

Ketamine differentially restores diverse alterations of neuroligins in brain regions in a rat model of neuropathic pain-induced depression

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Depression is present in a large proportion of patients suffering from chronic pain, and yet the underlying mechanisms remain to be elucidated. Neuroligins (NLs), as a family of cell-adhesion proteins, are involved in synaptic formation and have been linked to various neuropsychiatric disorders. Here, we studied the alterations in NL1 and NL2 in the medial prefrontal cortex (mPFC), the anterior cingulate cortex (ACC), and the hippocampus in a rat model of neuropathic pain-induced depression, and whether ketamine, a rapid and robust antidepressant, could restore these abnormalities. In the present study, we found that spared nerve injury induced significant mechanical allodynia and subsequent depressive-like symptoms, along with decreased NL1 and increased NL2 in the mPFC, decreased NL1 in the ACC, and decreased NL2 in the hippocampus. In addition, brain-derived neurotrophic factor (BDNF) was reduced in these brain regions. It is noteworthy that ketamine (10 mg/kg) relieved neuropathic pain-induced depressive behaviors and restored alterations of BDNF and NLs

in the mPFC and the hippocampus at 24 h and 72 h after the administration of ketamine, but only restored BDNF in the ACC. In conclusion, NLs showed diverse changes in different brain regions in the rat model of neuropathic pain-induced depression, which could be reversed differentially by the administration of ketamine. *NeuroReport* 29:863–869 Copyright © 2018 Wolters Kluwer Health, Inc. All rights reserved.

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Introduction

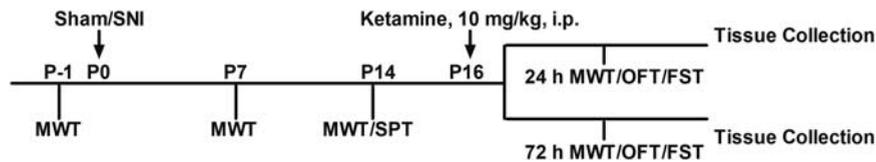
Depression is a debilitating disease with a high prevalence worldwide. Neuropathic pain is defined as a chronic pain resulting from a lesion or a disease affecting the somatosensory system [1]. Epidemiological studies showed that depression was present in 5–85% of patients with various pain conditions, whereas the incidence of depression increased considerably as the pain persisted [2]. Although this neuropathic pain-associated depression is well recognized in clinical practice, the pathogenesis remains largely to be determined.

Neuroligins (NLs) are a family of postsynaptic cell adhesion proteins. NLs bind to presynaptic neuroligin at the extracellular portion and postsynaptic density at the intercellular portion, respectively [3]. Among the NL family members in rodents (NL1–4), NL1 is localized preferentially to postsynaptic densities of excitatory synapses and NL2 is clustered at inhibitory synapses [3]. Therefore, NL1 and NL2 are believed to regulate excitatory and inhibitory synaptic numbers and functions, which play a pivotal role in maintaining the balance between excitation and inhibition (E/I) in the brain [3]. NLs are implicated in various neuropsychiatric disorders such as schizophrenia [4]. Currently,

few studies have investigated alterations of NLs in depression. One study showed that NL2 was reduced after chronic restraint stress for 3 weeks in the hippocampus [5]. NL1 was decreased after immobile restraint stress for 7 consecutive days in the hippocampus [6]. Another study observed that in the genetic depression model of Wistar Kyoto (WKY) rats, NL1 was higher in the prefrontal cortex (PFC), the motor frontal cortex, and the hippocampus [7]. Moreover, spinal NLs participated in the maintenance of chronic pain [8]. These findings suggest that NLs may play a key role in the neuropathic pain-induced depression.

The *N*-methyl-D-aspartate receptor antagonist ketamine exerts rapid, robust, and long-lasting antidepressant effects [9]. Enhanced synaptic plasticity driven by upregulated brain-derived neurotrophic factor (BDNF) after administration of ketamine is crucial for its persistent antidepressant effects [9]. Notably, a recent study reported that BDNF could regulate NL1 and NL2 during consolidation of fear memories [10]. Thus, we aimed to study alterations of NLs in three brain regions with close relevance to depression and pain, namely, the medial prefrontal cortex (mPFC), the anterior cingulate cortex (ACC), and the hippocampus, in rats with spared

Fig. 1



Schematic timeline of the experimental procedure. FST, forced-swim test; MWT, mechanical withdrawal threshold; OFT, open-field test; SPT, sucrose-preference test.

nerve injury (SNI)-induced depression. In addition, we hypothesized that ketamine could upregulate BDNF and rescue abnormal NLs expressions.

Materials and methods

Subjects

A total of 42 male Sprague–Dawley rats (200–250 g, 6–7 weeks) were obtained from the Animal Center of Jinling Hospital, Nanjing, China. All procedures were approved by the Ethics Committee of Jinling Hospital, and were performed according to the Guideline for the Care and Use of Laboratory Animals from the National Institutes of Health. Animals were housed in a standard condition (a 12 h light/dark cycle, room temperature of 22–24°C, and food and water available *ad libitum*).

Experimental paradigm and drug administration

Animals were divided into four groups: the Sham + Sal group ($n=10$), the SNI + Sal group ($n=12$), the SNI + Ket24 group ($n=10$), and the SNI + Ket72 group ($n=10$). For the Sham + Sal and SNI + Sal groups, the mechanical withdrawal threshold (MWT) was performed at 24 h and 72 h after saline injection, and the open-field test (OFT) and the forced-swim test (FST) were performed at 72 h after saline injection. For the SNI + Ket24 group, the MWT, OFT, and FST were performed at 24 h after ketamine injection. For the SNI + Ket72 group, the MWT, OFT, and FST were performed at 72 h after ketamine injection. For all four groups, MWT was performed before, and 7 days and 14 days after SNI. Before ketamine administration, the sucrose-preference test was performed to confirm the successful establishment of depressive-like behavior at 14 days. A single dose of ketamine (10 mg/kg in 1 ml; Hengrui Pharmaceutical Company, Jiangsu, China) or an equal volume of normal saline (0.9%) was injected intraperitoneally at 16 days after surgery. The flow chart of the study protocol is shown in Fig. 1.

Spared nerve injury

Rats were anesthetized by 2% of sodium pentobarbital (50 mg/kg, intraperitoneally; Wuhan Servicebio Technology Co. Ltd., Wuhan, China). The skin on the right thigh of the rat was incised, and then the biceps femoris muscle was bluntly dissected and the three terminal branches of the

sciatic nerve were exposed. The common peroneal and tibial nerves were isolated, tightly ligated with 4–0 silk, and sectioned distal to the knot, leaving the sural intact and untouched. Muscle and skin were then closed by 4–0 silk in two layers.

Mechanical withdrawal threshold

A classic up–down method with Von Frey filaments (Danmic Global, San Jose, California, USA) was used to measure mechanical allodynia [11]. In brief, rats were individually placed in transparent Plexiglas chambers over a metal mesh and habituated for at least 20 min. Von Frey filaments were applied to the lateral surface of the hindpaw (sural nerve territory) with a maximal cut-off of 8 s. Sudden paw withdrawal, flinching, or paw licking was defined as a positive response. Depending on the positive or the negative response, descending or ascending intensity of subsequent filament was applied, respectively. Then, 50% withdrawal thresholds were calculated as described previously [11].

Sucrose preference test

Rats were habituated to a 1% sucrose solution for 24 h before the test. During the test, rats were caged individually, with free access to both a 1% sucrose solution bottle and a tap water bottle for 24 h. Sucrose and tap water intakes were measured by volume before and after the test. Sucrose preference was defined as the percentage of sucrose solution consumed of the total consumption of sucrose and tap water during 24 h.

Open field test

The OFT was performed in a gray open top polyvinylchloride box (100 × 100 × 40 cm). Rats were gently placed in the center of the field and allowed to freely explore the area for 5 min. The movement was recorded with a video camera and the total distance was calculated by a computerized tracking system (XR-XZ301; Shanghai Xinruan Information Technology Co. Ltd., Shanghai, China). Between subjects, 75% ethanol was used to thoroughly clean the open-field area.

Forced swim test

The FST was composed of two sessions. Rats were placed individually into Plexiglas cylinders (height 65 cm;

diameter: 30 cm) filled with 40 cm height of water, conditioned at 22–24°C for a 15-min pretest session. Rats were then injected with ketamine 1 h later. During the 6-min test session, rats were placed in the same condition and recorded by a camera. Immobility is defined as floating in the water without struggling or only making necessary movements to keep its head above water. Immobility time during the last 5 min of the 6-min test was measured offline by an experimenter who was blinded to the treatment. The latency to the first immobility was the delay to the first bout of immobility after placing the rats in the cylinders. After the test, rats were dried and then moved back into the home cages.

Western blot

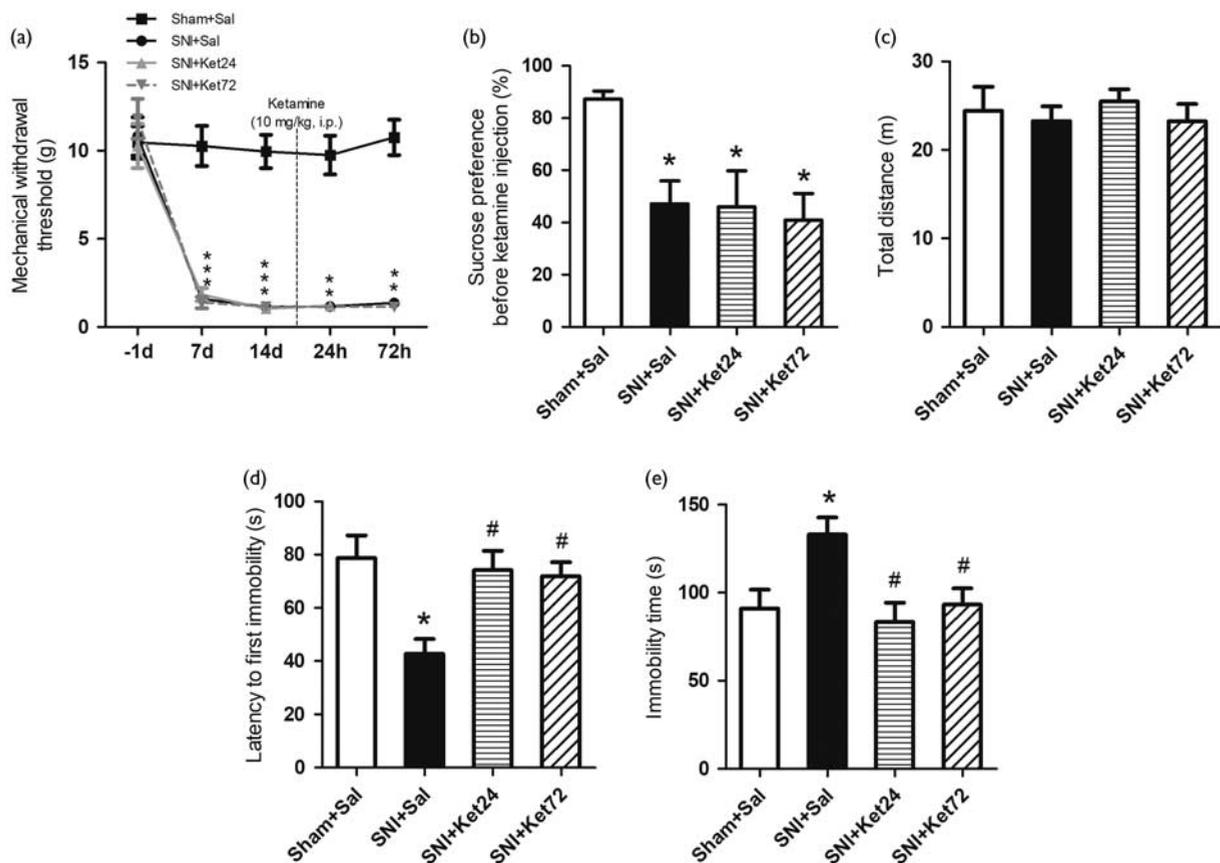
Rats were killed by anesthesia with 2% sodium pentobarbital (60 mg/kg intraperitoneally). Then, mPFC, ACC, and hippocampus were harvested on ice. Tissues were homogenized in an ice-cold RIPA lysis buffer (P0013B; Beyotime, Shanghai, China) plus protease inhibitors. Homogenates were centrifuged at 15 000 rpm for 10 min

at 4°C and supernatants were collected. Protein samples (35 µg/well) were separated on SDS-PAGE and transferred to a nitrocellulose membrane. The membranes were incubated with rabbit anti-BDNF (1 : 1000, ab203573; Abcam, Cambridge, UK), mouse anti-Neurologin1 (1 : 1000, 129111; Synaptic Systems, Gottingen, Germany), rabbit anti-Neurologin2 (1 : 1000, 129203; Synaptic Systems), and mouse anti-GAPDH (1 : 5000, 60004-1-Ig; Proteintech, Chicago, Illinois, USA) overnight at 4°C. Peroxidase-conjugated secondary antibodies were used as needed and chemiluminescence was used to detect the protein bands. The density of the western bands was quantified using ImageJ (National Institutes of Health, Bethesda, Maryland, USA).

Statistical analysis

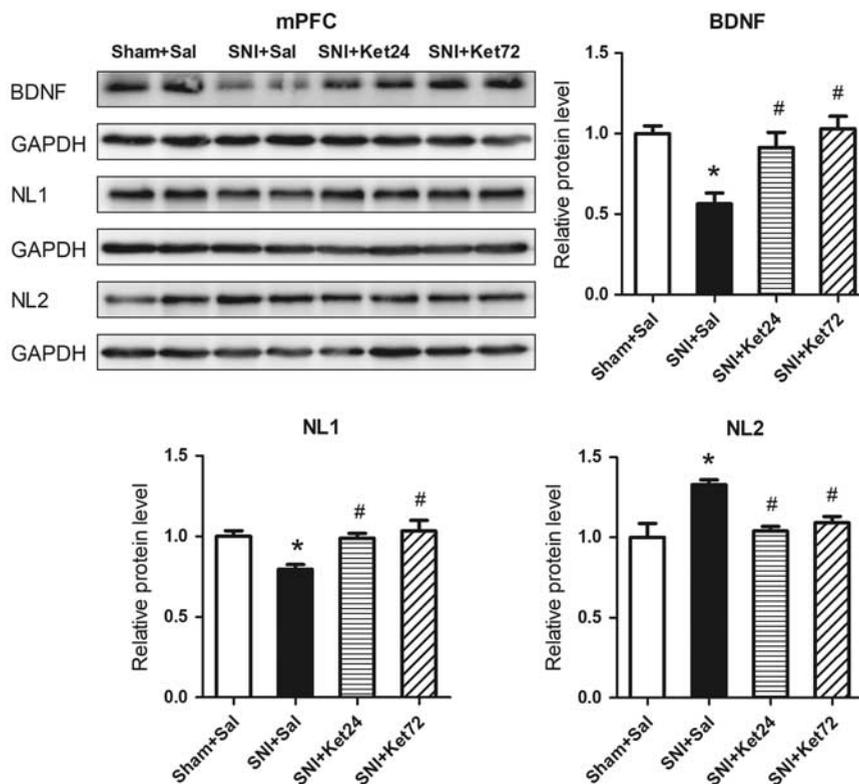
Data are presented as mean ± SEM. All analyses were carried out using the statistical package for social sciences (SPSS, version 17.0; SPSS Inc., Chicago, Illinois, USA). Comparisons were performed using one-way analysis of variance, followed by the Tukey test for

Fig. 2



(a) SNI elicited mechanical allodynia on 7 days and 14 days after surgery, and ketamine (10 mg/kg intraperitoneally) did not relieve mechanical allodynia. (b) SNI reduced sucrose preference on 14 days after surgery. (c) SNI or ketamine did not affect locomotor activity. SNI reduced the latency to first immobility (d) and increased the immobility time in the FST (e). The data are presented mean ± SEM ($n = 10-12$). FST, forced-swim test; SNI, spared nerve injury. * $P < 0.05$, vs. the Sham + Sal group; # $P < 0.05$, vs. the SNI + Sal group.

Fig. 3



Representative blots and quantitative analysis of BDNF, NL1, and NL2 in the medial prefrontal cortex. The data are expressed as the mean \pm SEM of four rats in each group. BDNF, brain-derived neurotrophic factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; mPFC, medial prefrontal cortex; NL, neuroligin; SNI, spared nerve injury. * $P < 0.05$, vs. the Sham + Sal group; # $P < 0.05$, vs. the SNI + Sal group.

post-hoc comparisons, except that mechanical allodynia was analyzed by repeated-measured one-way analysis of variance, followed by the Tukey test. A P value less than 0.05 was considered to indicate a statistically significant difference.

Results

Spared nerve injury induced mechanical allodynia, and depressive-like behavior and reversal by ketamine

SNI induced a significant reduction in MWTs in the ipsilateral hind paws of rats (Sham + Sal vs. SNI groups, all $P < 0.05$) (Fig. 2a). Sucrose preference was decreased in SNI groups compared with the Sham group on day 14 (all $P < 0.05$) (Fig. 2b). The total distance traveled in the OFT showed no significant difference among the four groups, indicating that surgery or administration of drugs did not influence locomotor activity (Fig. 2c). SNI decreased the latency to first immobility (Sham + Sal vs. SNI + Sal, $P = 0.002$) and increased the immobility time (Sham + Sal vs. SNI + Sal, $P = 0.023$) in the FST. Depressive-like behavior was reversed at 24 h (SNI + Sal vs. SNI + Ket24: latency, $P = 0.009$; immobility, $P = 0.006$) and 72 h (SNI + Sal vs. SNI + Ket72: latency, $P = 0.016$; immobility, $P = 0.035$) after a single low dose of ketamine

administration (Fig. 2d and e). However, ketamine did not attenuate mechanical allodynia (Fig. 2a).

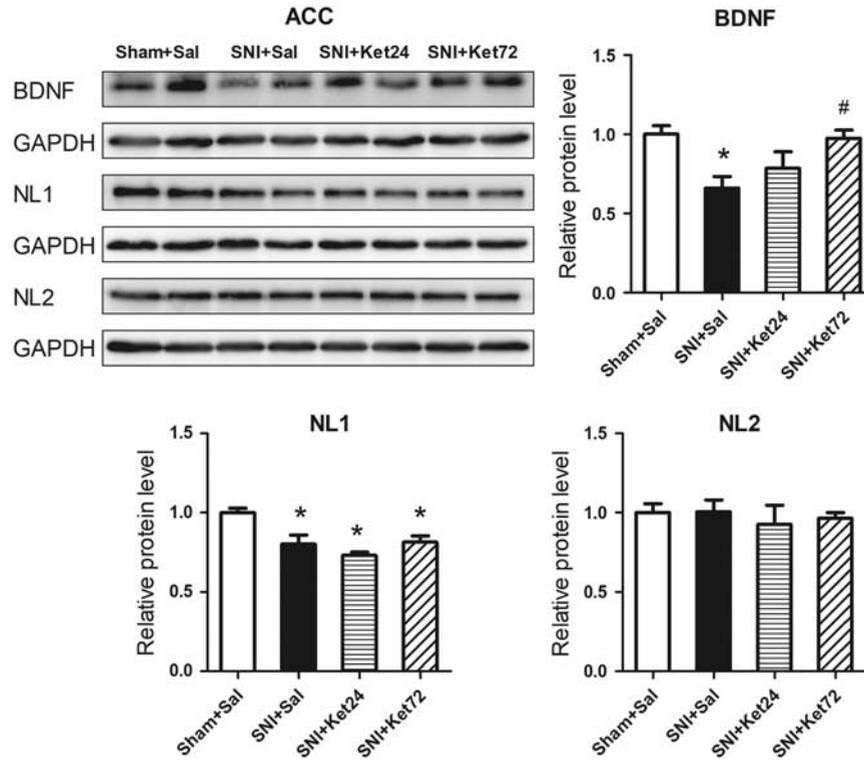
NL1 was decreased and NL2 was increased in the medial prefrontal cortex, both of which were restored by ketamine

Results from Western blot showed that NL1 expression was decreased (Sham + Sal vs. SNI + Sal, $P = 0.026$) and NL2 expression was increased (Sham + Sal vs. SNI + Sal, $P = 0.004$) in the mPFC in the rats with SNI-induced depression (Fig. 3). Moreover, BDNF expression was reduced in the mPFC (Sham + Sal vs. SNI + Sal, $P = 0.006$) (Fig. 3). These alterations were restored within 24 h and lasted up to at least 72 h after ketamine administration (Fig. 3).

NL1 was decreased in the anterior cingulate cortex, which was not restored by ketamine

NL1 expression was decreased (Sham + Sal vs. SNI + Sal, $P = 0.017$), but NL2 expression was not affected in the ACC (Fig. 4). Meanwhile, BDNF expression was reduced (Sham + Sal vs. SNI + Sal, $P = 0.030$). Interestingly, ketamine did not restore the decrease in NL1 expression at 24 h and 72 h, even though a gradual increase in BDNF expression was observed at 24 h and 72 h (Fig. 4).

Fig. 4



Representative blots and quantitative analysis of BDNF, NL1, and NL2 in the ACC. The data are expressed as the mean \pm SEM of four rats in each group. ACC, anterior cingulate cortex; BDNF, brain-derived neurotrophic factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NL, neuroigin; SNI, spared nerve injury. * $P < 0.05$, vs. the Sham + Sal group; # $P < 0.05$, vs. the SNI + Sal group.

NL2 was decreased in the hippocampus, which was restored by ketamine

NL2 expression was decreased in the hippocampus (Sham + Sal vs. SNI + Sal, $P = 0.017$), but not NL1 expression (Fig. 5). In the meantime, SNI decreased BDNF expression (Sham + Sal vs. SNI + Sal, $P = 0.025$). Ketamine rescued NL2 and BDNF expressions at 24 h and 72 h (Fig. 5).

Discussion

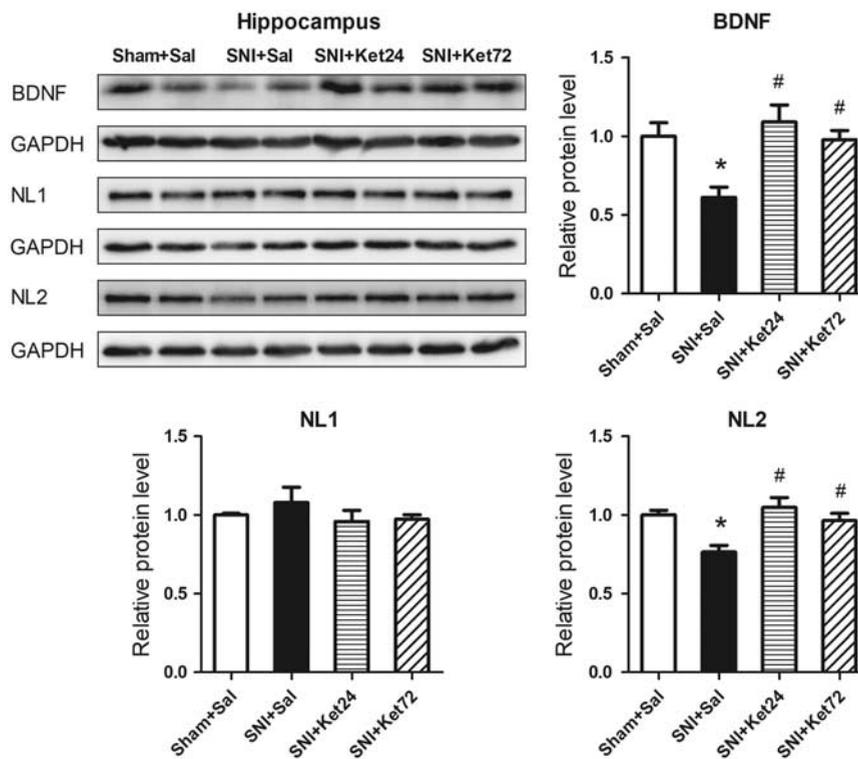
In the present study, we found decreased NL1 and increased NL2 in the mPFC, decreased NL1 in the ACC and decreased NL2 in the hippocampus in rats with SNI-induced depression, and the alterations in the mPFC and the hippocampus, but not ACC, were restored by ketamine within 24 h and lasted up to 72 h after injection. In addition, SNI-induced depression was accompanied by a significant reduction in BDNF expression in the three brain regions, which were also reversed by ketamine injection.

mPFC was selected for protein detection because of its critical involvement in depression, neuropathic pain, and the antidepressant effects of ketamine [12–14]. Substantial evidence has proven the deficit of neuronal excitability in the mPFC in depression [12,15,16]. Optogenetic stimulation

of the prelimbic cortex exerted potent antidepressant-like effects in mice susceptible to chronic social defeat stress [12]. Chronic variable stress increased inhibitory appositions and terminals onto excitatory glutamatergic neurons and the release of inhibitory neurotransmitter γ -aminobutyric acid (GABA) in the infralimbic cortex [15]. In addition, reduced glutamatergic currents and dendritic branching were observed in the rat model of SNI [16]. Accordingly, the decrease in NL1 and increase in NL2 in the mPFC in our study may imply decreased synaptic excitation and increased synaptic inhibition because of their specific synaptic function. Another study reported that in WKY depressive rats, NL1 mRNA and protein expressions were higher in the PFC [9]. As WKY rats are genetically depressed, compensatory mechanisms during development may distinguish WKY rats from depressed subjects in adulthood.

ACC is implicated in depression and neuropathic pain [17,18]. A recent study suggested that optogenetic stimulation of the ACC induced depressive-like behavior in naive mice, and the ACC lesion prevented the development of depressive symptoms of chronic pain without affecting the sensory mechanical allodynia [18]. In a human study, stimulation of ACC activity by presenting fearful faces showed that ACC activity was increased in depressed patients in comparison with healthy controls [19]. However, another

Fig. 5



Representative blots and quantitative analysis of BDNF, NL1, and NL2 in the hippocampus. The data are expressed as the mean \pm SEM of four rats in each group. BDNF, brain-derived neurotrophic factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NL, neuroigin; SNI, spared nerve injury. * $P < 0.05$, vs. the Sham + Sal group; # $P < 0.05$, vs. the SNI + Sal group.

study showed that baseline glutamate was decreased in the cingulum in depressed patients [20]. In the present study, we observed a decrease in NL1, but no change in NL2 in the ACC. These contradictory results suggested the complex role of ACC in the development of depression.

Similarly, the hippocampus plays an important role in depression, neuropathic pain, and ketamine's antidepressant effects [21,22]. A previous study showed that chronic restraint stress for 3 weeks downregulated NL2 in synaptosomes in the hippocampus and local administration of the mimic peptide that disrupted the interaction between NLS and neuroligin led to reduced sociability and increased aggression, whereas NL1 in the hippocampus and NL1 and NL2 in the PFC were not affected [5]. In WKY depressive rats, NL1 mRNA and protein expression was higher in the hippocampus and NL2 mRNA was lower in the hippocampus, without significant protein change [7]. In contrast, immobile restraint stress for 7 days decreased NL1 expression in the hippocampus, whereas treadmill exercise attenuated the decrease in NL1 [6]. In the present study, we showed a decrease in NL2, but no change of NL1 in the hippocampus, suggesting a possible deficiency in GABAergic neurotransmission in the hippocampus in SNI rats. Different alterations in NLS may be attributed to different animal models used and different time points for protein detection.

Surprisingly, a recent study has shown that astrocytes express NL1, NL2, and NL3 mRNAs at considerably high levels in both fetal and mature astrocytes, and juvenile astrocytic NL2 is essential for synapse development by controlling the formation and maintenance of excitatory synapses [23]. As the ratio of NL1/NL2 was used to represent the E/I in previous studies [7,10], the unexpected opposite functions of NLS on synaptosomes and astrocytes suggest that this ratio might not be accurate to represent E/I.

Consistent with the results of previous studies utilizing the animal model of neuropathic pain-induced depression, we observed broadly reduced BDNF in the mPFC, ACC, and hippocampus [24]. Similar to that in the classic stress-induced depression [14,22], BDNF was elevated in the mPFC and the hippocampus at 24 h and lasted up to 72 h after ketamine injection in the SNI-induced depression, which was consistent with the results of behavioral tests. Meanwhile, ketamine restored alterations of NLS in the mPFC and the hippocampus, but not ACC, suggesting the fundamental roles of the mPFC and the hippocampus in its antidepressant effects. Interestingly, BDNF showed a relatively delayed increase in the ACC, which might suggest that BDNF in the ACC did not play a causative role in ketamine's antidepressant effects. Nevertheless,

we cannot rule out the role of ACC in ketamine's antidepressant effects because a previous study showed that GABA was increased after ketamine injection in the ACC of rats [25]. Thus, further studies are needed to investigate whether the alterations in NLs that regulated by BDNF contribute to ketamine's antidepressant effects.

In summary, the present study showed distinct NLs changes along with decreased BDNF in the three regions in neuropathic pain-induced depression. Moreover, ketamine differentially restored the alterations among these regions. Future studies are needed to determine whether the altered NLs are mainly located on synaptosomes or astrocytes and the different functions of NLs on synaptosomes and astrocytes in synaptic transmission.

Acknowledgements

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Conflicts of interest

There are no conflicts of interest.

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