LC glutamate at 30 min: –36.2%; 95% CI: –49.3 to –23.2 and spinal noradrenaline at 60 min: –38.8%; 95% CI: –45.1 to –32.5).

LC Neuronal Activity. Photomicrographs in figure 6A depict pCREB-IR in noradrenergic LC neurons, identified by DβH-IR, in normal and SNL rats treated with forepaw injection of vehicle or capsaicin at the same dose used for NSIA. For pCREB-IR in the LC, there was a significant main effect of group ($F_{3, 40} = 31.07$; $P < 0.001$), but no main effect of side ($F_{1, 40} = 0.00003$; $P = 0.996$) or the group × side interaction ($F_{3, 40} = 0.11$; $P = 0.96$) (fig. 6B). *Post hoc* testing revealed that vehicle-treated SNL rats showed an increase in pCREB-IR in LC neurons compared with vehicle-treated normal rats ($P < 0.001$ for both sides) and
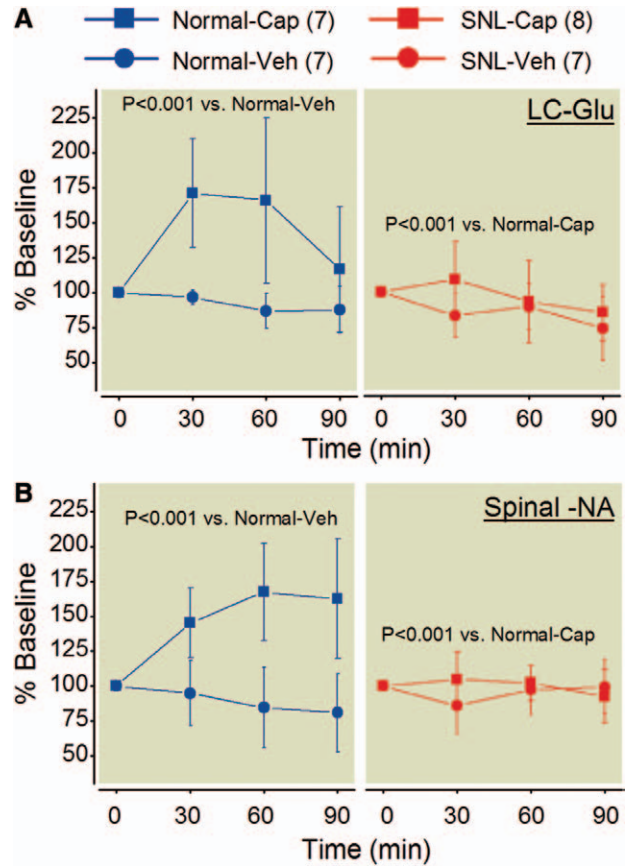

Fig. 5. Simultaneous microdialysis in the left locus coeruleus (LC) and left lumbar spinal dorsal horn. After two 30-min baseline samples, vehicle (Veh) or capsaicin (Cap, 150 μg/50 μl) was injected into the left forepaw in normal and spinal nerve ligated (SNL) rats. Changes in glutamate (Glu, *A*) and noradrenaline (NA, *B*) concentrations in microdialysates from the LC and spinal cord are presented over the time as percentage of baseline. Data are presented as mean ± SD. Groups were analyzed by two-way repeated-measures ANOVA (Time × Group).

that capsaicin increased pCREB-IR in LC neurons compared with vehicle in normal rats ($P < 0.001$ for both sides), whereas no such increase occurred in SNL rats ($P = 0.81$), consistent with current NSIA and the microdialysis results. These results suggest that SNL bilaterally increases tonic but reduces evoked LC neuronal activity, effectively taking the LC "off-line."

Expression of AMPA Receptor Subunits in the LC

Photomicrographs in figure 7, A and B depict GluR1-IR and GluR2-IR in spinally projecting LC neurons, identified by the spinally injected retrograde tracer-positive cells with DβH-IR or TH-IR, in normal and SNL rats. Since GluR1- and GluR2-IR showed no difference between the sides of LC from SNL rats in our preliminary experiment (data not shown), the current study summed cell counts from left and right LC to obtain a single value for each rat. For GluR1-IR in the LC, there was a significant main effect of
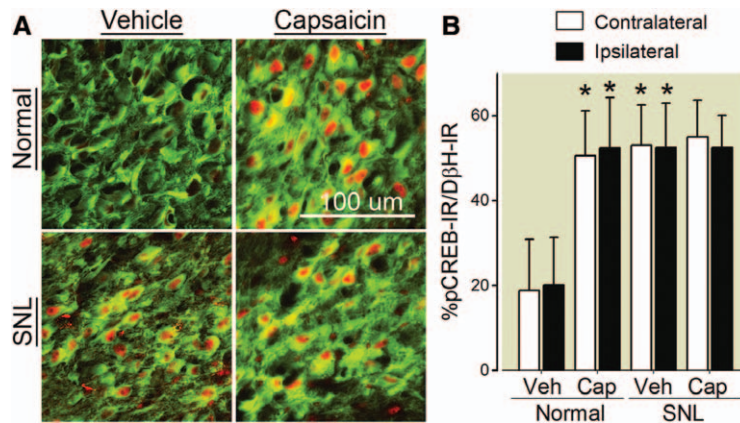
Fig. 6. Effects of spinal nerve ligation (SNL) on tonic and capsaicin-evoked phosphorylated cyclic adenosine monophosphate response element binding protein immunoreactivity (pCREB-IR) in the locus coeruleus (LC). (*A*) Photomicrographs of dopamine-β-hydroxylase (DβH)-IR (*green*) and pCREB-IR (*red*) in the LC collected from normal and SNL rats 30 min after the left forepaw injection of vehicle (Veh) or capsaicin (Cap) (150 μg/50 μl). (*B*) Quantification. Data (mean ± SD, n = 6 in each group) are presented as the percentage of pCREB-IR in DβH-IR neurons. Groups were analyzed by two-way ANOVA (Side × Group) with Tukey *post hoc* comparisons.*$P < 0.05$ *versus* normal vehicle.
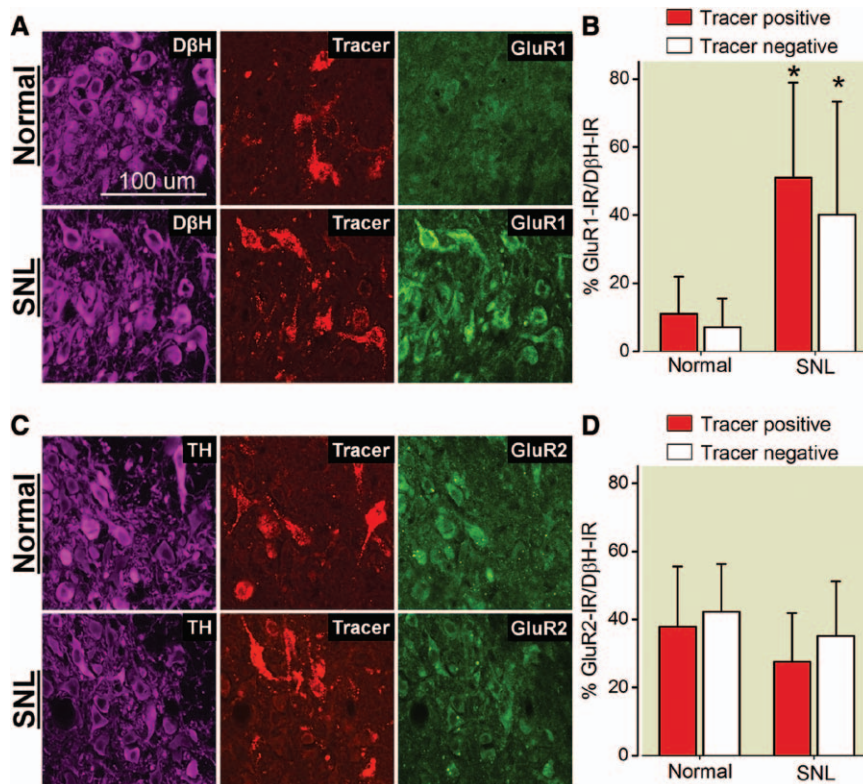

Fig. 7. Effects of spinal nerve ligation (SNL) on glutamate receptor 1 and 2 (GluR1 and GluR2)-immunoreactivity (IR) in spinally projecting locus coeruleus (LC) neurons. (*A*) Photomicrographs of dopamine-β-hydroxylase (DβH)-IR (*purple*), spinally injected retrograde fluorescent tracer (*red*), and GluR1-IR (*green*) in the brain stem from normal and SNL rats. (*B*) Data (mean ± SD, normal: n = 5, SNL: n = 6) are presented as the percentage of GluR1-IR in tracer-positive or negative DβH-IR neurons. (*C*) Photomicrographs of tyrosine hydroxylase (TH)-IR (*purple*), spinally injected fluorescent tracer (*red*), and GluR2-IR (*green*) in the brain stem from normal and SNL rats. Groups were analyzed by two-way ANOVA (Surgery × Tracer) with Tukey *post hoc* comparisons. *$P < 0.05$ *versus* normal. (*D*) Data (mean ± SD, n = 6 in each group) are presented as the percentage of GluR2-IR in tracer-positive or negative TH-IR neurons.

surgery ($F_{1, 18}$ = 12.94; $P < 0.01$), but no main effect of tracer ($F_{1, 18}$ = 0.53; $P = 0.48$) or the surgery × tracer interaction ($F_{1, 18}$ = 0.11; $P = 0.74$) (fig. 7C). *Post hoc* testing revealed

that SNL significantly increased GluR1-IR in LC neurons compared to normal (fig. 7D; $P < 0.01$). For GluR2-IR in the LC, there was no significant main effect of surgery

($F_{1,20}$ = 1.85; P = 0.19), tracer ($F_{1,20}$ = 0.88; P = 0.36), or surgery × tracer interaction ($F_{1,20}$ = 0.06; P = 0.81) (fig. 7D). These results suggest that SNL increases rather than decreases expression of GluR1 in overall LC neurons and do not support the idea that down-regulation of AMPA receptors in LC neurons contributes to impaired NSIA after nerve injury.

Discussion

Peripheral nerve injury leading to hypersensitivity is associated with a plethora of changes in pain processing at many levels of the nervous system, from the primary sensory afferent to the spinal cord to the brain stem and cortex. In the past 15 yr, pain research has primarily focused on facilitatory changes in pain processing after nerve injury, including spinal neural and glial activation and descending facilitation, yet the clinical relevance of these phenomena in animals is unknown. By contrast, descending inhibition important to endogenous analgesia has been shown to be reduced in patients with neuropathic pain,[2] yet its mechanisms have been less studied. Interestingly, although LC activity in neuropathic pain may facilitate supraspinal pain pathways,[4–6] many drugs approved to treat neuropathic pain, including gabapentinoids, opioids, noradrenaline re-uptake inhibitors, and clonidine, activate, augment, or mimic the LC-spinal descending noradrenergic pathway to produce analgesia,[17,23–25] suggesting that this pathway is a key pharmacological target to treat neuropathic pain. As presented in the scheme (fig. 8), the current results demonstrate that down-regulation of astroglial glutamate transporters in the LC after nerve injury dysregulates descending noradrenergic activation, which is important to endogenous and exogenous analgesia.

To measure endogenous analgesia in rats, we chose forepaw capsaicin injection remote from nerve injury as the stimulus because this models conditioned pain modulation in humans.[2,3,6] Our results with this test in SNL rats mirror the reduction in conditioned pain modulation in patients with chronic neuropathic pain.[2] We recognize we could have applied the conditioning noxious stimulus to the nerve injured hindpaw,[26,27] but it would be difficult to match the nociceptive input from this site after SNL to that in the normal animal in which peripheral and central sensitization are not present. We also recognize that all behavioral measures in the current study reflect threshold withdrawal to an external stimulus. Although this is especially relevant to NSIA, future studies should examine more complex behaviors that may better reflect pain itself. The current and previous studies showed that unilateral peripheral nerve injury affects both LCs, demonstrated by a bilateral increase in neuronal activation markers in the LC after injury.[17,28,29] These results provide a rationale to study LC dysregulation in the side contralateral to injury.

Previous studies in rats demonstrated that noxious stimuli evoke glutamate release in the LC to activate noradrenergic
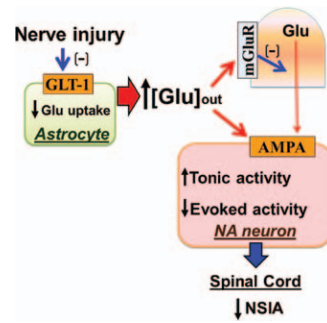

Fig. 8. Nerve injury increases basal extracellular glutamate (Glu) concentration in the locus coeruleus (LC) by down-regulating glutamate transporter-1 (GLT-1), and this inhibits evoked glutamate release by enhanced presynaptic inhibition *via* presynaptic metabotropic glutamate receptors (mGluRs). The increased basal extracellular glutamate concentration results in increased tonic activity of noradrenergic (NA) neurons and the reduced evoked glutamate release that diminishes α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor-mediated NA neuronal activation important to noxious stimulation–induced analgesia (NSIA).

neurons.[9,30] The current study confirmed these observations by demonstrating that forepaw injection of capsaicin in normal rats increased LC glutamate and spinal noradrenaline release and LC neuronal activity, consistent with NSIA which was reversed by the blockade of AMPA receptors in the LC or α2-adrenoceptors in the spinal cord. By contrast, SNL diminished these effects of capsaicin, while it increased basal extracellular LC glutamate and spinal noradrenaline levels and tonic LC neuronal activity. Although several peripheral and central mechanisms are involved in endogenous analgesia,[4–6] these results suggest that the descending noradrenergic pathway is essential to pain-evoked endogenous analgesia in normal conditions and that SNL bilaterally increases tonic activity of the LC-spinal noradrenergic pathway and impairs evoked LC activity and NSIA, effectively taking the LC "off-line." This is consistent with previous work in animals showing that cortical responses to nonnoxious sensory stimuli are modulated by tonic LC activity, with reduced response in the presence of high tonic activity,[31] consistent with the adaptive gain concept.[15] Similar effects of LC tonic activity as inferred by pupillometry in humans on the role of the LC in decision-making[32,33] support this theory.

In contrast to the stable hypersensitivity in the ipsilateral hindpaw, NSIA was reduced in the contralateral hindpaw in a delayed manner, present at 4 and 6 weeks, but not at 2 weeks after SNL surgery. A previous study demonstrated that sciatic nerve injury in rats results in increased bursting activity of LC neurons in a delayed manner, 4 weeks after injury.[34] Similarly, SNL increased pCREB expression in the LC and basal extracellular noradrenaline concentrations in the spinal cord 6 weeks after nerve injury. By contrast, early after peripheral nerve injury (1 to 2 weeks), there is no change in spontaneous discharge rate of LC neurons.[26,27] These results suggest the delayed appearance of functional

plasticity in tonic and evoked LC neuronal activity after nerve injury. They also suggest that phasic responses of the LC to noxious events which occur during activity daily living may be more important to recovery from injury than increased tonic activity, even though both processes result in norepinephrine release in the spinal cord. Future studies should examine this relationship more directly *via* LC neuronal recording to confirm the inferred activity from pCREB expression.

Similar to our previous observations in the spinal cord,[14,22] the current results showed that SNL down-regulated GLT-1 expression and increased basal extracellular glutamate concentrations in the LC. Although glutamate is the primary excitatory neurotransmitter on noradrenergic neurons in the LC,[9] increased synaptic concentrations of glutamate can activate presynaptic mGluRs to autoinhibit glutamate release from its terminals.[10] As such, intra-LC mGluR blockade had no effect on withdrawal threshold in normal rats, but restored impaired NSIA and reduced hypersensitivity in SNL rats, and this is dependent on downstream release of noradrenaline in the spinal cord, acting on α2-adrenoceptors. Restoring down-regulated GLT-1 expression in the LC by HDAC inhibition restored NSIA in SNL rats. Knockdown of GLT-1 by a selective siRNA, which increases basal extracellular glutamate concentrations in the LC,[13] mimicked the effect of SNL on NSIA in normal rats. These results strongly suggest a causal relationship between increased basal extracellular glutamate concentrations in the LC and impairment of NSIA after SNL. Taken together, the current observations support the hypothesis that peripheral nerve injury increases extracellular glutamate by down-regulating GLT-1 to inhibit evoked glutamate release in the LC *via* activation of mGluRs, thereby diminishing evoked LC neuronal activity important to NSIA.

Based on previous data in the rat, GluR1, GluR2, GluR3, and GluR4 AMPA receptor subunits are all expressed in the LC.[35] The current study also examined an alternative hypothesis that impaired NSIA after nerve injury is due to reduced expression of AMPA receptor subunits in noradrenergic LC neurons, rendering them less responsive to glutamate. However, SNL *increased* GluR1 expression and did not affect GluR2 expression in both spinally projecting and other LC neurons. Although we did not examine GluR3-4 subtypes and expression of phosphorylated GluR1, which is a more relevant marker for AMPA receptor activity,[36,37] the current results suggest that down-regulation of AMPA receptors in the LC after SNL is unlikely to occur.

In summary, down-regulation of astroglial glutamate transporters in the LC is critical to impairment of pain-evoked endogenous analgesia after peripheral nerve injury in rats. Since the descending noradrenergic pathway is not only essential to endogenous analgesia but also an important target for many drugs approved to treat neuropathic pain,[17,23–25] restoring impaired responsiveness of descending noradrenergic pathway may have considerable clinical significance.

Competing Interests

The authors declare no competing interests.

References

1. Melzack R, Wall PD: Pain mechanisms: A new theory. Science 1965; 150:971–9

2. Lewis GN, Rice DA, McNair PJ: Conditioned pain modulation in populations with chronic pain: A systematic review and meta-analysis. J Pain 2012; 13:936–44

3. Yarnitsky D, Crispel Y, Eisenberg E, Granovsky Y, Ben-Nun A, Sprecher E, Best LA, Granot M: Prediction of chronic post-operative pain: Pre-operative DNIC testing identifies patients at risk. Pain 2008; 138:22–8

4. Ferrari LF, Gear RW, Levine JD: Attenuation of activity in an endogenous analgesia circuit by ongoing pain in the rat. J Neurosci 2010; 30:13699–706

5. Gear RW, Aley KO, Levine JD: Pain-induced analgesia mediated by mesolimbic reward circuits. J Neurosci 1999; 19:7175–81

6. van Wijk G, Veldhuijzen DS: Perspective on diffuse noxious inhibitory controls as a model of endogenous pain modulation in clinical pain syndromes. J Pain 2010; 11:408–19

7. Peters CM, Hayashida K, Suto T, Houle TT, Aschenbrenner CA, Martin TJ, Eisenach JC: Individual differences in acute pain-induced endogenous analgesia predict time to resolution of postoperative pain in the rat. Anesthesiology 2015; 122:895–907

8. Millan MJ: Descending control of pain. Prog Neurobiol 2002; 66:355–474

9. Singewald N, Philippu A: Release of neurotransmitters in the locus coeruleus. Prog Neurobiol 1998; 56:237–67

10. Pan HL, Wu ZZ, Zhou HY, Chen SR, Zhang HM, Li DP: Modulation of pain transmission by G-protein-coupled receptors. Pharmacol Ther 2008; 117:141–61

11. Robinson MB: The family of sodium-dependent glutamate transporters: A focus on the GLT-1/EAAT2 subtype. Neurochem Int 1998; 33:479–91

12. Rothstein JD, Dykes-Hoberg M, Pardo CA, Bristol LA, Jin L, Kuncl RW, Kanai Y, Hediger MA, Wang Y, Schielke JP, Welty DF: Knockout of glutamate transporters reveals a major role for astroglial transport in excitotoxicity and clearance of glutamate. Neuron 1996; 16:675–86

13. Suto T, Severino AL, Eisenach JC, Hayashida K: Gabapentin increases extracellular glutamatergic level in the locus coeruleus *via* astroglial glutamate transporter-dependent mechanisms. Neuropharmacology 2014; 81:95–100

14. Yoshizumi M, Eisenach JC, Hayashida K: Valproate prevents dysregulation of spinal glutamate and reduces the development of hypersensitivity in rats after peripheral nerve injury. J Pain 2013; 14:1485–91

15. Aston-Jones G, Cohen JD: An integrative theory of locus coeruleus-norepinephrine function: Adaptive gain and optimal performance. Annu Rev Neurosci 2005; 28:403–50

16. Kim SH, Chung JM: An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. Pain 1992; 50:355–63

17. Hayashida K, Obata H, Nakajima K, Eisenach JC: Gabapentin acts within the locus coeruleus to alleviate neuropathic pain. ANESTHESIOLOGY 2008; 109:1077–84

18. Paxinos G, Watson C: The Rat Brain in Stereotaxic Coordinates. San Diego, Academic Press, 2007

19. Randall LO, Selitto JJ: A method for measurement of analgesic activity on inflamed tissue. Arch Int Pharmacodyn Ther 1957; 111:409–19

20. Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72:248–54

21. Hayashida K, Clayton BA, Johnson JE, Eisenach JC: Brain derived nerve growth factor induces spinal noradrenergic fiber sprouting and enhances clonidine analgesia following nerve injury in rats. Pain 2008; 136:348–55

22. Hobo S, Eisenach JC, Hayashida K: Up-regulation of spinal glutamate transporters contributes to anti-hypersensitive effects of valproate in rats after peripheral nerve injury. Neurosci Lett 2011; 502:52–5

23. Eisenach JC, DuPen S, Dubois M, Miguel R, Allin D: Epidural clonidine analgesia for intractable cancer pain. The Epidural Clonidine Study Group. Pain 1995; 61:391–9

24. Sindrup SH, Otto M, Finnerup NB, Jensen TS: Antidepressants in the treatment of neuropathic pain. Basic Clin Pharmacol Toxicol 2005; 96:399–409

25. Xu Z, Tong C, Pan HL, Cerda SE, Eisenach JC: Intravenous morphine increases release of nitric oxide from spinal cord by an alpha-adrenergic and cholinergic mechanism. J Neurophysiol 1997; 78:2072–8

26. Alba-Delgado C, Mico JA, Sánchez-Blázquez P, Berrocoso E: Analgesic antidepressants promote the responsiveness of locus coeruleus neurons to noxious stimulation: Implications for neuropathic pain. Pain 2012; 153:1438–49

27. Viisanen H, Pertovaara A: Influence of peripheral nerve injury on response properties of locus coeruleus neurons and coeruleospinal antinociception in the rat. Neuroscience 2007; 146:1785–94

28. Brightwell JJ, Taylor BK: Noradrenergic neurons in the locus coeruleus contribute to neuropathic pain. Neuroscience 2009; 160:174–85

29. Mao J, Mayer DJ, Price DD: Patterns of increased brain activity indicative of pain in a rat model of peripheral mononeuropathy. J Neurosci 1993; 13:2689–702

30. Ennis M, Aston-Jones G, Shiekhattar R: Activation of locus coeruleus neurons by nucleus paragigantocellularis or noxious sensory stimulation is mediated by intracoerulear excitatory amino acid neurotransmission. Brain Res 1992; 598:185–95

31. Devilbiss DM, Waterhouse BD: Phasic and tonic patterns of locus coeruleus output differentially modulate sensory network function in the awake rat. J Neurophysiol 2011; 105:69–87

32. Jepma M, Nieuwenhuis S: Pupil diameter predicts changes in the exploration-exploitation trade-off: Evidence for the adaptive gain theory. J Cogn Neurosci 2011; 23:1587–96

33. Murphy PR, Robertson IH, Balsters JH, O'connell RG: Pupillometry and P3 index the locus coeruleus-noradrenergic arousal function in humans. Psychophysiology 2011; 48:1532–43

34. Alba-Delgado C, Llorca-Torralba M, Horrillo I, Ortega JE, Mico JA, Sánchez-Blázquez P, Meana JJ, Berrocoso E: Chronic pain leads to concomitant noradrenergic impairment and mood disorders. Biol Psychiatry 2013; 73:54–62

35. Tohyama M, Oyamada H: Gene expression of neuroreceptors in the locus coeruleus of the rat. Microsc Res Tech 1994; 29:200–3

36. Fang L, Wu J, Lin Q, Willis WD: Protein kinases regulate the phosphorylation of the GluR1 subunit of AMPA receptors of spinal cord in rats following noxious stimulation. Brain Res Mol Brain Res 2003; 118:160–5

37. Lu Y, Sun YN, Wu X, Sun Q, Liu FY, Xing GG, Wan Y: Role of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptor subunit GluR1 in spinal dorsal horn in inflammatory nociception and neuropathic nociception in rat. Brain Res 2008; 1200:19–26