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Hippocampal Glutamatergic Synapses Impairment Mediated Novel-object Recognition Dysfunction in rats with Neuropathic Pain

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Abstract

Cognitive impairment is one of the most common complications associated with chronic pain. Almost 20% of chronic pain patients suffer from cognitive impairment, which may substantially influence their quality of life. Levels of major excitatory neurotransmitters in the central nervous system, and alterations in the glutamatergic system may influence cognitive function and the pain sensory pathway. In the present study, we adopted the spare nerve injury model to establish the progress of chronic pain and investigated the mechanism underlying the cognitive aspect related to it. At behavioral level, using the novel-object recognition test, mechanical hypersensitivity was observed in peripheral nerve injured rats as they exhibited recognition deficits. We showed a dramatic decrease in hippocampal glutamate concentration using nuclear magnetic resonance and reduced glutamatergic synaptic transmission using whole-cell recordings. These were associated with deficient hippocampal long-term potentiation induced by high-frequency stimulation of the Schaffer collateral afferent. Ultra-high-performance liquid chromatography revealed lower levels of D-serine in the hippocampus of SNI rats and that D-serine treatment could restore synaptic plasticity and cognitive dysfunction. The reduction of excitatory synapses was also increased by administering D-serine. These findings suggest that chronic pain has a critical effect on synaptic plasticity linked to cognitive function and may built up a new target for the development of cognitive impairment under chronic pain conditions.

Keywords: Neuropathic pain, hippocampus, cognitive impairment, glutamatergic synaptic transmission, D-serine

Introduction

Pain is a multi-dimensional experience, which includes sensory, affective and cognitive components which interact with each other [7; 14].Research has shown many objective adverse effects of chronic pain on cognition, such as processing speed, psychomotor speed, executive and general cognitive functioning [3; 5; 15]. Memory impairments have also been reported in chronic pain syndromes that largely affect therapy adherence, daily functioning, capacity for work, relationships, leisure activities, mood and quality of life. Unfortunately, the mechanisms of chronic pain that induce cognitive changes are extremely complicated and there is lack of preventive and therapeutic measures.

To date, some studies have indicated that nerve injury can induce changes in neurotransmitters and affect the synaptic plasticity [17; 21]. Glutamate is a well- known excitatory neurotransmitter of the central nervous system and recently, many studies have focused on how the glutamatergic system impacts on cognitive dysfunction [29; 38]. An abnormal glutamatergic system has been implicated in pain induced learning and memory deficits or emotional dysfunction [40; 41; 56]. However, except for a few studies reporting the alterations of glutamatergic receptors in some brain areas after nerve injury [18; 56], little is known about the alterations in the functions of glutamatergic neurocircuits and how they are affected.

Previous studies have demonstrated that chronic pain arising from peripheral nerve injury induces alterations in various areas of the brain including the hippocampus [42; 52]. In addition, the hippocampus is a major part of the limbic system that has been identified as the most important region for processing learning and memory [11; 51; 57]. Therefore, it is necessary to explore the hippocampal cellular and molecular changes in cognition during persistent pain conditions. Additionally, recent preclinical studies found that neuropathic pain rats exhibited structural and functional alterations in the hippocampus following experimental nerve injury [4; 10; 19; 22].

To further understand the pattern of changes in glutamatergic synaptic transmission within the hippocampus of the SNI rats, we investigated the activity of the glutamatergic system combining proton nuclear magnetic resonance, whole-cell patch-clamp, molecular and behavior approaches. Our results propose a role for D-serine as a

regulator of the glutamatergic system, which may be related to glutamatergic synapses loss and contribute to memory deficits caused by neuropathic pain.

Methods

Animals

Adult male (230-250g) Sprague–Dawley rats were obtained from Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China. Rats were raised under controlled conditions (22–25 °C, 12-h alternate circadian rhythm, food and water were usable ad libitum). All animal studies were approved by the Animal Care and Use Committee of Tongji Medical College and performed to comply strictly with the guidelines accepted by the International Association for the Study of Pain.

Induction of neuropathic pain

The neuropathic pain was produced by spared nerve injury in keeping with the procedure described by Decosterd and Woolf [9]. In brief, under pentobarbital sodium anaesthesia (50mg/kg, *i.p.)*, exposure of the left sciatic nerve including the three branches was made through an incision of the lateral surface of the thigh. 5.0 silk thread (or sutures) was tightly ligated to the two branches (common peroneal and the tibial nerves) of the sciatic nerve and sectioned distal to the ligation, removing 2-4 mm of the distal nerve stump. For the sham-operate rat, we just exposed the sciatic nerve without ligation.

Pain-related behaviors

All procedures were conducted between 8:30 and 11:30 am. The rats were placed into Plexiglas chambers with a wire net floor for 30 min in order to adapt to the surroundings as previously described [48; 49]. The mechanical thresholds were measured using an electro Von-Frey (UGO, 38450, Italy).

Novel-object recognition test

The novel-object recognition test was performed in a Plexiglas box (60*60*50) 3 hours later after last testing the mechanism of paw withdrawal threshold. The floor of the box was covered with grey paper. The rats were handled for 6 days before habituation. The rats were placed into the box for 20min on the first day without objects.

Four hours later, two objects were placed into the testing box which allowed the rats to explore the objects for 5min. The time spent exploring each object was recorded. A test was performed in the same box after a retention interval of 24 hours, when one of the two objects used during exploration was replaced with a novel object. The rats could explore the objects freely for 5min, and the time spent with each object recorded. A recognition index was represented by (C-A)/ (A+C), a ratio of the difference between the time spent exploring the familiar object and the novel object over the total time spent exploring both objects, was used to evaluate cognitive function.

Western blotting

The rats were anesthetized with pentobarbital sodium (50mg/kg, *i.p.*), rapidly dissociated and the hippocampus collected at 4°C and then stored at -80°C. The tissues were mixed in RIPA lysis buffer supplemented with proteinase inhibitor, then the supernatant was harvested and centrifuged at 13,000 r/min for 15 min. The protein concentrations were measured using the BCA Protein Assay Kit. The samples were denatured in water for 10 min at 95°C in loading buffer. Equal amounts of protein samples were separated in 10% SDS-PAGE and transferred onto the polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA) using electrophoresis. The membranes were blocked in 5% BSA in TBST (0.1%) for 1 hour at room temperature, then incubated overnight at 4°C with the primary antibodies. The primary antibodies used in this study include mouse anti-GAPDH (1:3000, Proteintech, China), rabbit anti-NMDAR1(1:500, ABclonal, China), rabbit anti-NMDAR2A (1:800, Cell Signaling Technology, USA), rabbit anti-NMDAR2B (1:1000, Cell Signaling Technology, USA), rabbit anti-GluR1 (1:1000, Proteintech, China), rabbit anti-GluA2 (1:1000, Cell Signaling Technology, USA), guinea pig anti- vGluT1 (1:500, Millipore, USA), and rabbit-anti PSD95 (1:1000, GeneTex, USA). Then the membranes were washed three times with TBST and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1.5 hours at room temperature. The second antibodies were HRP-conjugated goat-anti-mouse (1:5000, Promoter, Wuhan, China), goat-anti-guinea pig (1:10000, Jackson, USA) and goat-anti-rabbit secondary antibody (1:5000, Promoter, Wuhan, China). Then the protein bands were detected with

chemiluminescence (Pierce ECL Western Blotting Substrate, Promotor, Wuhan, China) and measured using the ChemiDocXRS chemiluminescence image analysis system (Bio-Rad, Hercules, CA, USA). The intensity of the bands were analyzed by software Image Lab 5.2.1(BIO-RAD).

Immunohistochemistry

The rats were deeply anesthetized with pentobarbital sodium (50mg/kg, *i.p.*), then transcardially perfused with 250ml ice-cold 0.1M PBS followed by 4% PFA in 0.1 M PBS. The brain tissues were collected, and postfixed in 4% PFA/PBS overnight and cryoprotected with 30% sucrose/PBS for 48 hours at 4°C. Coronal 20μm thick sections were cut on a cryostat (CM1900, Leica, Wiesbaden, Germany) serially and the sections of the hippocampus were collected from each brain. After washing in the PBS, the sections were permeabilized with 0.3% TritonX-100/PBS for 15 min and blocked with 5% Donkey serum for 45 min at room temperature. Then the sections were incubated for 24 hours with guinea pig anti vGluT1 (1:200, Millipore, USA) and rabbit anti PSD95 (1:100, GeneTex, USA). After rinsing the sections three times with PBS for 5 min, the sections were treated with Alexa Fluor 594-labeled donkey anti-rabbit guinea pig (1:300, Jackson, USA) and Alexa Fluor 488-labeled donkey anti-rabbit (1:200, Abbkine, USA) secondary antibodies for 2 hours at room temperature. They were then washed again 3 times in PBS and cover-slipped with 30% glycerol.

The image acquisition and analysis were blinded to the experimenters. We imaged tissue sections with a Leica DMI 3000B microscope, controlled by LASX software. We imaged the CA1, CA3 and DG areas of the dorsal hippocampus, using the same light intensity and exposure settings for all slices within each image set. High magnification 63x/1.4 N.A. oil objective plus 1.4x software zoom Z-stack images were acquired. Synaptic puncta quantification was presented as density like synaptic puncta counts/100μm^2. Then synaptic densities were quantified by co-localization of vGluT1 (Red) and PSD95 (Green) puncta using Image J software. All image stacks were displayed as 2-dimensional extended-focus images collapsed along the z-axis.

Proton nuclear magnetic resonance (1H–NMR) spectroscopic analysis

Proton nuclear magnetic resonance (1H-NMR) spectroscopic analysis was used to

access the relative concentrations of neurotransmitters in hippocampus between the SNI and the Sham groups. The anesthetized rats were microwaved for 19s in a microwave machine, then the hippocampus quickly removed into a 2ml EP tube. The tissue was weighed and immediately frozen at -80℃ for further processing.

0.1% HCl/methanol 200μL mixed with 800μL 60% ethanol was added into the tissues to extract the substance, then homogenized with Tissuelyser for 1.5min at a 20Hz frequency (Tissuelyser II, QIAGEN, German). The mixture was centrifuged at 14000rpm for 15min, and the supernatant harvested to a 5ml EP tube. These steps were repeated twice with 1000 μ L 60% ethanol. The supernatants were collected together, and then desiccated with the centrifugal drying apparatus (Thermo Scientific 2010, Germany) and the solid product was retained for further studies.

60ul of phosphate buffer solution and 540ul of double distilled water were added to a 5ml EP tube and the dried products dissolved. The solution was standardized with TSP then the solution vibrated with a high-speed vortex until the products dissolved, and then centrifuged at 12000r for 10min. The supernatant was collected and transferred to an NMR tube for analysis.

Electrophysiology

Both Sham and SNI rats were deeply anesthetized with pentobarbital sodium (50mg/kg, *i.p.)* and then decapitated. Hippocampal slices (320μm) were prepared as described previously [37; 46; 58]. Slices were transferred to a holding chamber filled with ACSF including (in mM): 124NaCl, 26$NaHCO_3$, 3KCl, 1.2$MgCl_2 \cdot 6H_2O$, 1.25$NaH_2PO_4 \cdot 2H_2O$, 10$C_6H_{12}O_6$ and 2$CaCl_2$ at PH=7.3-7.4, 305mOsm. Slices were recovered at 31.5°C for 30 minutes and then at room temperature for at least 1 hour. The acute slices were then transferred to the recording chamber perfused with ACSF (1-2ml/min).

We recorded the CA1 pyramidal neurons for the whole-cell patch-clamp. When stable whole-cell recordings were achieved with good access resistance (~20$_M\Omega$), the basic electrophysiological properties were recorded. All the data were acquired at 10 kHz and filtered with a low-pass filter at 2 kHz. An internal solution containing (in mM): 140 potassium gluconate, 0.2EGTA, 10HEPES, 2NaCl, 2MgATP and 0.3NaGTP,

and an external solution containing 10μM bicuculline, 1μM TTX or using 10μM CNQX and 50μM AP V (spontaneous excitatory postsynaptic currents (sEPSCs) without TTX) were used to record the miniature excitatory postsynaptic currents (mEPSCs)

For the long-term potentiation (LTP), we recorded at the stratum radiatum of the CA1 area after electrical stimulation of the Schaffer collateral-commissural pathway. The stimulation intensity was set to 40% of the maximum responses. LTP was induced in the slices by high frequency stimulation (HFS, 100Hz, 50 pulse, four trains at 20s interval). The postsynaptic potentials (fPSPs) were recorded at least 30 minutes before HFS and 60 minutes after HFS.

Ultra-high-performance liquid chromatography tandem mass spectrometry

The samples were added to the extract solvent (acetonitrile-methanol-water precooled at -20^{o}C), and then the samples were vortexed for 30s, homogenized at 45Hz for 4 min, and sonicated in ice-water bath for 5min. The homogenization and sonication was repeated 3 times, and then the samples incubated at -20^{o}C for 1hour and centrifuged at 12000rpm at 4^{o}C for 15min. Derivatization was performed according to previous reports with a few modifications [20; 34], in order to extract clear supernatant which could be subjected to LC-MS/MS analysis. The separation of UHPLC was implemented by using an Agilent 1290 Infinity II series UHPLC System, containing a Waters ACQUITY UPLC BEH C18 column (100 × 2.1mm, 1.7μm). Mobile phase A consisted of 0.1% formic acid in water, while acetonitrile was used in phase B. For assay development, An Agilent 6460 triple quadrupole mass spectrometer containing an AJS electrospray ionization (AJS-ESI) interface was applied. Agilent Mass Hunter Workstation Software was employed for MRM data collection and processing. All analyses were performed by a researcher blinded to the groups.

Drug treatment

In order to verify the role of D-serine in glutamatergic transmission after SNI, D-serine (Sigma-Aldrich, USA) was dissolved in 0.9% saline. D-serine was administered intraperitoneally at a dose of 50mg/kg per day, and continuously injected for 21 days. The Sham group received intraperitoneal injections of 0.9% saline (Vehicle).

Statistical analyses

All data were analyzed by SPSS 20.0 and represented as means ±S.E.M. To compare the differences between two groups, Student *t* test was used. Two-way ANOVA was applied to calculate the values of MPWT and novel-object recognition test. Statistical figures were drawn using GraphPad prism 7. Statistical significance was indicated by $P < 0.05$.

Results

3.1 Spared nerve injury induced mechanical allodynia and cognitive impairment in rats

In order to evaluate nociceptive symptoms, the mechanical paw withdrawal threshold (MPWT) was tested from day0 to day21 after the operations (Fig. 1A). As expected, mechanical allodynia developed after SNI surgery. The MPWT was lower on day3 after the operation in the SNI groups and lasted until 21 days after SNI.

We next used a novel-object recognition test to evaluate the hippocampus-dependent memory in each group on day21 after operation (Fig. 1B) [23]. In the training session no significant difference was observed in the time spent exploring each object between the two groups (Fig. 1C), indicating that they had the same motivational state and interest in the objects. By contrast, the Sham group spent more time with the novel object 1 day (24 hours) after the training session, whereas the exploring time spent with the two objects was the same as the SNI rats (Fig. 1D).The recognition index, defined as the proportion of the amount of time taken in exploring the novel object in the test session and the total time spent in exploring objects, was greater in the Sham rats than in the SNI rats (Fig. 1E), indicating that recognition memory was impaired in the neuropathic pain rats.

3.2 Hippocampal glutamate levels were decreased after spared nerve injury in rats.

Glutamate and GABA are the most important neurotransmitters in the central nervous system [12; 40]. We used the proton nuclear magnetic resonance spectroscopic analysis to detect whether the concentrations of these two neurotransmitters were changed after SNI. In order to estimate the differences between the Sham group and SNI group, absolute concentrations of glutamate and GABA were calculated and

compared to normalized spectra by the weight of samples (Fig. 2A). The raw data of the average and deviation of these two chemicals are exhibited (see Fig. 2B). As shown in Fig. 2C, the glutamate concentration in the hippocampus was decreased after 21 days in SNI compared to the Sham group. However, there was no significant difference in the content of GABA between the Sham and SNI groups (Fig. 2D).

3.3 Hippocampal synaptic glutamatergic activity and synaptic plasticity were decreased in the SNI rats.

We performed whole-cell patch-clamp, recording the sEPSCs of the Sham and SNI rats in hippocampal slices, to compare the glutamatergic transmission between the two groups (Fig. 3A). The frequency of the SNI rats was lower than in the Sham group (Fig. 3B). Nevertheless, there was no significant difference in the amplitude (Fig. 3C). We recorded the miniature EPSCs (mEPSCs) in order to assess the single synaptic response to individual synaptic vesicles (Fig. 3D). The frequency and amplitude of mEPSCs were significantly reduced in the SNI neurons (Fig. 3E-F). In addition, the expression of glutamate receptor subunits were evaluated using western blot analysis. We observed that the expressions of NR1 and NR2B were clearly downregulated in the SNI group. However, no statistical differences were observed about other protein expressions (Fig. 3G-H).

Long-term potentiation (LTP) plays a critical role in synaptic transmission plasticity, which is often used to study learning and memory [28; 55; 60]. To research whether the LTP in the hippocampus of the SNI group was impaired, we recorded Schaffer collateral and evoked fPSP of hippocampal slices from the Sham and SNI rats. In slices from the Sham group, the amplitude of the fPSPs was absolutely increased (Fig. 3I-J). In contrast, LTP was impaired and had only a slight increase at baseline in slices from the SNI group (Fig. 3I-J). These results further explain that SNI could induce the impairment of learning and memory.

3.4 The numbers of hippocampal excitatory synapses were decreased in the SNI rats.

Because the glutamatergic transmission was markedly deceased in the SNI rats, we next investigated the number of excitatory synapses. Firstly, we detected the protein

levels of vGluT1 and PSD95, the pre- and postsynaptic markers of excitatory synapses [1]. Reduced vGluT1 and PSD95 expressions were observed in the SNI group (Fig4. A-B). We next quantified the synaptic density within the areas of the hippocampus in the SNI rats and compared it with the density of synapses in the same areas of the Sham rats (Fig. 4C). The excitatory synapses were labelled by the co-localization of the vGluT1 and PSD95. The density of excitatory synapses within the CA1 and CA3 areas of the hippocampus in the SNI rats was significantly decreased compared to the Sham group (Fig. 4D-4E), whereas SNI did not affect the excitatory synaptic density in the DG (Fig. 4F). These observations are consistent with our findings verified by electrophysiology. Collectively, our data shows that SNI may impair the formation of excitatory synapses in the hippocampus.

3.5 Hippocampal D-serine levels were decreased after SNI and exogenous D-serine administration prevented the development of novel object recognition dysfunction in the SNI rats.

Because D-serine is the key controller of neuronal excitability, it may participate in synaptogenesis [44; 50]. We tested whether the SNI induced memory deficits are related to the D-serine levels in the hippocampus. HPLC was used to detect the changes in D-serine levels in the hippocampus of the SNI rats. The levels of D-serine were significantly decreased in the SNI rats compared with the Sham rats (Fig. 5A), which suggests that D-serine may play an important part in the pathological process of SNI. D-serine was injected daily for 21 days (50mg/kg *i.p.*) after SNI (Fig. 5B). Both vehicle and D-serine application had no effect on the allodynia in SNI rats (Fig. 5C).

To detect whether the reduced levels of D-serine were responsible for the recognition memory deficits, we used a novel-object recognition test to determine the cognitive function after administration of vehicle and D-serine in the SNI rats. During the training session, no significant difference was observed in the time spent exploring each object between the two groups (Fig. 5D). But D-serine treated rats spent more time exploring the novel object than vehicle treated rats in the test session (Fig. 5E-F). These results indicate that D-serine may be responsible for inducing memory deficits in the SNI and may provide a new target for the treatment of learning and memory deficits after SNI.

3.6 Exogenous D-serine administration prevented loss of excitatory synapses in the SNI rats.

As D-serine administration is associated with the rescue of glutamatergic transmission, we further investigated the relationship between D-serine treatment and the density of excitatory synapses in the SNI rats. The protein expressions of vGluT1 and PSD95 in the hippocampus were assessed at 21 days after D-serine treatment in the SNI rats. The expressions of vGluT1 and PSD95 were notably up-regulated in the D-serine treated group (Fig.6A, B). The synaptic density within the areas of the hippocampus were labelled by the co-localization of vGluT1 and PSD95 (Fig. 6C). The density of excitatory synapses within CA1 and CA3 areas after D-serine application were remarkably increased compared to the saline treated group, whereas D-serine administration did not affect the excitatory synaptic density in the DG area (Fig. 6D-F). Taken together, these results suggest an important role of D-serine in locally controlling the density of excitatory synapses in the SNI hippocampus.

3.7 Exogenous D-serine administration rescued the glutamatergic transmission impairment and restored the LTP in the SNI rats

To directly test whether D-serine impaired recognition memory through its effect on glutamatergic transmission, we performed the whole-cell patch-clamp to record the sEPSCs in hippocampal slices of D-serine and vehicle treated SNI rats (Fig. 7A). In the SNI rats, D-serine application increased both the amplitude and frequency of sEPSCs compared to the vehicle treated neurons (Fig. 7B, C). At the same time, the amplitude and frequency of mEPSCs were also increased in the SNI group after administration of D-serine (Fig. 7D-F).

Studies consider LTP in the hippocampus to be partly dependent on D-serine, and LTP important for studying learning and memory [16]. We tested whether we could restore the LTP in the SNI rats by the application of D-serine. In hippocampal slices, the amplitude of fPSPs increased after D-serine administration (Fig. 7G, H). These results suggest that D-serine is involved in the dysfunction of glutamatergic transmission induced by SNI, and it maybe the critical mechanism that regulates the density of excitatory synapses.

Discussion

Clinical studies have demonstrated that more than half of patients who suffer from chronic pain also experience cognitive impairment [2; 36], but the actual mechanisms remain largely unclear. As a major excitatory neurotransmitter in the central nervous system, glutamate has great effects on the pathogenesis and treatment of cognitive disorders [17; 35; 45; 47]. Here by addressing a spared nerve injury model, novel object recognition impairment, which highly resembles clinical recognition dysfunction, was presented in rats. Our results demonstrated that hippocampal glutamatergic synapses were significantly impaired. Furthermore, we speculated that endogenous concentrations of D-serine downregulation in the hippocampus could be responsible for the glutamatergic synaptic damage.

The mechanisms underlying memory deficits in chronic pain patients remains largely elusive. Finn proposed a model based on three theories: (1) neuroplasticity, (2) competing limited resources and (3) dysregulated neurochemistry, to explain the potential mechanisms involved in pain-related cognitive impairment [30]. In this study, we found that periphery nerve injury resulted in glutamatergic synapses damage in the hippocampus which supports the neuroplasticity theory. Our results demonstrated that in the SNI rats, the expression levels of pre- and postsynaptic markers of excitatory synapses, vGluT1 and PSD95, were significantly downregulated. Puncta numbers from vGluT1 and PSD95 double staining and representing synaptic counts, were reduced. Furthermore, the glutamate levels in the hippocampus were decreased under the neuropathic pain condition while the GABA concentration remained unchanged. The evidence revealed that SNI caused loss of glutamatergic synaptic expression in the hippocampus. Ren's studies also showed that the presynaptic boutons were reduced in the CA1 region in the SNI model [39]. In addition, clinical neuroimaging research illustrated hippocampal atrophy in fibromyalgia patients [27]. Thus, glutamatergic synaptic loss could be the reason for hippocampal volume reduction after periphery nerve injury. It is also well known that structural synaptic plasticity, which refers to alterations in synaptic architecture and number, is a vital biological basis of learning

and memory [24]. Thus, it is reasonable to suggest that cortical plasticity may be a very important composition of synaptic/cellular substrate for pain related recognition dysfunction. Here, we found that the expressions of NR1 and NMDAR2B, which are very important for synaptic plasticity, were significantly decreased in neuropathic pain rats, and this is consistent with Wang et al's findings [56]. And electrophysiological studies revealed that SNI not solely altered the long-term plasticity of synapses, but also changed the frequency facilitation substantially, a presynaptic form of short-term plasticity. These findings are in harmony with Ren and Musto's study [33; 39]. To sum up, SNI caused hippocampal glutamatergic synaptic loss, and functionally synaptic plasticity changes. Since the hippocampus is essential for recognition, here glutamatergic synapses impaired the expression of novel objection recognition dysfunction at a behavior level.

In the mature brain, the number of gained synapses is balanced with that of loss of synapses. Any pathological conditions which block synaptogenesis or cause synaptic damage will result in the number of synaptic losses. There are high levels of D-serine in the mature brain and it is an amino acid found in the brain which plays a pivotal role in the formation of excitatory synapses in adult born neurons [50]. In this study, we observed a more prominent decline of D-serine in the hippocampus of the SNI rats. D-Serine could originate from both astrocytes and neurons, but is enriched in astrocytes, raising the possibility that it is mainly released from astrocytes. A recent study reported hippocampal astrocyte atrophy changes following peripheral nerve injury [13]. Hence it is reasonable to speculate that low levels of D-serine result from astrocyte atrophy after never injury. It is interesting to note that the administration of exogenous D-serine could restore the recognition memory. Further, we found that exogenous administered D-serine could reverse the reduction of excitatory synaptic numbers, the frequency and amplitude of EPSCs and the production of LTP impairment in the SNI rats. D-serine is a neuromodulator and is often considered to be an endogenous agonist of the NMDA receptors [6]. Studies have shown that D-serine can induce LTP effects both in vivo and in vitro. More importantly, D-serine oxidase interferes with its endogenous release, which can inhibit the activity of NMDA receptors and the production of LTP [31; 59].

Reduced D-serine levels hinder the induction of NMDA receptor-dependent synaptic plasticity [8]. Nevertheless, D-serine contributes to synaptogenesis and glutamatergic synaptic stability [25; 53]. Thus, giving exogenous D-serine could block periphery never injury induced glutamatergic synaptic impairment in the hippocampus and be a potential way to prevent pain related recognition dysfunction.

D-serine may also play an important role in the transmission of pain signals [43]. D-serine pretreatment with chronic compression nerve injury in animal models can produce significant antinociceptive responses [32]; and in the transgenic mice lacking D-serine oxidase, formalin-induced pain response is significantly increased [54]. However, our results demonstrated that D-serine treatment had no significant effect on mechanical hypersensitivity. Our observation is similar to the results for formalin-induced pain behavior using intrathecal treatment of D-serine [26]. These conflicting results may be attributed to the different approaches of drug administration.

In summary, the glutamatergic system is a key constituent of many neuropsychiatric diseases. Our data described the dysfunction of the glutamatergic system after peripheral nerve injury that may be related to the reduction of D-serine, which should be considered as a new effective strategy of treatment of cognitive impairment in patients with chronic pain.

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Figure legends

Figure 1. The exploratory and recognition memory in the neuropathic condition. (A) Mechanical paw withdrawal threshold was measured by an electroVonFrey, and spare nerve injury induced the decreases in MPWT starting at day3 and continuing to day21 post operation; n=10 rats per group. (B) The schematic presentation for the protocol of novel-object recognition test. Habituation, training and test, represented by three phases: S0, S1, S2. A, B and C represent the different objects used during the test. In the training section, A and B were placed in the box. In the test section, a novel object C replaced object B. (C, D) There were no significant differences in time spent exploring both objects (object A and B) during the training section (S1) between the Sham and SNI groups. And in each group, the two groups of rats spent the same amount of exploration time in A or B on average. When exposed to the novel object (object C), the SNI rats demonstrated a significant reduction in exploration time to the novel object compared with the Sham rats; n=8 rats per group. (E) Recognition index represented the recognition memory; n=8 rats per group. Data are presented as means ± S.E.M. $^{**}P < 0.01$; $^{***}P < 0.001$; N.S. $P > 0.05$, compared to the Sham group, A and E: Student's *t* test; C and D: Two-way Repeated Measures ANOVA; n=8-10 rats per group.

Figure 2. Effect of neuropathic pain on the levels of glutamate and GABA in the hippocampus. (A) The average normalized ^{1}H–NMR spectra of the hippocampus

extracts in the Sham and SNI groups. (B) The average normalized spectra of glutamate and GABA is different in the Sham and SNI groups (means ± S.E.M). (C, D) The concentrations of the glutamate and GABA in the hippocampus. Neuropathic pain induced a marked reduction of the concentration of glutamate (C), but no difference was observed in GABA (D). n=13 rats per group. Data are presented as means ± S.E.M. ***$P < 0.001$; N.S. $P > 0.05$, compared to the Sham group. Student's *t* test; n= 13 rats per group.

Figure 3. Neuropathic pain induced hypofunction of glutamatergic synaptic activity and synaptic plasticity in the hippocampus. (A) sEPSCs paths from hippocampal CA1 pyramidal neurons in the Sham and SNI rats. (B, C) Compared with the Sham group, the SNI rats exclusively decreased the frequency of sEPSCs (B), but there was no difference in amplitude (C); n=9 rats per group. (D) Representative traces of mEPSCs from the Sham and SNI groups. (E, F) In the SNI group, both thefrequency and amplitude of mEPSCs were clearly reduced. n=6 rats per group. (G) Representative bands of western blot for NR1, NR2A, NR2B, GluR1, GluR2 and GAPDH of the Sham and SNI groups. (H) At protein levels, the NR1 and NR2B were dramatically reduced in the SNI rats, but not any other proteins; n=4 rats per group. (I) The LTP of CA3-CA1 pyramidal cells synapse was recorded. The potentiation of HFS was induced in the SNI group. (J) The summarized data of recording; n=5 rats per group. Data are presented as means ± S.E.M. **$P < 0.01$; ***$P < 0.001$; N.S. $P > 0.05$, compared to the Sham group. Student's *t* test; n=4-9 rats per group.

Figure 4. The number of glutamatergic synapses were altered in the SNI rats. (A) The bands and bar graphs show that the expression of PSD95 was markedly reduced after nerve injury; n=4 rats per group. (B) The expression of vGluT1 was significantly decreased in the SNI group; n=4 rats per group. (C) Images of glutamatergic synapses in different hippocampal areas, including CA1, CA3 and DG (VGluT1 (red), PSD95 (green) and Merge (yellow)). (D, F) Quantifications of average hippocampal synaptic co-localized puncta within the Sham and SNI rats; n= 4 rats per group. Data are

presented as means ± S.E.M. *$P < 0.05$; **$P < 0.01$; N.S. $P > 0.05$, compared to the Sham group. Student's *t* test; n=4 rats per group.

Figure 5. D-serine level reduction in the hippocampus of the SNI rats connected with the cognitive impairment but not to pain behaviors. (A) Neuropathic pain caused a significant decrease in D-serine expression on day 21 after surgery; n= 4 rats per group. (B) The schedule of administering the D-serine and behavioral assessment. (C) Both of the vehicle and D-serine treatment induced the decrease in MPWT. D-serine treatment did not reverse the allodynia induced by nerve injury; n= 6 rats per group. (D, E) D-serine administration did not alter the exploration time to both objects during the training section compared with the vehicle treatment group. And within each group, the rats spent the same amount of time exploring A or B on average (D). When exposed to the novel object, D-Serine treatment rats demonstrated a significant increase in exploring time to the novel object compared with the vehicle rat (E); n= 8 rats per group. (F) Administration of D-serine restored impaired recognition memory in the SNI rats; n=8 rats per group. Data are presented as means ± S.E.M. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; N.S. $P > 0.05$. A: Student's *t* test, compared to the Sham group; C: Two-way Repeated Measures ANOVA, compared to the SNI +Vehicle group; D and E: Two-way ANOVA, compared to the SNI +Vehicle group; F: Student's *t* test, compared to the SNI+Vehicle group; n= 4-8 rats per group.

Figure 6. D-serine administration restored the number of glutamatergic synapses in the SNI rats. (A, B) At protein levels, D-serine administration significantly increased the expressions of PSD95 and vGlutT1 compared with the vehicle group; n=5 rats per group. (C) Images of glutamatergic synapses in different hippocampal areas, including CA1, CA3 and DG (vGluT1 (red), PSD95 (green) and Merge (yellow)). (D, F) Quantifications of average hippocampal synaptic co-localized puncta within the vehicle and D-serine treatment rats of SNI; n=4 rats per group. Data are presented as means ± S.E.M. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; N.S. $P>0.05$, compared to the Vehicle group. Student's *t* test; n=4-5 rats per group.

Figure 7. D-serine treatment increased the glutamatergic synaptic transmission efficiency. (A) sEPSCs traces from hippocampal CA1 pyramidal neurons of the SNI rats by applying vehicle or D-serine. (B, C) Compared with the vehicle group, treatment with D-serine significantly increased the frequency and amplitude of sEPSCs; n=5 rats per group. (D) Representative traces of mEPSCs from the vehicle and D-serine groups. (E, F) The frequency and amplitude of the D-serine group were increased compared with the vehicle group; n=5 rats per group. (G) Administration D-serine enhanced the LTP in the SNI rats as shown by the amplitude in fPSP; n=5 rats per group. (H) The summarized data of the recording; n=5 rats per group. Data are presented as means ± S.E.M. $^{**}P < 0.01$; $^{***}P < 0.001$; N.S. $P > 0.05$, compared to the Vehicle group. Student's *t* test; n=5 rats per group.