Hypofunction of Glutamatergic Neurotransmission in the Periaqueductal Gray Contributes to Nerve-Injury-Induced Neuropathic Pain

Yu-Cheng Ho,¹ Jen-Kun Cheng,^{5,6,7} and Lih-Chu Chiou^{1,2,3,4}

¹Graduate Institute, ²Department of Pharmacology, ³Graduate Institute of Brain and Mind Sciences, College of Medicine, ⁴Neurobiology and Cognitive Science Center, National Taiwan University, Taipei 10051, Taiwan; ⁵Department of Anesthesiology, Mackay Memorial Hospital, Taipei 10449, Taiwan, ⁶Mackay Medicine, Nursing and Management College, Taipei 11260, Taiwan, and ⁷Department of Medicine, Mackay Medical College, New Taipei City 25245, Taiwan

Neuropathic pain, a chronic pain due to neuronal lesion, remains unaltered even after the injury-induced spinal afferent discharges have declined, suggesting an involvement of supraspinal dysfunction. The midbrain ventrolateral periaqueductal gray (vlPAG) is known to be a crucial supraspinal region for initiating descending pain inhibition, but its role in neuropathic pain remains unclear. Therefore, here we examined neuroplastic changes in the vlPAG of midbrain slices isolated from neuropathic rats induced by L5/L6 spinal nerve ligation (SNL) via electrophysiological and neurochemical approaches. Significant mechanical hypersensitivity was induced in rats 2 d after SNL and lasted for >14 d. Compared with the sham-operated group, vlPAG slices from neuropathic rats 3 and 10 days after SNL displayed smaller EPSCs with prolonged latency, less frequent and smaller miniature EPSCs, higher paired-pulse ratio of EPSCs, smaller AMPAR-mediated EPSCs, smaller AMPA currents, greater NMDAR-mediated EPSCs, greater NMDA currents, lower AMPAR-mediated/NMDAR-mediated ratios, and upregulation of the NR1 and NR2B subunits, but not the NR2A, GluR1, or GluR2 subunits, of glutamate receptors. There were no significant differences between day 3 and day 10 neuropathic groups. These results suggest that SNL leads to hypoglutamatergic neurotransmission in the vlPAG resulting from both presynaptic and postsynaptic mechanisms. Upregulation of NMDARs might contribute to hypofunction of AMPARs via subcellular redistribution. Long-term hypoglutamatergic function in the vlPAG may lead to persistent reduction of descending pain inhibition, resulting in chronic neuropathic pain.

Introduction

Neuropathic pain is one of leading causes of intractable pain. It is caused by a lesion or dysfunction in the CNS or/and peripheral nervous systems and manifests with persistent hyperalgesia and allodynia (Sandkühler, 2009). Although both central and peripheral mechanisms have been proposed, it remains unclear how neuropathy modulates neural circuits and leads to behavioral hypersensitivity.

In the periphery, ectopic discharges in injured primary afferent nerves was initially proposed to contribute to both the initiation and maintenance of neuropathic pain (Wall and Gutnick, 1974a, 1974b; Chaplan et al., 1994). However, it was later suggested that enhanced discharges in primary afferents are insufficient for maintaining long-term neuropathic pain status without other central mechanisms, because ectopic discharges in injured

nerves occurred 1 d after nerve lesion and declined gradually over time (Liu et al., 2000).

Increased descending pain facilitation at supraspinal nuclei has been demonstrated in neuropathic pain models. In rats with spinal nerve ligation (SNL), Burgess et al. (2002) found that microinjection of lidocaine, a Na⁺ channel blocker, into the rostral ventral medulla (RVM) impeded SNL-induced mechanical hypersensitivity and thermal hyperalgesia 6 d, but not 3 d, after SNL, suggesting that descending pain facilitation arising from neuroplastic changes in the RVM is necessary for maintaining, but not initiating, neuropathic pain. Consistently, 7 d after SNL, the spontaneous firing rate was significantly increased and decreased, respectively, in ON- and OFF-cells in the RVM, the cell types for pronociceptive and antinociceptive responses, respectively (Carlson et al., 2007; Gonçalves et al., 2007). Wei et al. (2008) found that glial-neuronal interactions in the RVM may contribute to the descending pain facilitation after nerve injury. Neuronal loss, possibly OFF-cell, in the RVM after SNL may shift the descending pain inhibition to facilitation (Leong et al., 2011). In the anterior cingulate cortex (ACC), presynaptic glutamate release and postsynaptic GluR1 expression were increased in mice 7 d after common peroneal nerve injury (Xu et al., 2008).

In addition to the RVM and ACC, the midbrain periaqueductal gray (PAG) plays an important role in pain regulation. It integrates inputs from a variety of higher centers, such as the hypothalamus and amygdala, and sends excitatory outputs to the

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Correspondence should be addressed to Lih-Chu Chiou, PhD, Professor, Department of Pharmacology, College of Medicine, National Taiwan University, No. 1, Jen-Ai Rd., Section 1, Taipei 10051, Taiwan. E-mail: lcchiou@ntu.edu.tw.

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RVM, which sends inhibitory outputs to the spinal dorsal horn, forming the PAG-RVM-spinal descending pain inhibitory pathway (Fields et al., 1976; Basbaum and Fields, 1984; Millan, 2002; Heinricher et al., 2009). Downregulation of this descending pain inhibition circuitry may also contribute to the pathogenesis of neuropathic pain. However, except for a few studies reporting changes of glutamate receptor expression in the PAG after nerve injury (Renno, 1998; Hu et al., 2009; Terashima et al., 2012), there has thus far been no study investigating neuroplastic changes and their underlying mechanism(s) in the PAG in neuropathic pain models.

In this study, we examined functional and cellular changes in the vPAG in rats receiving SNL, a model of neuropathic pain, to determine whether alteration of descending pain inhibition from the PAG is involved in the development or/and maintenance of nerve-injury-induced neuropathic pain.

Materials and Methods

Animals. All experiments adhered to the guidelines approved by the Institutional Animal Care and Use Committee of College of Medicine, National Taiwan University and followed the guidelines of the International Association for the Study of Pain. Efforts were made to minimize the suffering of the animals and the number of animals used in this study.

L5/L6 SNL. The neuropathic pain model induced by right L5/L6 SNL was established in male Sprague Dawley rats (6–8 weeks of age; LASCO) as described previously (Chu et al., 2011). Briefly, in rats anesthetized with isoflurane (5% for induction and 2% for maintenance), the dorsal vertebral column from L4 to S2 was exposed after a midline incision above the lumbar spine. The L5 and L6 spinal nerves were tightly ligated with 4-0 silk suture. The incision was then closed and the rat was allowed to recover and returned to its home cage. Sham-operated rats underwent the same operation and handling as nerve-ligated rats except that they did not receive SNL. Rats that exhibited motor impairment such as paw dragging or dropping or no hypersensitivity to the subsequent innocuous mechanical stimulation were excluded from further experiments.

von Frey filament testing. Mechanical hypersensitivity in rats after SNL was evaluated by the von Frey filament testing as described previously (Cheng et al., 2003). Rats were kept in suspended wire-mesh cages individually and allowed to acclimate for 30 min. The paw-withdrawal threshold to the punctate mechanical stimulation induced by calibrated von Frey filaments with logarithmically incremental stiffness from 0.6 to 26 g (Stoelting) was measured in the hindpaw ipsilateral or contralateral to the ligation side. Beginning with a 4 g probe, the filament was applied vertically to the plantar surface of the rat paw for 6 s while the filament was bent. Brisk withdrawal or paw flinching was considered as a positive response. If a positive response was observed, the filament with the next lower force

Figure 1. Schema demonstrating the locations of stimulating and recording electrodes in a vPAG slice dissected from a neuropathic rat induced by L5/L6 SNL. **A**, Coronal midbrain slice containing the vPAG dissected from a sham-operated rat and from an NP3 or NP10 rat. **B**, Left: Photomicrograph of a representative coronal midbrain slice where the vPAG region, the area between two white dotted lines, was selected for conducting electrophysiological recordings. The stimulation electrode (S) was placed 50–200 μ m away from the recording electrode. IPAG indicates lateral PAG; Aq, central aqueduct. Right: Higher-power image demonstrating a vPAG neuron visualized under an IR-DIC microscope, where the whole-cell recording was conducted with a glass recording microelectrode (R). **C**, Development of mechanical hypersensitivity in rats receiving L5/L6 SNL. The ordinate is the paw withdrawal threshold to von Frey filament stimulation, expressed as gram force, and recorded as described in Materials and Methods. Note that mechanical hypersensitivity occurred within 2 d after SNL and lasted for 2 weeks in the ipsilateral ($\bullet$), but not the contralateral ($\blacksquare$), hindpaw to the injured side. The sample size (n) is denoted in parentheses for all figures. Two-way ANOVA with repeated measures over time analysis indicated significant differences with a main effect of group ($F_{(3,144)} = 171.5$; $p < 0.001$) and time ($F_{(9,144)} = 4.329$; $p < 0.001$) and in the interaction of group by time ($F_{(27,144)} = 3.769$; $p < 0.001$). ** $p < 0.01$, *** $p < 0.001$ compared with the sham group (post hoc Bonferroni's comparisons of the data points denoted).

was applied; otherwise, the next stiffer filament was used. The stimulus force (in g) producing a 50% likelihood of paw withdrawal was determined as the withdrawal threshold using the Dixon “up-down” method, as described previously (Chaplan et al., 1994).

Brain slice preparations. Under anesthesia with isoflurane, 6- to 7-week-old rats were decapitated with a guillotine. Coronal midbrain slices (300 μ m) containing the PAG (Fig. 1A) were dissected, as described previously (Chiou and Huang, 1999) with modification, from neuropathic rats that had received SNL for 3 d (NP3 group) or 10 d (NP10 group) or from sham-operated rats. After dissection, slices were equilibrated in artificial CSF (aCSF) at room temperature for at least 1 h before

recording. The aCSF consisted of the following (in mM): 117 NaCl, 4.5 KCl, 2.5 CaCl₂, 1.2 MgCl₂, 1.2 NaH₂PO₄, 25 NaHCO₃, and 11.4 dextrose bubbled with 95% O₂/5% CO₂, pH 7.4. During recordings, one slice was mounted on a submerged recording chamber and continuously perfused with oxygenated aCSF at 2–3 ml/min.

Electrophysiological recordings. Visualized patch-clamp whole-cell recordings were performed in neurons located in the ventrolateral area of PAG slices as described previously (Ho et al., 2011; Liao et al., 2011) with modification. The vIPAG neurons were visualized under a stage-fixed upright IR-DIC microscope (BX51WI; Olympus) equipped with a 40× water-immersion objective (Fig. 1B). Recording microelectrodes were filled with a Cs⁺-based internal solution except the experiments for measuring neuronal excitability, in which a K⁺-based internal solution was used. The Cs⁺-based internal solution consisted of the following (in mM): 110 Cs⁺ gluconate, 5 TEA, 5 QX314, 0.5 CaCl₂, 5 BAPTA, 10 HEPES, 5 MgATP, and 0.33 GTP-Tris (liquid junction potential = 14.6 mV). The K⁺-based solution contained the following (in mM): 125 K⁺ gluconate, 5 KCl, 0.5 CaCl₂, 5 BAPTA, 10 HEPES, 5 MgATP, and 0.33 GTP-Tris, pH 7.3, 280 mOsm/L (liquid junction potential = 11.4 mV). The electrode resistance was 4–6 MΩ.

Neuronal excitability. The membrane excitability of the recorded neuron was measured in the current-clamp mode by the number of action potentials elicited by intracellular injection of 20, 40, and 60 nA depolarizing currents for 200 ms.

EPSCs. In vIPAG slices, we have proven that glutamatergic EPSCs can be evoked by local stimulation and that fast EPSCs, which are mediated by AMPARs, can be recorded at −70 mV after blocking GABAergic transmission by (−)-bicuculline methiodide (bicuculline), a GABA_A receptor antagonist (Chiou and Chou, 2000). Therefore, EPSCs were recorded at −70 mV in the presence of 10 μM bicuculline. They were evoked at 0.05 Hz by square wave pulses (10–50 V, 150 μs) from a stimulator (S88; Grass Telefactor) through a bipolar concentric electrode (Frederick Haer) placed 50–200 μm away from the recording electrode (Fig. 1B). In each neuron, the stimulation voltage was adjusted to elicit an EPSC with an amplitude 70–80% of the maximal amplitude, except the experiment for establishing the input–output (I–O) relationship (see below).

Miniature EPSCs. Miniature EPSCs (mEPSCs) were recorded in the presence of bicuculline and tetrodotoxin (TTX; 1 μM), a Na⁺ channel blocker that inhibits action-potential-driven spontaneous neurotransmitter release. Mini Analysis 6.0 software (Synaptosoft) was used to analyze the amplitude and frequency of mEPSCs. The Kolmogorov–Smirnov test was used to compare the cumulative probability of the frequency and amplitude of mEPSCs.

I–O relationship. The I–O relationship of glutamatergic transmission in vIPAG slices was constructed by the stimulation intensity (input) and the amplitude of EPSCs (output). In each neuron, 12 EPSCs were evoked by 10, 20, 30, 40, and 50 V and averaged. When the latency of the EPSC was measured, it was defined as the time difference between the stimulation artifact and the takeoff of the EPSC.

Paired-pulse ratio of EPSCs. When the paired-pulse ratio (PPR) of EPSCs was examined, paired pulses with interstimulus intervals of 25, 50, 75, 100, and 200 ms were given every 20 s as described previously (Xu et al., 2008). Every three pairs of EPSCs evoked at each interval were averaged and the PPR was defined as the ratio of the averaged amplitude of the second EPSC (EPSC₂) to that of the first EPSC (EPSC₁).

Current–voltage curves of EPSC_{AMPA} and EPSC_{NMDA}. AMPA-receptor-mediated EPSCs (EPSC_{AMPA}s) were recorded in the presence of 50 μM 2-amino-5-phosphonopentanoic acid (APV), an NMDAR blocker, and 10 μM bicuculline. NMDAR-mediated EPSCs (EPSC_{NMDA}s) were recorded in the presence of 20 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), an AMPAR blocker, and 10 μM bicuculline. EPSC_{AMPA}s and EPSC_{NMDA}s were recorded at −60, −40, −20, 0, +20, and +40 V and nine EPSCs at each voltage were averaged to establish their current–voltage (I–V) curves.

EPSC_{AMPA}/EPSC_{NMDA} ratio. To compare the postsynaptic response of glutamatergic transmission among different groups, the ratio of EPSC_{AMPA}/EPSC_{NMDA} recorded in the same neuron was examined as described previously (Ungless et al., 2001). In the presence of bicuculline

(10 μM), the EPSCs in a neuron were first recorded at −70 mV where NMDARs are blocked by the Mg²⁺ in the aCSF to assess the EPSC_{AMPA}s; then, in the same neuron, 20 μM CNQX was also added and EPSCs were recorded at +40 mV to assess the EPSC_{NMDA}s. In every neuron, nine EPSC_{AMPA}s and EPSC_{NMDA}s were averaged and the ratio of EPSC_{AMPA}/EPSC_{NMDA} was calculated.

AMPA and NMDA currents. AMPA- and NMDA-induced currents were elicited by puff application of AMPA and NMDA, respectively, to the surface of the recorded vIPAG neuron and were recorded at −70 mV and +40 mV, respectively, as described previously (Lee et al., 2008). Briefly, the AMPA (1 μM) or NMDA (10 μM) solution was loaded into a glass pipette (2 μm tip diameter) and ejected by a Picospritzer (General Valve). The solution was ejected onto the recorded neuron for 5 s by applying an 8 psi pressure via the glass pipette, which was 10–20 μm above the recorded neuron. In the presence of TTX (1 μM) and bicuculline (10 μM), AMPA and NMDA currents were recorded with APV (50 μM) and CNQX (20 μM), respectively.

Western blotting. Glutamate receptor subunits in the rat vIPAG were measured as described previously (Bagley et al., 2005) with modifications. Briefly, the midbrain block containing the PAG was isolated from three groups of rats and sliced into 300-μm-thick sections. The vIPAG region was microdissected bilaterally from each section. The solubilization of membrane receptors was conducted as described previously (Sheng et al., 2002). Microdissected vIPAG samples from each rat were collected, lysed in a nondenaturing-detergent-containing tissue protein extraction reagent (T-PER, catalog #78510; Pierce Biotechnology), sonicated, and centrifuged at 15,000 × g at 4°C for 30 min. The supernatant was then assayed for the total protein amount using a protein assay kit (Bio-Rad). Each brain tissue sample taken from a rat was separated in an 8% SDS-PAGE gel for GluR1, GluR2, and NMDAR1 subunit analysis or in a 6% gel for NMDAR2A and NMDAR2B subunit analysis. After transferring onto nitrocellulose membranes, blots were blocked with a buffer solution containing 5% milk and 0.1% Tween 20 in PBS at pH 7.2 for 1 h. The blot was incubated with the primary antibody recognizing the respective subunit of glutamate receptors overnight at 4°C, including GluR1 (1:10,000, catalog #3861-1, lot YH122305C; Epitomics), GluR2 (1:1000, catalog #3520-1, lot YH081712D; Epitomics), NR1 (1:1000, catalog #2824-1, lot YG060310C; Epitomics), NR2A (1:1000, catalog #4205S, lot 1; Cell Signaling Technology), or NR2B (1:1000, catalog #4212S, lot 1; Cell Signaling Technology). All antibodies are rabbit monoclonal antibodies except the NR2A antibody, which is polyclonal. The specificity of the antibodies purchased was not reconfirmed in this study because it was already confirmed by Cell Signaling Technology with preabsorption control experiments. The membrane was then washed with Tris-buffered saline and incubated for 1 h with HRP-conjugated secondary antibody. Immunoreactivity was detected using enhanced chemiluminescence (PerkinElmer Life Sciences). The loading and blotting of equal amounts of protein were verified by reprobing the membrane with anti-β-actin antiserum (1:1000, catalog #MAB1501, lot LV1435643; Millipore). Immunoblots were quantified using Image Quant 5.2 software (Molecular Dynamics).

Statistical analysis. Data are expressed as the mean ± SEM; *n* indicates the number of the animals sampled in behavioral or Western blotting experiments or the number of neurons recorded in electrophysiological experiments. From each rat, 4–5 slices were dissected and 1–2 neurons were sampled (1 neuron from 1 slice). Because the neuropathic pain (NP) groups had fewer healthy neurons, successful recordings were usually obtained after having examined 4–5 slices from a neuropathic rat but only 1–2 slices from a sham-control rat. Statistical comparison was performed by one-way ANOVA (using Dunnett's correction for *post hoc* analysis), two-way ANOVA (using Bonferroni correction for *post hoc* analysis), or unpaired Student's *t* test where appropriate. Differences were considered significant if *p* < 0.05.

Chemicals. (−)-Bicuculline methiodide, TTX, CNQX, AMPA, NMDA, and APV were purchased from Tocris Bioscience. All drugs were prepared as a 1000-fold concentrated stock solution and diluted to the final concentration with aCSF. CNQX was dissolved in dimethylsulfoxide. The final concentration of dimethylsulfoxide was <0.1%, which had no effect *per se*. Other drugs were dissolved in deionized water.

Figure 2. SNL led to low efficiency of I–O transfer of glutamatergic transmission in vPAG slices. **A**, I–O relationship of glutamatergic transmission established by the stimulation intensity (synaptic input) and the amplitude of EPSC (output) in slices isolated from sham-operated (Sham), NP3, and NP10 rats. Representative EPSCs evoked by 10, 20, 30, 40, and 50 V, respectively, were recorded in a vPAG neuron of a slice isolated from the sham, NP3, or NP10 group. **B**, Averaged I–O curves of glutamatergic transmission in sham, NP3 and NP10 groups. The I–O curve was constructed by the percentage increment of the EPSC evoked at each stimulation intensity in each neuron, taking the EPSC evoked by 10 V as 100%. Note that the slope of the I–O curve in NP3 (Δ) and NP10 ($\square$) groups was significantly smaller than that in the sham group ($\circ$). Two-way ANOVA with repeated measures over intensity analysis indicated significant differences with a main effect of group ($F_{(2,72)} = 20.43$; $p < 0.001$) and intensity ($F_{(4,72)} = 42.93$; $p < 0.001$) and in the interaction of group by intensity ($F_{(8,72)} = 7.986$; $p < 0.001$). $^{**}p < 0.01$, $^{***}p < 0.001$ compared with the sham group (*post hoc* Bonferroni's comparisons of the data points denoted). n in the parentheses are the number of tested neurons in all the figures with electrophysiological experiments. Usually, 1 neuron was recorded from 1 slice and 1–2 slices were sampled from a sham-operated rat, whereas 1–2 neurons were successfully recorded from 4–5 slices isolated from an NP rat.

Results

Establishment of mechanical hypersensitivity in SNL rats

After SNL, the hindpaw withdrawal threshold to mechanical stimulation induced by the von Frey filament in rats was significantly decreased in the ipsilateral, but not contralateral, side compared with the sham-operated group (Fig. 1C). This mechanical hypersensitivity reached steady state 2 d after SNL and lasted for at least 14 d (Fig. 1C). The paw withdraw threshold on day 3 (2.26 ± 0.23 g, $n = 5$) after SNL was not significantly different from that on day 10 (2.25 ± 0.34 g, $n = 5$) or that on day

Figure 3. SNL led to reduced presynaptic glutamate release, revealed by increased PPR of EPSCs, in vPAG slices. Paired EPSCs were evoked by paired pulses with intervals of 25, 50, 75, 100, and 200 ms. PPR was measured by the amplitude ratio of the second EPSC to the first one. **A**, Representative paired EPSCs evoked by 25, 50, and 75 ms-separated paired pulses in sham, NP3, and NP10 groups. **B**, PPR evoked at various intervals in the sham ($\circ$), NP3 (Δ), and NP10 ($\square$) groups. Two-way ANOVA with repeated measures over interstimulus interval analysis indicated significant differences with a main effect of group ($F_{(2,84)} = 11.25$; $p < 0.001$) and interstimulus interval ($F_{(4,84)} = 6.054$; $p < 0.001$) and in the interaction of group by interstimulus interval ($F_{(8,84)} = 2.799$; $p < 0.01$). One-way ANOVA analysis indicated significant differences at 25 and 50 ms intervals in the NP groups compared with the sham group (25 ms interval, $F_{(2,21)} = 8.682$, one-way ANOVA, $p < 0.01$, *post hoc* Dunnett's test; 50 ms interval, $F_{(2,21)} = 6.438$, one-way ANOVA, $p < 0.01$, *post hoc* Dunnett's test) $^{*}p < 0.05$, $^{**}p < 0.01$ compared with the sham group.

14 (1.74 ± 0.13 g, $n = 5$), suggesting that sustained ipsilateral neuropathic pain is induced from day 3 after SNL in rats. This time course is consistent with findings in which neuroplastic changes in the RVM were studied in the same model (Burgess et al., 2002). Therefore, in the subsequent electrophysiological studies, PAG slices were isolated from rats 3 and 10 d after SNL defined as the NP3 and NP10 groups, respectively.

SNL leads to low I–O transfer efficiency in vPAG glutamatergic transmission

Compared with the sham group, it was more difficult to obtain successful whole-cell recordings and elicit EPSCs in the vPAG slices isolated from neuropathic rats. Because fewer healthy neurons were found in the brain slices dissected from NP groups, the chances of obtaining successful whole-cell configuration in the NP groups were less than those in the sham group. The following data were obtained in neurons with successfully whole-cell recordings. The I–O relationships of the glutamatergic transmission established by the magnitude of EPSC versus the stimulation intensity in vPAG slices isolated from sham, NP3, and NP10

Figure 4. SNL led to less frequent and smaller miniature EPSCs in vIPAG slices. mEPSCs were recorded at -70 mV in the presence of $1 \mu\text{M}$ TTX and $10 \mu\text{M}$ bicuculline. **A**, Representative trace of mEPSCs recorded in a vIPAG slice isolated from a sham, NP3, or NP10 rat or a rat from the sham group treated with $20 \mu\text{M}$ CNQX. **B**, **C**, Cumulative probability histograms of the interevent interval (**B**) or amplitude (**C**) of mEPSCs in the three groups. Note that the interevent interval of mEPSCs (**B**) was significantly longer and the amplitude (**C**) was smaller in NP3 and NP10 groups than in the sham group ($p < 0.01$, Kolmogorov–Smirnov test). **D**, **E**, Bar charts of averaged frequency, ($F_{(2,25)} = 26.23$, one-way ANOVA, $p < 0.001$; **D**), and amplitude, ($F_{(2,25)} = 5.647$, one-way ANOVA, $p < 0.01$; **E**) of mEPSCs in the sham, NP3, and NP10 groups. * $p < 0.05$, ** $p < 0.01$ compared with the sham group (*post hoc* Dunnett's test).

groups were compared. The amplitude of EPSCs increased gradually with increasing stimulation intensity from 10 to 50 V in all the three groups (Fig. 2); however, compared with the sham group, smaller EPSCs were evoked with the same stimulation intensity in both the NP3 and NP10 groups (Fig. 2). Increasing stimulation intensity induced limited increases in the amplitude of EPSCs in both the NP3 and NP10 groups. NP groups showed a significant decrease in stimulus-induced responses. This led to a flat slope in the I–O curve in either NP3 or NP10 group, which was markedly different from the steep slope in the sham group (Fig. 2B). The percentage increment of EPSCs by increasing stimulation intensity from 10 to 50 V in the sham group ($924 \pm 124\%$, $n = 6$) was significantly higher than that in the NP3 ($444 \pm 84\%$,

$n = 9$) or NP10 ($289 \pm 55\%$, $n = 6$) groups, whereas the increments between the NP3 and NP10 groups were not significantly different ($p = 0.19$; Fig. 2B). These results suggest that the I–O transfer efficiency of excitatory synaptic transmission is impaired in the vIPAG of rats after SNL.

SNL leads to hypofunction of glutamatergic neurotransmission in the vIPAG via both presynaptic and postsynaptic mechanisms

Increased PPR in SNL groups

To determine whether the impairment of the glutamatergic I–O relationship in the vIPAG could be attributed to presynaptic or postsynaptic changes after SNL, we compared the PPR of EPSCs,

which depends on the presynaptic release machinery (Zucker and Regehr, 2002). In the sham group, no paired-pulse facilitation of EPSCs was elicited when the inter-pulse interval was 25 ms ($p = 0.63$, one sample t test) and 50 ms ($p = 0.54$, one sample t test). However, significant paired-pulse facilitation was observed at 25 and 50 ms intervals in both the NP3 and NP10 groups (Fig. 3). PPRs in the NP3 and NP10 groups were significantly greater than those in the sham group (Fig. 3B). There was no significant difference in the PPRs at 25 ms ($p = 0.31$) and 50 ms ($p = 0.84$) intervals between the NP3 and NP10 groups, suggesting that the presynaptic glutamate release in the vPAG of neuropathic rats is impaired.

Lower mEPSC frequency and amplitude in SNL groups

To further investigate the presynaptic and postsynaptic contributions in SNL-induced hypofunction of vPAG glutamatergic transmission, we compared mEPSCs in slices from the three groups. The mEPSCs recorded in the vPAG slices isolated from the sham group were blocked by CNQX, suggesting that they are AMPAR mediated (Fig. 4A). In both the NP3 and NP10 groups, mEPSCs were smaller and less frequent than in the sham group (Fig. 4A). The mean frequency of mEPSCs in the NP3 and NP10 groups was only $21.9 \pm 6\%$ and $27.6 \pm 5\%$ of the sham group, respectively (Fig. 4D) and their mean amplitude was $69.6 \pm 6\%$ and $68.5 \pm 6\%$ of the sham group, respectively (Fig. 4E). The finding of markedly lower mEPSC frequency in the NP groups, although it might be confounded by indistinguishable small mEPSCs, suggests a presynaptic contribution in SNL-induced hypoglutamatergic function in the vPAG.

The smaller mEPSCs in the NP groups could have been caused by a time-associated degradation of the slices: because there were fewer healthy neurons in the NP groups, more slices had to be examined. However, this possibility can be excluded by the result that the NP3 and NP10 groups still had significantly smaller mEPSCs than the sham group when only the data from the first recorded slices were compared. The average mEPSC amplitude in the first recorded slices in the sham, NP3, and NP10 groups, respectively, was 22.94 ± 3.62 pA ($n = 5$), 14.92 ± 1.55 pA ($n = 8$), and 15.71 ± 1.33 pA ($n = 7$) ($F_{(2,17)} = 5.096$, one-way ANOVA, $p < 0.05$, *post hoc* Dunnett's test comparisons).

Reduced AMPA currents and enhanced NMDA currents in the NP groups

The finding of smaller AMPAR-mediated mEPSCs in the NP groups (Fig. 4A) suggests an impairment in the function or/and number of postsynaptic AMPARs or a decrease in the presynaptic vesicle size in the vPAG after SNL. Therefore, in these three

Figure 5. SNL resulted in smaller AMPA currents and greater NMDA currents in vPAG slices. AMPA and NMDA currents were induced by puff application (8 psi) of AMPA (1 μ M) and NMDA (10 μ M) for 5 s, respectively, onto the recorded neurons. AMPA currents were recorded at -70 mV in the presence of 10 μ M bicuculline and 50 μ M APV. NMDA currents were recorded at $+40$ mV with 20 μ M CNQX and 10 μ M bicuculline. **A, B**, Representative AMPA (**A**) and NMDA (**B**) currents induced by AMPA and NMDA (horizontal bars), respectively, were recorded in vPAG slices isolated from sham, NP3, and NP10 groups. **C, D**, Normalized AMPA (**C**) and NMDA (**D**) currents recorded in the sham (○), NP3 (△), and NP10 (□) groups. Normalized AMPA (**C**) and NMDA (**D**) currents were obtained by normalizing the currents recorded every 1 s during puff application of AMPA and NMDA in each neuron to the baseline current at -70 mV and $+40$ mV, respectively. **E, F**, Averaged AMPA (**E**) and NMDA (**F**) currents recorded from vPAG slices taken from sham, NP3, and NP10 rats. Note that the AMPA current was significantly smaller ($F_{(2,23)} = 7.596$, one-way ANOVA, $p < 0.01$; **E**) but the NMDA current was larger ($F_{(2,21)} = 6.296$, one-way ANOVA, $p < 0.01$; **F**) in the NP groups compared with the sham group. * $p < 0.05$, ** $p < 0.01$ compared with the sham group (*post hoc* Dunnett's test).

groups, we further compared AMPAR-mediated currents induced by exogenous AMPA and the current mediated by NMDARs, which are also important postsynaptic glutamatergic receptors in the vPAG (Chiou and Chou, 2000).

Puff application of AMPA (1 μ M) for 5 s onto the vPAG neurons held at -70 mV induced an inward AMPA current (Fig. 5A). These AMPA-induced currents in the sham group were significantly larger than those in both the NP3 and NP10 groups, but were not significantly different between the NP3 and NP10 groups (Fig. 5A, C, E).

Puff application of NMDA (10 μ M) for 5 s onto the vPAG neurons held at $+40$ mV induced an outward current (Fig. 5B). In contrast to AMPA currents, NMDA-induced currents in the NP groups were significantly larger than those in the sham group

Figure 6. SNL resulted in reduced EPSC_{AMPA}s and enhanced EPSC_{NMDA}s in vPAG slices. EPSC_{AMPA}s were recorded with 50 μ M APV (**A**) and EPSC_{NMDA}s were recorded with 20 μ M CNQX (**B**) in the presence of 10 μ M bicuculline at -60 , -40 , -20 , 0 , $+20$, and $+40$ mV. The I - V curves of EPSC_{AMPA}s (**C**) and EPSC_{NMDA}s (**D**) in sham ($\circ$; gray line), NP3 ($\triangle$; blue line), and NP10 ($\square$; black line) groups. **E**, **F**, Averaged EPSC_{AMPA}s recorded at -60 mV (**E**) and EPSC_{NMDA}s at $+40$ mV (**F**) in the three groups. Note that the EPSC_{AMPA} was significantly smaller ($F_{(2,17)} = 5.742$, one-way ANOVA, $p < 0.05$) but the EPSC_{NMDA} was larger ($F_{(2,17)} = 5.369$, one-way ANOVA, $p < 0.05$) in the NP groups than in the sham group. * $p < 0.05$ compared with the sham group (*post hoc* Dunnett's test).

(Fig. 5B,D,F). Similarly, no significant difference in NMDA-induced currents was observed between the NP3 and NP10 groups. These results suggest that the AMPAR response to exogenous ligand is decreased but the NMDAR response is enhanced after SNL.

Reduced EPSC_{AMPA}s and enhanced EPSC_{NMDA}s in the NP groups
The finding that SNL inversely altered the responses of AMPARs and NMDARs to their respective exogenous ligands is interesting. We further examined whether SNL also affected the responses of glutamate receptors in the vPAG to the endogenous ligand glutamate, which mediates EPSCs. EPSC_{AMPA}s and EPSC_{NMDA}s evoked by local stimulation can be isolated by APV (50 μ M) and CNQX (20 μ M), respectively, in the presence of bicuculline (10 μ M; Chiou and Chou, 2000). As shown in Figure 6A, C, E, EPSC_{AMPA}s in both the NP3 and NP10 groups were significantly smaller than those in the sham group. The reversal potential of the I - V curve of EPSC_{AMPA}s in the sham group, being ~ 0 mV, was not significantly different from that in either the NP3 or NP10 group (Fig. 6C). The average amplitudes of EPSC_{AMPA}s recorded at -60 mV, as demonstrated in Figure 6E, in the NP3

and NP10 groups were significantly smaller than that in the sham group (Fig. 6E).

EPSC_{NMDA}s in both the NP3 and NP10 groups were larger than those in the sham group, unlike EPSC_{AMPA}s, which were smaller (Fig. 6B). The reversal potential of the I - V curve of EPSC_{NMDA}s in the sham group, being ~ 0 mV, was also not significantly different from that in either the NP3 or NP10 group (Fig. 6D). The average amplitudes of EPSC_{NMDA}s recorded at $+40$ mV in the NP3 and NP10 groups were significantly larger than those in the sham group (Fig. 6F). Neither EPSC_{AMPA}s (Fig. 6E) nor EPSC_{NMDA}s (Fig. 6F) of the NP3 and NP10 groups were significantly different. These results confirm that SNL led to decreased AMPAR response and enhanced NMDAR response to both exogenous and endogenous ligands.

We further compared the ratio of EPSC_{AMPA}/EPSC_{NMDA} in the same neuron with the same stimulation voltage, which corrects the variations from pre-synaptic release conditions due to different stimulation voltages among slices. In the presence of 10 μ M bicuculline, EPSC_{AMPA}s (Fig. 7A, downward deflection) were recorded at -70 mV first, and then in the same neuron the EPSC_{NMDA}s (Fig. 7A, upward deflection) were recorded at $+40$ mV after further addition of 20 μ M CNQX. The ratios of EPSC_{AMPA}/EPSC_{NMDA} in the NP3 and NP10 groups were significantly lower than that in the sham group (Fig. 7B), whereas there was no significant difference between the NP3 and NP10 groups ($p > 0.05$; Fig. 7B).

To investigate whether the neuroplastic change in the vPAG is injury-side specific, we compared changes of EPSC_{AMPA}/EPSC_{NMDA} ratio in the vPAG ipsilateral and contralateral to the injury side in neuropathic rats. The ratios of EPSC_{AMPA}/EPSC_{NMDA} in ipsilateral and contralateral vPAG neurons were not different in either the NP3 (ipsilateral: 1.92 ± 1.22 , $n = 3$; contralateral: 1.79 ± 0.27 , $n = 6$; $p > 0.05$) or NP10 (ipsilateral: 2.49 ± 0.4 , $n = 4$; contralateral: 1.86 ± 0.61 , $n = 5$; $p > 0.05$) group, whereas they were significantly lower than the ratio of EPSC_{AMPA}/EPSC_{NMDA} 5.7 ± 1.03 ($n = 12$) in the sham group. This suggests that unilateral SNL causes bilateral impairment of glutamatergic neurotransmission in the vPAG.

Upregulation of NR1 and NR2 subunits of NMDARs, but not the GluR1 or GluR2 subunit of AMPARs, in neuropathic rats

The changes in AMPAR and NMDAR responses can be attributed to an alteration in receptor expression, receptor sensitivity, and/or receptor function (Perkel and Nicoll, 1993). Therefore, we further quantified the protein levels of the constitutive subunits of the AMPARs GluR1 and GluR2 and the NMDARs NR1, NR2A, and NR2B in the vPAG region of three groups of rats. The protein level of either the GluR1 (Fig. 8A) or the GluR2 (Fig. 8B) subunit of AMPARs in the vPAG of the NP3 or NP10 group was

Figure 7. The ratio of EPSC_{AMPA}/EPSC_{NMDA} recorded from the same neuron was significantly reduced in the vPAG of rats after SNL. The EPSC_{AMPA}s and EPSC_{NMDA}s were recorded in the same neuron at -70 and $+40$ mV, respectively. **A**, Representative trace of EPSC_{AMPA}s (downward) and EPSC_{NMDA}s (upward) recorded from a vPAG neuron of the slice dissected from the sham, NP3, and NP10 groups. **B**, Averaged ratio of EPSC_{AMPA}/EPSC_{NMDA} in the sham, NP3, and NP10 groups. The average of the ratio of EPSC_{AMPA}/EPSC_{NMDA}, which was calculated from each recorded neuron, in the NP groups was significantly lower than in the sham group ($F_{(2,27)} = 8.354$, one-way ANOVA, $p < 0.01$). ** $p < 0.01$ compared with the sham group (*post hoc* Dunnett's test).

not significantly different from that in the sham group. However, the expression levels of the NR1 (Fig. 8C) and NR2B (Fig. 8D), but not the NR2A (Fig. 8E), subunits of NMDARs in the vPAG were significantly higher in both the NP3 and NP10 groups than in the sham group. These results suggest that the increased NMDAR response in the vPAG after SNL is attributed to an upregulation of NR1 and NR2B proteins, whereas the decreased AMPAR response is due to a decrease in the function, but not protein level, of AMPARs.

SNL results in longer EPSC latency and lower excitability in vPAG neurons

The latencies of EPSCs in vPAG slices in both the NP3 and NP10 groups were longer than that in the sham group (Fig. 2, traces). Although the latency varied depending on the distance between stimulation and recording electrodes, we kept it within 50–200 μ m in each slice and plotted the distribution of the latency of the EPSC recorded from all experiments described above in Figure 9A. The averaged latencies of EPSCs in the NP3 (4.04 ± 0.29 ms, $n = 25$) and NP10 (3.82 ± 0.29 ms, $n = 22$) groups were significantly longer (2.90 ± 0.21 ms, $n = 23$) than that in the sham group, whereas there was no significant difference between the NP3 and NP10 groups. Therefore, SNL may lead to nerve conduction block in glutamatergic transmission in the vPAG.

We further examined whether the neuronal excitability in the NP groups was reduced, which may lead to conduction block. Indeed, the neuronal excitability, as measured by the number of action potentials elicited by injecting a depolarizing current, was significantly lower in either the NP3 or NP10 group than in the control group (Fig. 9B). The numbers of action potentials induced by a 60 nA-current injection for 200 ms in both the NP3

(1.92 ± 0.65 , $n = 12$) and NP10 (1.8 ± 0.81 , $n = 15$) groups were significantly lower than that (7.82 ± 1.03 , $n = 11$) in the sham group ($F_{(2,35)} = 15.76$, one-way ANOVA, $p < 0.0001$, *post hoc* Dunnett's test comparisons). However, the passive membrane properties, including the membrane potential (sham: -67.9 ± 2.3 mV, $n = 11$; NP3: -66.34 ± 3.5 mV, $n = 12$; NP10: -67.29 ± 2.8 mV, $n = 15$), input resistance (sham: 780 ± 76 M Ω , $n = 11$; NP3: 792 ± 94 M Ω , $n = 12$; NP10: 782 ± 97 M Ω , $n = 15$), and membrane capacitance (sham: 48.9 ± 5.1 pF, $n = 11$; NP3: 39.9 ± 3.8 pF, $n = 12$; NP10: 47.0 ± 4.8 pF, $n = 15$) were not significantly different among these three groups.

Discussion

In this study, we found reduced glutamate release and an impaired AMPAR response in the vPAG of neuropathic rats 3 and 10 d after SNL. These neuroplastic changes may result in hypofunction of glutamatergic neurotransmission in the vPAG and reduction of descending pain inhibition, which may contribute to the initiation and maintenance of neuropathic pain.

Hypofunction of glutamatergic neurotransmission is attributed to both presynaptic and postsynaptic mechanisms

The reduced I–O relationship of EPSCs in the NP groups suggests that SNL induced hypofunctioning of glutamatergic neurotransmission in the vPAG, which may be attributed to both presynaptic and postsynaptic mechanisms. The findings of higher PPR and lower mEPSC frequency in the NP groups compared with the sham group support the contribution of presynaptic reduction of glutamate release in this hypofunctioning of neurotransmission. In the vPAG, EPSCs are mainly mediated by AMPARs (Chiou and Chou, 2000). The findings of smaller EPSCs, mEPSCs, EPSC_{AMPA}s, and AMPA currents in the NP groups suggest that SNL results in postsynaptic impairment of AMPAR function in the vPAG, because the expression of the AMPAR subunits was not altered.

Hypofunction of glutamatergic neurotransmission in the vPAG results in decreased descending pain inhibition, leading to neuropathic pain

Activation of the vPAG initiates the descending inhibitory pathway, leading to antinociception (Millan, 2002; Heinricher et al., 2009). It has been reported that the projection neurons in the vPAG form excitatory connections with OFF-cells in the RVM (Morgan et al., 2008), activation of which is associated with antinociceptive behaviors (Tortorici and Morgan, 2002; Maione et al., 2006). Therefore, hypofunctioning of glutamatergic neurotransmission in the vPAG after nerve injury may lead to reduced OFF-cell activity in the RVM and thus decreased descending inhibition onto the spinal dorsal horn, leading to neuropathic pain.

In various pain models, long-term neuroplastic changes in glutamatergic transmission have been reported in the spinal cord and several supraspinal pain-related nuclei, including the RVM, amygdala, and ACC (Seltzer et al., 1991; Coutinho et al., 1998; Neugebauer et al., 2003; Wu et al., 2005; Iwata et al., 2007; Xu et al., 2008; Zhang and Hammond, 2009; Da Silva et al., 2010). However, all of these studies found hyperfunctioning of glutamatergic transmission in these pain-related regions, which leads to increased descending pain facilitation. This is in contrast to our finding in a neuropathic pain model that glutamatergic transmission in the vPAG was decreased, resulting in decreased descending pain inhibition.

Figure 8. SNL resulted in upregulation of the NR1 and NR2B subunits of NMDARs, but not the GluR1 or GluR2 subunit of AMPARs in the vPAG. Representative Western blots and summarized bar graphs depicting the expression level of GluR1 (**A**), GluR2 (**B**), NR1 (**C**), NR2A (**D**), or NR2B (**E**) subunit protein in vPAG samples micropunched from sham, NP3, and NP10 rats. The top blots are the immunoreactive bands against the respective antibody against the GluR1, GluR2, NR1, NR2A, or NR2B subunit protein and the bottom blots are the bands for the housekeeping protein β -actin. The ordinate of the base graph is the expression level of the receptor protein, taking the sham group as 100%. * $p < 0.05$ compared with the sham group as 100% (one-sample t test).

Neuroplastic changes of glutamate receptors in chronic pain models

NMDARs

The findings of upregulation of the NR1 and NR2B subunits, but not the NR2A subunits, of postsynaptic NMDARs may explain the increased EPSC_{NMDA}s and NMDA currents in the vPAG of neuropathic rats. Although we did not reconfirm the specificity of the antibodies used, the same findings in NMDAR subunit changes have also been reported in the PAG of mice after SNL

(Renno, 1998) or inflamed with complete Freund's adjuvant (CFA; Renno, 1998; Hu et al., 2009). We found upregulated NMDARs and reduced glutamate release in the vPAG in the same neuropathic pain model. Glutamate release reduction could be the cause or effect of NMDAR upregulation after SNL. If it were the cause, then AMPARs might also be upregulated. Because AMPARs were not upregulated in the NP groups, it is more likely that reduced glutamate release is a compensatory change due to NMDAR upregulation.

Upregulation of the NR2B subunit, but not the NR2A subunit, was also found in the ACC of CFA-inflamed mice (Wu et al., 2005) and in the mouse spinal dorsal horn after SNL (Iwata et al., 2007). In the RVM, increased NR2A subunit phosphorylation in CFA-inflamed rats (Guo et al., 2006), increased NR1 subunit phosphorylation via glial–neuronal interactions in rats with infraorbital nerve injury (Wei et al., 2008), and NR1 subunit upregulation in an acid-induced muscle pain model (Da Silva et al., 2010) have been reported to contribute to descending pain facilitation. However, NMDAR subunit upregulations or phosphorylation in these areas, unlike the PAG, may increase nociceptive responses. Compared with other NR2 subunits, the NR2B and NR1 subunits seem to be more vulnerable to being upregulated.

The NMDAR, especially the NR2B subunit, in the vPAG has been suggested to be involved in pain processing, because CFA-induced inflammatory pain was blocked by intra-PAG injection of a selective NR2B blocker (Hu et al., 2009). Similarly, NMDAR activation in the RVM was also implicated in mediating nociceptive responses in inflammatory (Urban et al., 1999; Guan et al., 2002) and visceral (Coutinho et al., 1998) pain models. In the spinal dorsal horn, NMDAR activation is also believed to mediate neuropathic pain (Iwata et al., 2007). Therefore, NMDAR may contribute to long-term changes in pain processing whether as a cause or an effect.

In this study, upregulated NR1 and NR2B subunits may have resulted in excitotoxicity due to intracellular calcium overload through excessive activation of NMDARs (Choi, 1988), leading to neuronal loss in the vPAG after SNL. Neuronal loss is suggested from the findings of prolonged EPSC latency (possibly due to conduction block) and fewer healthy neurons recorded in the NP groups. The NMDAR-mediated neuronal loss has been attributed to several neurological disorders, including pain (Whiteside and Munglani, 2001; Leong et al., 2011; Mor et al., 2011). Neuronal loss of OFF-cells in the RVM (Leong et al.,

Figure 9. SNL resulted in a prolongation of the latency of EPSCs in vPAG slices. **A**, Latency of the EPSC measured as the time difference between the stimulation artifact and the takeoff of EPSC in the sham (○), NP3 (△), and NP10 (□) groups. Each data point represents the latency of one EPSC recorded in a neuron from the sham, NP3, and NP10 groups ($F_{(2,67)} = 5.137$, one-way ANOVA, $p < 0.01$). ** $p < 0.01$ compared with the sham group (post hoc Dunnett's test). **B**, Whole-cell current-clamp recordings of action potentials generated by current injection in vPAG neuron of the sham, NP3, and NP10 groups. Top: Representative recordings of action potentials induced by a current of 0, 20, 40, and 60 nA for 200 ms in the sham, NP3, and NP10 groups. Bottom: Averaged spike number induced by current injection in vPAG sham (○), NP3 (△), and NP10 (□) groups. Two-way ANOVA with repeated measures over current analysis indicated significant differences with a main effect of group ($F_{(2,105)} = 20.11$; $p < 0.0001$) and of current ($F_{(3,105)} = 47.14$; $p < 0.0001$), and in the interaction of group by current ($F_{(6,105)} = 12.08$; $p < 0.0001$). ** $p < 0.01$, *** $p < 0.001$ compared with the sham group (post hoc Bonferroni's comparisons of the data points denoted).

2011), GABAergic neurons in the spinal dorsal horn (Whiteside and Munglani, 2001; Moore et al., 2002), and possibly glutamatergic neurons in the PAG (Mor et al., 2011) have been reported in various neuropathic pain models.

AMPA

The smaller EPSC_{AMPA}s and AMPA currents observed in vPAG slices from neuropathic rats could have been due to downregulation or endocytosis of AMPARs or to reduced sensitivity or efficacy of AMPARs. The findings of unaltered GluR1 and GluR2 immunoreactivity suggest no downregulation or intracellular degradation of AMPARs in the vPAG of neuropathic rats. Although the total number of AMPARs was not changed, there might be a subcellular redistribution of AMPARs. In the hippocampus, intracellular calcineurin activation after NMDAR activation can lead to a clathrin-dependent internalization of

AMPA (Beattie et al., 2000; Carroll et al., 2001). Jarzylo and Man (2012) also demonstrated that stimulation of NR2B-NMDARs induced AMPAR internalization in cortical and hippocampal neurons. Activation of the vPAG of neuropathic rats with more available NMDARs may lead to internalization of AMPARs. Postsynaptic AMPAR trafficking is involved in the learning, memory, and synaptic plasticity of the hippocampus (Malinow and Malenka, 2002; Sheng and Kim, 2002; Brecht and Nicoll, 2003). It remains to be elucidated whether postsynaptic AMPAR redistribution, which may occur after nerve injury, contributes to the synaptic plasticity of the vPAG, leading to hyper-sensitive behaviors.

Neuroplastic changes in the vPAG during the development and maintenance of neuropathic pain

Burgess et al. (2002) found a descending pain facilitation mechanism originated from the RVM at 6 d, but not 3 d, after SNL, and proposed that this mechanism may contribute to the maintenance of neuropathic pain but not the initiation phase, which was considered to have originated from afferent ectopic discharges (Han et al., 2000). However, the neuroplastic changes in the mouse ACC may contribute to both the development and maintenance of chronic pain, because upregulation of glutamate receptor was found after 3 d (Wu et al., 2005) and 7 d (Xu et al., 2008), respectively, in inflammatory and neuropathic pain models. In the vPAG, we found neuroplastic changes 3 d after SNL, which lasted for at least 10 d. Similarly, in the PAG, upregulation of NR2B subunits occurred 3 d after hindpaw CFA injection (Hu et al., 2009) and an increased EPSC_{NMDA}/EPSC_{non-NMDA} ratio occurred 5 d after sciatic nerve ligation (Terashima et al., 2012). These results suggest that neuroplastic changes in the PAG, which lead to decreased descending pain inhibition, contribute to both the development and maintenance of neuropathic pain.

Unilateral peripheral injury induces bilateral changes in the PAG

The findings that unilateral SNL led to ipsilateral mechanical hypersensitivity but bilateral neuroplastic changes in the vPAG may have been due to the fact that the PAG is a midline structure that receives input projections in the bulbospinal tract from both sides of the body and extremities (Wiberg et al., 1987; Lima and Coimbra, 1989). Similarly, unilateral peripheral injury induced bilateral changes in the vPAG were also reported in inflammatory and neuropathic pain models (Renno, 1998) and in the ACC in an amputation pain model (Wei et al., 1999). Unilateral SNL also led to comprehensive neuroplastic changes that were not injury-side specific in the RVM, which is also a midline nucleus (Gonçalves et al., 2007). Burgess et al. (2002) showed that bilateral lesions of the dorsolateral funiculus completely reversed ipsilateral SNL-induced mechanical hypersensitivity/hyperalgesia, suggesting an important role of bilateral descending pain modulation in maintaining neuropathic pain.

In conclusion, SNL resulted in bilateral long-lasting hypo-functioning of glutamatergic neurotransmission in the vPAG due to reduced glutamate release and impaired AMPAR response, which may result from upregulation of NR1 and NR2B subunits, leading to AMPAR internalization and possible neuronal loss. This SNL-induced hypoglutamatergic neurotransmission in the PAG may impair descending pain inhibition and contribute to the development and maintenance of neuropathic pain.

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