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Menin regulates spinal glutamate-GABA balance through GAD65 contributing to neuropathic pain

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ABSTRACT

Background: Our previous work found that tumor suppressor menin potentiates spinal synaptic plasticity in the context of peripheral nerve injury-induced neuropathic hypersensitivity, but the underlying molecular mechanisms are not clear. We hereby assessed the role of menin in regulating the spinal balance between glutamate and GABA and its contribution to the pathological condition of nerve injury-induced hypersensitivity.

Methods: In spared nerve injury induced C57BL/6 mice, mechanical withdrawal threshold was measured with von Frey filaments after intrathecal administration of small interfering RNA (siRNA) of *MEN1* or/and subcutaneous histone deacetylase (HDAC) inhibitors to control the level of glutamic acid decarboxylase 65 (GAD65). Immunoblotting and high-performance liquid chromatography were used to detect the level of protein expression and spinal glutamate and GABA, respectively.

Results: Genetic knockdown of spinal menin alleviated nerve injury evoked mechanical hypersensitivity, which was strongly associated with the alteration of the spinal level of GAD65 that resulted in an imbalance of glutamate/GABA ratio from the baseline ratio of 5.8 ± 0.9 ($\times 10^{-4}$) to the peak value of 58.6 ± 11.8 ($\times 10^{-4}$) at the day 14 after SNI ($p < 0.001$), which was reversed by *MEN1* siRNA to 14.7 ± 2.1 ($\times 10^{-4}$) at the day 14 after nerve injury ($p < 0.01$). In further, selective inhibitors of HDACs considerably reversed the ratio of spinal glutamate and GABA, and also alleviated the mechanical withdrawal threshold markedly.

Conclusion: Our findings provide mechanistic insight into the contribution of the upregulated spinal menin to peripheral nerve injury induced neuropathic hypersensitivity by regulating glutamate-GABA balance through deactivating GAD65.

Introduction

Estimates on the prevalence of neuropathic pain range from 2% to ~60% on different neuropathies [1,4,6,7,19,21], and over 50% patients encountered refractory pain that is attributed to its intractable characteristics leading to frequent recurrence and hyporesponsiveness to over-the-counter analgesics [22,29]. Spinal cord, the first relay station where primary sensory inputs integrate and magnify to the higher levels of the nervous system, takes an essential part in the transmission pathway of nociception [25]. Nerve injury induced noxious signals affects synaptic plasticity resulted from the production of multiple neurotransmitters and

Abbreviations: aCSF, artificial cerebral spinal fluid; CNS, central nervous system; EPSCs, excitatory post-synaptic currents; GABA, γ-aminobutyric acid; GAD, glutamic acid decarboxylase; GAD65-rAAV2, glutamic acid decarboxylase 65-recombinant adeno-associated virus vector 2; GAPDH, glyceraldehydes 3-phosphate dehydrogenase antibody; HDAC, histone deacetylase; HPLC, high-performance liquid chromatography; IPSCs, inhibitory post-synaptic currents; MEN1, multiple endocrine neoplasia type 1; q-PCR, quantitative polymerase chain reaction; SD, standard deviation; siRNA, small interfering RNA; SNI, spared nerve injury.

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the transmission of intracellular signaling, through which the expression or functional changes occurred in the excitatory and inhibitory receptors [8].

In vitro study with molluscan neurons (soma–soma synapses) showed that postsynaptic expression of menin, a 610-amino acid tumor suppressor protein encoded by the multiple endocrine neoplasia type 1 (*MEN1*) gene, is necessary for the proper formation of both excitatory and inhibitory central synapses [27]. Zhang and colleagues reported that the expression of spinal menin upregulated after nerve injury [33], and our previous study in further showed that spinal menin plays a pivotal role in potentiating synaptic plasticity in the context of peripheral nerve injury induced neuropathic pain [32]. Although such a change in the expression of spinal menin contributes to the pathogenesis of neuropathic hypersensitivity [33] *via* affecting synaptic plasticity [32], the precise synaptic mechanisms of the upregulated menin in peripheral nerve injury induced neuropathic pain is still so far unclear.

Glutamate and γ-aminobutyric acid (GABA) are respectively two major excitatory and inhibitory neurotransmitters of the central nervous system (CNS), and they control over and balance the excitation level of the neural homeostasis [17]. Contents of both neurotransmitters in the context of pain are changed [20]. Abnormally high levels of glutamate and lower levels of GABA coordinately get the CNS fired up which leads to hypersensitive to noxious stimuli [23]. In terms of the balance control between glutamate to GABA, glutamic acid decarboxylase (GAD) is one major component through which glutamate is catalyzed to GABA through decarboxylation [28]. Even though GAD65 contributes to the pathogenesis of chronic pain [34], and enhancement of the GAD65 expression possesses analgesic effect [9,11], it is still unknown the upstream molecules regulating the content of GAD65 in the context of pain. Our previous study [32] showed that intrathecal antisense oligonucleotide of *MEN1* can alleviate nerve injury-induced changes in the frequency and amplitude of excitatory post-synaptic currents (EPSCs) suggesting spinal menin may be involved in the regulation of both pre- and post-synaptic transmission. We, therefore, proposed that spinal menin is an essential contributor to neuropathic pain *via* affecting the composition or quantity of presynaptic neurotransmitters and then we investigated whether peripheral nerve injury-induced upregulation of spinal menin mediates pain by unbalancing neurotransmission between glutamate and GABA at the site of spinal cord.

Materials and methods

Subjects and peri-surgical care

Male C57BL/6 mice were used for all behavioral tests in accordance to the Ethical Guidelines for Investigations of Experimental Pain in Conscious Animals. The peri-surgical treatment of the animals was reported elsewhere [30]. Briefly, animals were housed to a plastic cage with soft bedding on a reverse 12:12 h light/dark cycle with lights on at 8:00 am and maintained in climate with 23 ± 1 °C housing temperature, and free access to food and water for at least three days before the experiments. Animal assignment to each testing group was randomly blinded. The random numbers were generated by means of the QuickCalcs (GraphPad Software Inc., La Jolla, San Diego, CA; Online Calculators for Scientists, available at http://www.graphpad.com/quickcalcs/RandMenu.cfm. Last accessed July 16, 2012). Test sessions took place during the light phase between 10:00 am and 6:00 pm in a quiet room maintained at 22–24 °C. Prior to testing, each animal was placed in a test box with the dimensions of 30 cm × 30 cm × 15 cm having three mirrored sides for a 10-min habituation period to minimize stress. No food or water was available to the mice during the experiment. Each animal was used only once and was euthanized at the end of the behavioral experiment by administrating a lethal dose of pentobarbital.

siRNA and chemicals

We genetically knocked-down spinal menin expression by using small interfering RNA (siRNA) of *MEN1* synthesized by the Sangon Biotech (Shanghai, China). The sequence of the siRNA of *MEN1* was based on the previous report [15]. The sequences of the RNAi use were as follows: sense sequence 5′-GCU GUA UGA CCU GGA CAU UU-3′ and anti-sense sequence 5′-PUU CGC GAC UAG AAA CAC CUU U-3′, and non-targeting RNAi 5′-UGG UUU ACA UGU CGA CUA A-3′ was used as a control. All siRNAs were delivered using Dharma*FECT* transfection agent 3 (Dharmacon, Lafayette, CO). Besides, following potent histone deacetylase (HDAC) inhibitors were used to reverse GAD65 contents: 0.5 μg (intrathecal, *it*) or 1.0 μg (subcutaneous, *sc*) of MS-275 (Selleck, Houston, TX), and 10 μg (intrathecal, *it*) or 20 μg (subcutaneous, *sc*) of suberoylanilide hydroxamic acid (SAHA; Cayman Chemical, Ann Arbor, MI).

Study protocol

All animals underwent intrathecal catheterization seven days before nerve injury. The mechanical behavioral tests were performed at the day 1 before nerve injury, and days of 0, 1, 3, 7, 8, 9, 14, 21 and 35 after nerve injury. For the intrathecal treatment, we administered 10 μM siRNA according to the dose-responses curve (ED_{50} = 9.86 μM; range 6.37–17.41 μM) developed by using gradient doses of siRNA (in μM): 0.5, 1, 5, 10, 20 and 50 intrathecally in another preliminary set of different animals (n = 6) and the ED50 was calculated using maximum likelihood estimation (MLE) and logistic regression with Firth's correction, or control of *MEN1* in 5 μl transfection agent was injected daily for three consecutive days since the post-injured day 7. All experimental drugs were injected intrathecally using a microsyringe in a volume of 5 μl over 30 s, and then a 10 μl of saline was used to flush the catheter dead space. For the vehicle control animals, a total of 15 μl of transfection agent only was given.

Intrathecal catheterization

Mice were implanted with an intrathecal catheter (ALZET Osmotic Pumps, Cupertino, CA, USA) for drug delivery as described previously [32]. In brief, after shaving and sterilizing the cephalic-cervical area, a midline incision was made followed by dissection of the paravertebral muscles from the spinous processes. Under the guidance of a surgical microscope, a hole (1 mm × 1 mm) was drilled manually until the dura was exposed. The dura was slit and the catheter was inserted for 2.5 cm caudal from the dural slit, and the catheter was fixed with a drop of tissue glue (Histoacryl®; B. Braun, Tuttingen, Germany) and further was secured on the fascia of paravertebral muscle. Finally, sodium penicillin 10,000 IU (Shanghai AoBopharmtech, Shanghai, China) was given intramuscularly against infection. The mice would be excluded (~10%) neurological deficits were exhibited after catheterization.

Animal model and behavioral detection

The spared nerve injury (SNI) induced model was used as described elsewhere [2]. In brief, animals were anesthetized with isoflurane, and the tibial and common peroneal branches of the sciatic nerve were ligated and sectioned distally, but the sural nerve was left intact. For sham surgeries, the sciatic nerve was merely exposed but not ligated and desected. Sham operated animals were used as the comparison. Mechanical pain thresholds

were assessed using the von Frey filaments (Stoelting Co., Wood Dale, IL) prior to surgical procedures and again at different time points thereafter. The testing protocol has been described in our previous study [32]. In brief, the filaments were applied to the central surface of the hind paw plantar for a maximal 10 s to determine the threshold of the stimulus through evoking a withdrawal response. The stimulation was initiated from the weakest filament (0.407 g). The increment of stimulus was based on the response of the mouse to the current filament, if the paw withdrew, the same filament was again used 60 s later; but if not, the next stronger filament was presented. If the mouse withdrew its paw in two consecutive trials with the same filament value, no further filaments were tested. Withdrawal responses were used to determine the absolute threshold, *i.e.* the 50% withdrawal threshold, by fitting to a Gaussian Integral Psychometric Function *via* a Maximum Likelihood method.

RNA extraction and quantitative polymerase chain reaction

The expression levels of specific transcripts were determined using quantitative PCR (q-PCR). The detailed procedures were described elsewhere [30]. In summary, cDNAs were made using a Thermo-Script reverse-transcriptase kit (Invitrogen Co., Carlsbad, CA) and oligo-dT primers according to manufacturer's protocol. q-PCR amplification and quantitation were carried out using an iCycler machine (Bio-Rad Laboratories, Inc., Hercules, CA). Sample amplification was done in a volume of 25 μl containing 12.5 μl of 2 × IQ SybrGreen Mix (Bio-Rad), 1 μmol/L of each primer, and a volume of cDNA corresponding to 80 ng of total RNA. Cycling conditions were as follows: 95 °C for 5 s, 30 s at the appropriate annealing temperature for each primer, and 72 °C for 30 s. The expression of each gene was normalized relative to 18S expression levels for each sample. The $2^{-\Delta\Delta Ct}$ method was used to calculate the expression of each gene relative to untreated control. Primers were designed using Beacon Designer (Premier Biosoft International, Palo Alto, CA). Primer sequences are as follows: 18S, 5′-CGC CGC TAG AGG TGA AAT TC-3′, 5′-TTG GCA AAT GCT TTC GCT-3′; *MEN1* exon 1, 5′-CGA CGG CAT CTG CAA AAT GGG-3′, 5′-GGG TTT GGG TAG AGG TGA GG-3′. All samples were tested in triplicate.

Western blotting detection

The detailed methods of western blotting were presented in our previous studies [30,32]. The following primary antibodies were used: menin (Bethyl Laboratories Inc., Montgomery, TX): 1:2000, rabbit polyclonal against mouse; glutamic acid decarboxylase (GAD65; Abcam Inc., Cambridge, MA): 1:1000, goat polyclonal against mouse; a polyclonal anti-mouse glyceraldehydes 3-phosphate dehydrogenase antibody (GAPDH; 1:10,000, GenScript USA Inc., Piscataway, NJ); a monoclonal anti-mouse β-actin antibody (1:10,000, Sigma–Aldrich Inc.) and a polyclonal rabbit-anti-mouse β-tubulin (1:10,000, Abcam, MA). After repeated washing, goat anti-rabbit secondary antibody (1:4000, horseradish peroxidase-conjugated; Vector Laboratories, Peterborough, UK) or rabbit anti-goat secondary antibody (Sigma–Aldrich Inc.) incubation was performed, developed with a chemiluminescence system, and followed with film exposure and relative density analysis with the Typhoon Imaging System (GE Healthcare, Piscataway, NJ).

Microdialysis and high-performance liquid chromatography

Microdialysis studies were performed with normal, sham and SNI animals. In brief, after induction with 10–20 g/kg of pentobarbital sodium intraperitoneally and then maintained with 1.5% isoflurane in 100% oxygen through a nose cone. After shaving the hair from vertebra T12–L1, the skin was incised and muscle was cleared away above the vertebral column. Two holes (diameter 0.2 mm) were drilled at T13 level till to the dorsal horn. A linear dialysis probe with a 1 mm active length membrane (OD 218 μm; BAS, West Lafayette, IN) was placed transversely through the spinal cord dorsal cord. Epoxy glue was used to cover the dialysis membrane, and then the end of the dialysis tube was connected to tubing that was exteriorized and fastened in the neck. When the mice were recovered from surgeries, a microdialysis pump (M Dialysis, Solna, Sweden) was connected to the dialysis probe and perfused with Ringer's solution (NaCl 147 mM, KCl 4 mM, $CaCl_2$ 2.3 mM) at a flow rate of 5 μL/min. After 1-hour's equilibration, six dialysis samples were collected every 10 min over 1 h at each observing time points, *i.e.* at the days of 1, 4, 7, 9, 14, 21, and 35 after SNI.

In addition, two subgroups were set to validate the microdialysis technique in mice spinal cord dorsal horn by analyzing the influence of changing the perfusing solution (from aCSF to Ringer's solution) and the flow rate (from 1 to 5 μl/min) on basal glutamate and GABA release. After completing each experiment, an overdose of pentobarbital was used to kill the animal, and the position of the microdialysis probe in the dorsal horn was confirmed microscopically *via* injecting methylene blue through the catheter. Given we catheterized the animals with two intrathecal tubes (one for microdialysis probe connection and another for drug administration), the behavioral responses with von Frey filaments were assessed to verifying influence of these catheters on normal nerve function.

The 10-min perfusate fractions were collected into an auto injector (Eicom Co., San Diego, CA), and the glutamate and GABA concentrations were analyzed using high-performance liquid chromatography (HPLC) with electrochemical detection using an HTEC-500 analyzing system (Eicom Co.). The chromatographic conditions were as follows: The mobile phase comprised 0.1 M/L ammonium acetate buffer (pH = 6.0), 0.05 M/L sodium sulfonate in methanol (7:3, v/v), and 50 mg/L Na_2-EDTA, and the column was an Eicompak FA-30DS (3.0 ID × 50 mm; Eicom Co.). The working electrode was glassy carbon (WE-GC with Gasket, GS-25; Eicom Co.); flow rate, 500 μL/min. The detector voltage was set at 600 mV (*vs* Ag/AgCl) and the temperature was set at 40 °C. The retention time was 12 min. The detection limit of this assay in the present study was 100 nM for glutamate and 10 nM for GABA with a 10 μL injection.

Data analyses

Data are presented as the means ± standard deviation (SD), and analyzed using GraphPad Prism version 5.0 (GraphPad Software Inc., San Diego, CA) or PASW Statistics v18.0 (IBM Co., Armonk, NY). Nociceptive data and multiple group results were analyzed with two-way analysis of variance where treatment and nociceptive status or other multiple factors were considered as independent variables in the model, and all of the factors were between-subjects factors. The analysis of variance was followed by the Bonferroni *post hoc* tests for multiple comparisons among different time points. When there were two testing groups, Student's *t*-test was used to analyze the intergroup difference. The expressions of proteins detected with immunohistochemistry were analyzed with paired *t*-test. All reported *p*-values are two-sided and a *p*-value of less than 0.05 is accepted for statistical significance.

Results

Time-dependent expression of spinal menin after peripheral nerve injury

The time course of mechanical withdrawal threshold in the spared nerve injured animals showed that the nerve injury induced

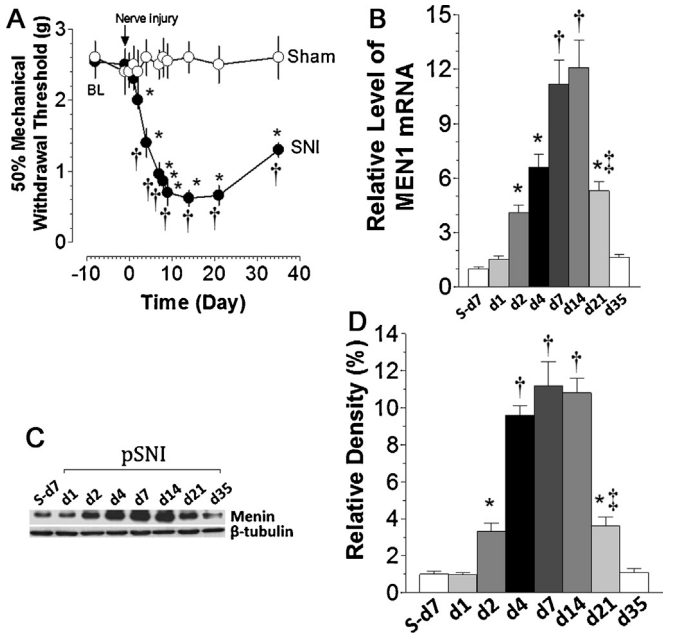

Fig. 1. Expression of spinal menin after nerve injury. The pain trajectory showed that mechanical withdrawal threshold recovered 41 ± 6% at day 35 after SNI (A) $^{*}p < 0.05$ *vs* Sham; $^{\dagger}p < 0.05$ *vs* baseline; $n = 12$. (B–D) Both of the expression of *MEN1* mRNA and menin protein peaked during the day 7 to day 14, but returned to the baseline at day 35 ($^{*}p < 0.05$ *vs* S-d7; $^{\dagger}p < 0.01$ *vs* S-d7; $^{\ddagger}p < 0.05$ *vs* d7; $n = 7$).

a time-dependent reduction of mechanical threshold and peaked at the 14th day after injury and then started recovery (Fig. 1A; $n = 12$). Besides, both of the expression of *MEN1* mRNA and menin protein peaked during the day 7 to day 14, and lasted up to 21 days after nerve injury, but returned to the baseline level at day 35 (Fig. 1B–D; $n = 7$). This suggested that spinal expression of menin protein changed time-dependently after peripheral nerve injury.

Knockdown of spinal menin alleviates pain and alters spinal balance between glutamate and GABA

As expected that spinal *MEN1* siRNA reversed ipsilateral mechanical withdrawal threshold markedly, and this effect lasted at least one week after a three-day consecutive injection one bolus every day (Fig. 2A and B). In consideration of the balance between excitatory and inhibitory neurotransmitters at synapses [31] and their key roles playing in homeostatic synaptic regulation [24], plus the role of spinal menin in synaptic plasticity [32], we then measured the spinal contents of glutamate and GABA with HPLC at different time points after SNI treated with *MEN1* siRNA, and found that SNI induced converse alteration in the levels of glutamate and GABA, *i.e.* increase in glutamate but decrease in GABA (Fig. 2C and D; $n = 10$). The levels of both glutamate and GABA could be reversed by the intrathecal *MEN1* siRNA but not by the *MEN1* msRNA suggesting spinal menin involves in the pathogenesis of neuropathic pain by elevating synaptic ratio of glutamate/GABA (Fig. 2E; $n = 10$). Since the rate-limiting step in GABA biosynthesis is the decarboxylation of glutamate by GAD65 through which the neural level of glutamate and GABA is balanced [14], we then also measured the expression of spinal GAD65 and found that SNI-induced a significant decrease in the spinal level of GAD65, which could be reversed by *MEN1* knockdown (Fig. 2F and G; $n = 6$). Thus we inferred from these preliminary data and our previous work [32] that spinal menin is a critical contributor to SNI-induced neuropathic pain through increasing glutamate/GABA ratio *via* reducing GAD65 contents by which a considerable increase in neural excitability was resulted from.

Menin elevates glutamate/GABA ratio by suppressing GAD65

Our preliminary data indicated that spinal menin influenced the assumed pre-synaptic level of GAD65 which results in a synaptic alteration of the glutamate/GABA ratio (Fig. 2F and G), we next tested this role of menin by using different types of inhibitors of HDAC to increase GAD65 level in SNI mice. As reported by Zhang et al. [34] that epigenetic suppression of GAD65 expression at brainstem nucleus raphe is a crucial supraspinal step in maintaining hypersensitive behavior in chronic pain animals, and considering the effectiveness of GAD65-rAAV2 (recombinant adeno-associated virus vector 2) administration to dorsal root ganglion and sciatic nerve in neuropathic pain [9,10], we first administered HDAC inhibitors subcutaneously (*sc*) or intrathecally (*it*) to observe their reversing role in spinal GAD65 expression and alleviating effect on pain sensitivity. Immunoblotting data showed that SNI induced a considerable downregulation of spinal GAD65 expression, but this downregulation could be attenuated in part by both subcutaneously and intrathecally administered HDAC inhibitors at day 14 after nerve injury (Fig. 3A and B; $p < 0.05$ *vs* SNI; $n = 8$) indicating that subcutaneous administration of HDAC inhibitors is an effective means to increase spinal GAD65 level. Additionally, simultaneous administration of *MEN1* siRNA and subcutaneous suberoylanilide hydroxamic acid (SAHA, also known as Vorinostat) reversed GAD65 expression to 60% to the sham (Fig. 3A and B; $p < 0.0001$ *vs* SNI + SAHA *sc*). These HDAC inhibitors could not alter the expression of menin after SNI injury (Fig. 3A and B). To observe the functional effect of the reversed GAD65 in the spinal cord, we measured the mechanical threshold after injection of the HDAC inhibitors and found that the intrathecal MS-275 (also known as Entinostat) and SAHA produced a more significant alleviating effect which lasted up to 7 days than that of the subcutaneous drug administration (Fig. 3C and D; $p < 0.05$ intrathecal *vs* subcutaneous; $n = 8$). Interestingly, simultaneous administration of both *MEN1* siRNA and SAHA *sc* produced a more significant elongation of the alleviating effect on mechanical hyperalgesia than single *MEN1* siRNA or SAHA *sc* (Fig. 3E; $n = 8$). In the article from Zhang et al. [34], they did not measure the contents of glutamate and GABA after HDAC inhibition but the GABAergic inhibitory post-synaptic currents (IPSCs), however taking into account of the rate-limiting role of GAD65 in catalyzing glutamate to GABA to keep the levels of both excitability and inhibitory transmitters into normal range [26], we measured the spinal levels of glutamate and GABA and found that both *sc* and *it* inhibitors of HDAC all produced reversing effect on glutamate and GABA at day 14 (Fig. 3F and G; $n = 8$). These data suggested that in the context of neuropathic pain, the rescued GAD65 by inhibiting its rate-limiting enzyme of HDAC attenuates noxious pain responses through reversing glutamate/GABA ratio to suppress SNI-induced sensitization.

Discussion

In the present study, we provided evidence that menin in the spinal cord contributes to peripheral nerve injury induced neuropathic pain *via* regulating spinal glutamate-GABA balance as (i) the expression of spinal menin displayed a time-dependent manner after nerve injury; (ii) knockdown of spinal menin with *it* *MEN1* siRNA alleviates mechanical pain and alters spinal balance between glutamate and GABA; (iii) SNI-induced a significant decrease in the spinal level of GAD65, which could be reversed by *MEN1* knockdown; and (iv) blockade of GAD65 with HDAC inhibitors produced a combined alleviation of pain with *MEN1* siRNA. These data suggest that spinal menin causes neuropathic hyper-excitability by elevating synaptic ratio of glutamate/GABA through decreasing the level of GAD65.

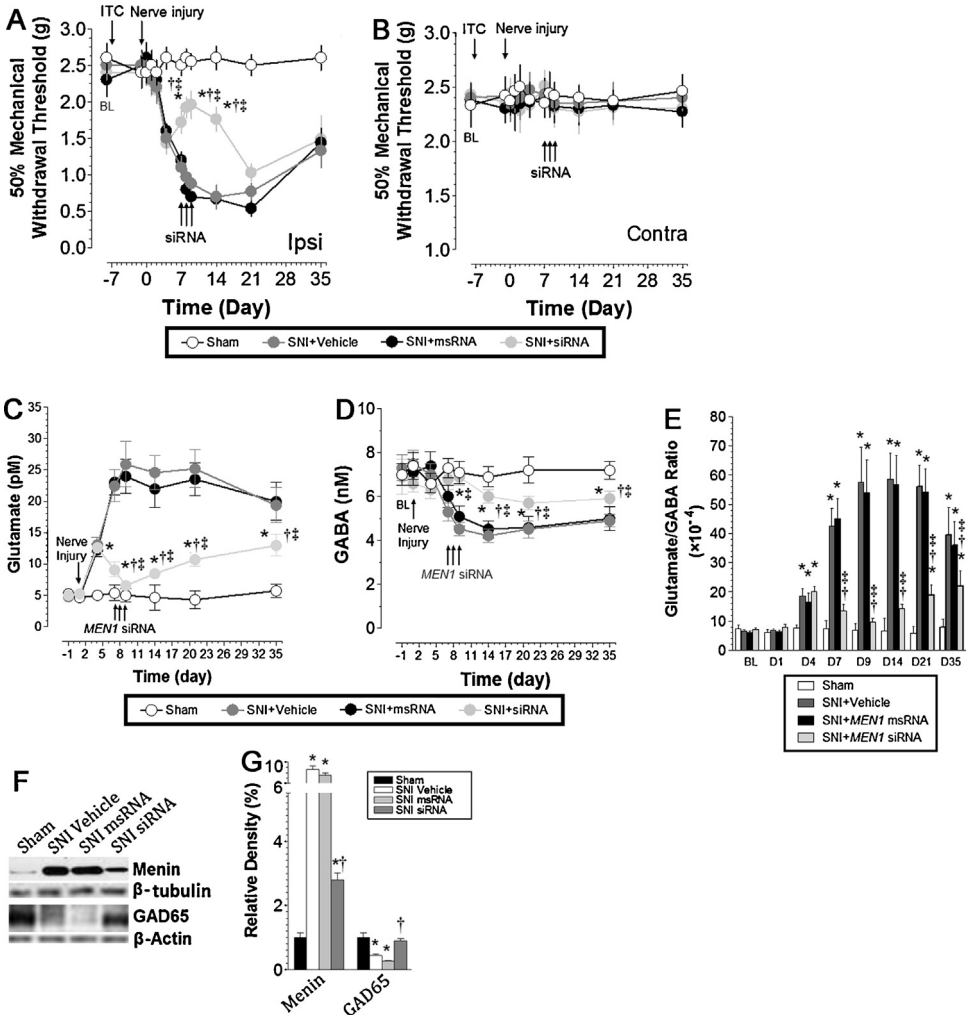

Fig. 2. Knockdown of spinal menin alleviates pain and alters spinal balance between glutamate and GABA. (A and B) Intrathecal administration of siRNA of *MEN1* reversed ipsilateral mechanical withdrawal threshold markedly, which lasted at least one week ($^{*}p < 0.05$ *vs* Sham; $^{\dagger}p < 0.05$ *vs* SNI + Vehicle; $^{\ddagger}p < 0.05$ *vs* SNI + msRNA; $n = 12$). (C–E) SNI induced increase in glutamate but decrease in GABA, which could be reversed by the intrathecal *MEN1* siRNA but not by the *MEN1* msRNA ($^{*}p < 0.01$ *vs* Sham; $^{\dagger}p < 0.05$ *vs* SNI + Vehicle; $^{\ddagger}p < 0.05$ *vs* SNI + msRNA; $n = 10$), and the ratio of glutamate/GABA was elevated ($^{*}p < 0.05$ *vs* Sham; $^{\dagger}p < 0.05$ *vs* SNI + Vehicle; $^{\ddagger}p < 0.05$ *vs* SNI + msRNA; $n = 10$). (F and G) SNI-induced a significant decrease in the spinal level of GAD65, which could be reversed by *MEN1* knockdown ($^{*}p < 0.05$ *vs* Sham; $^{\dagger}p < 0.05$ *vs* SNI + Vehicle and SNI + msRNA; $n = 6$ for each sample).

In the study of Zhang et al. [33], the expression of spinal menin was upregulated merely at the first week in the SNI animals, although they recorded a similar time-course of mechanical withdrawal threshold as that we did, we found further that the threshold recovered 41 ± 6% at day 35 post SNI. Besides, both of the expression of *MEN1* mRNA and menin protein peaked during the day 7 to day 14, and lasted up to 21 days after nerve injury, but returned to the baseline level at day 35. We used mice that differ to rats for which may explain this difference in part, but we still suggested that further studies are needed to verify this in different types of animals. Meanwhile, Zhang and colleagues measured the level of actin as the loading control of menin protein [33]; this in our point view should be reconsidered because menin is a nuclear factor, the cytoplasmic protein of actin cannot reflect the accurate change occurred in the nucleus, so the difference in extracting proteins from cytoplasm or nucleus is likely to result in significant differences in the levels of proteins.

Although two major isoforms of GAD (GAD67 and GAD65) exist encoded by two different genes-*gad1* and *gad2*, respectively, they share substantial similarities and interactions and each of them synthesizes GABA for both of the transmitter pool and the metabolic pool in the CNS [13]. The synthesis of GABA within pre-synaptic vesicles of central neurons is the preferred target of GAD65, and the active synaptic release of GABA also needs GAD65 [18,26]. Loss of GABAergic inhibition caused by impaired GABA synthesis and release results in neuronal hyperactivity which is strongly associated with nerve injury induced hypersensitivity, and on the contrary enhancing GABA inhibition is an effective means in pain control [5,16]. In this study, we only observed the change of GAD65 that was associated with spinal menin, but we still cannot preclude a role of cytoplasmic GAD67 in pain-related neural excitability, and the precise role of GAD67 and its possible relationship to menin in pain warrants further studies. Our findings collectively demonstrate that spinal menin is an alternative pivotal suppressor of synaptic GAD65 which results in a decrease in GABAergic inhibition and an enhancement of glutamatergic transmission in nerve injury induced hyperalgesic condition.

Our data and other reports suggest that targeting GAD65 through inhibiting HDAC may be a new promising type of analgesics [3]. These evidences give us hope to control, at least in part, neuropathic pain by targeting on GAD65. However, given the fact as showed in our study that both GAD65-associated epigenetic control on the transmitters' synthesis and release and glutamatergic receptors-related neuronal hypersensitivity were regulated by the nuclear factor menin, so it is probably inadequate

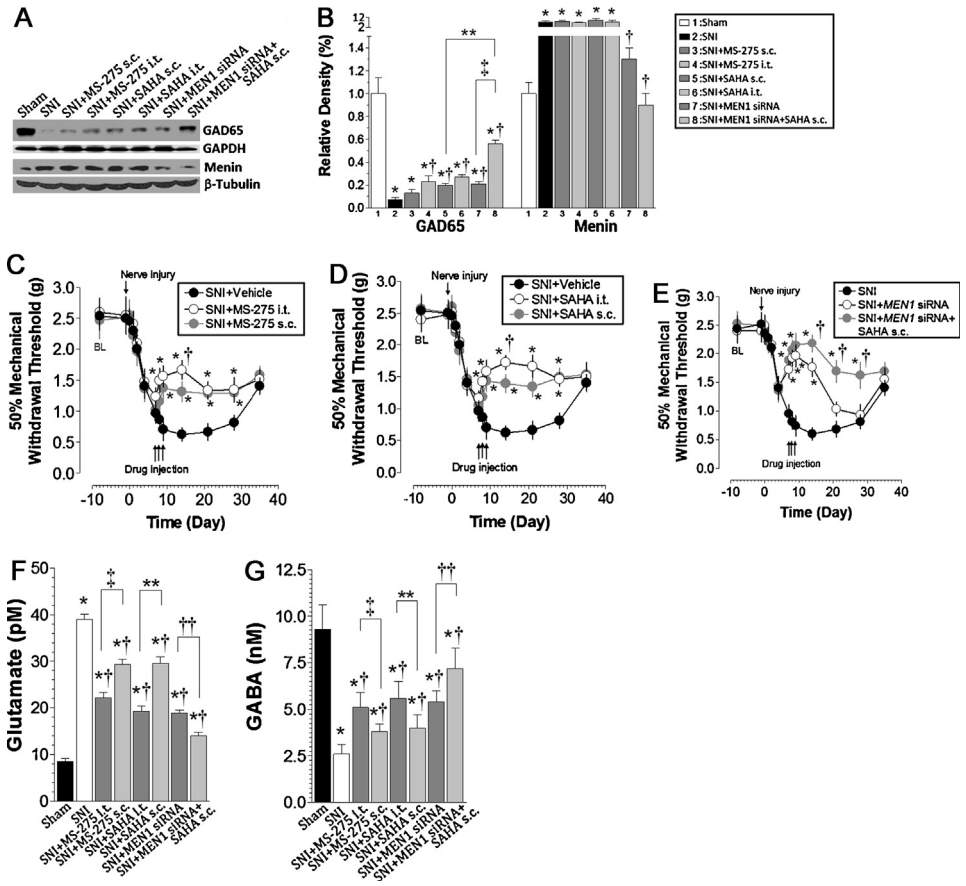

Fig. 3. Blockade of GAD65 produces combined alleviation of pain with *MEN1* siRNA. (A and B) SNI induced a considerable downregulation of spinal GAD65 expression, but this downregulation could be in part attenuated by both *sc* and *it* administered HDAC inhibitors at day 14 after nerve injury. However, HDAC inhibitors played no role in menin expression ($^{*}p < 0.05$ *vs* Sham; $^{\dagger}p < 0.05$ *vs* SNI; $^{\ddagger}p$ and $^{**}p < 0.05$; $n = 8$). (C and D) Intrathecal MS-275 and SAHA produced a more significant alleviating effect on mechanical threshold which lasted up to 7 days than that of the subcutaneous drug administration ($^{*}p < 0.05$ *vs* SNI + Vehicle; $^{\dagger}p < 0.05$ intrathecal *vs* subcutaneous; $n = 8$). (E) Simultaneous administration of both *MEN1* siRNA and SAHA *sc* produced a more significant elongation of the alleviating effect on mechanical hyperalgesia than single *MEN1* siRNA or SAHA *sc* ($^{*}p < 0.05$ *vs* SNI; $^{\dagger}p < 0.05$ *MEN1* siRNA *vs* *MEN1* siRNA + SAHA *sc*; $n = 8$). (F and G) Both *sc* and *it* inhibitors of HDAC all produced reversing effect on glutamate and GABA at day 14 ($^{*}p < 0.05$ *vs* Sham; $^{\dagger}p < 0.05$ *vs* SNI; $^{\ddagger}p$, $^{**}p$ and $^{\ddagger\ddagger}p < 0.05$; $n = 8$).

to control nerve injury-induced pain if only concentrating on single HDAC or glutamatergic receptors. The major reason for such a possibility is that the abovementioned two pathways were activated simultaneously under pain condition and formed a self-enhancing circle which was mediated by nuclear factor menin. Besides, taking into account of the nuclear location of menin, it is difficult to find a suitable drug that can pass through nuclear membrane and target to menin or *MEN1*. In our study we used *MEN1* siRNA to knockdown *MEN1* translation and menin expression, and the results showed it is effective in controlling the expression of menin and subsequently its downstream effector GAD65. Although so, its maximal effectiveness (*i.e.* potency) still was affected finally by cellular metabolism, we initially based our study on the presumption that RNAi was an efficient and satisfactory method of gene knockdown without considerable toxic side effects. In fact, these issues should be noted when interpreting the data and great care has to be taken before initiating therapeutic studies with humans. Although an siRNA has been considered as a single molecule for target discovery, target validation and therapeutic application [12], it is still a challenge for drug development.

The levels of glutamate and GABA in the CNS are generally associated with the changes in (1) the production of their corresponding genes; (2) the function of GAD65; and (3) efficacy of both uptake transporters. In our study, we merely observed the role of GAD65 in SNI animals and found an opposite expression of spinal menin and GAD65 after nerve injury, and further behavioral detection showed that HDAC inhibitors and *MEN1* siRNA can produce combined effect on the alleviation of mechanical pain, plus the protein expression measurement showing that *MEN1* siRNA upregulated GAD65 expression, but HDAC inhibitors did not change the level of menin suggesting that GAD65 is a downstream effector of menin activation after peripheral nerve injury. However, we still cannot exclude the possible role of menin in affecting the uptake transporters of both glutamate and GABA that may contribute to the alteration of glutamate/GABA ratio after SNI. Of course, the function of these two transmitters' corresponding genes also were not detected and then the probable relationship with the upregulated menin expression after nerve injury was not measured, these should be verified by further studies. Another limitation should be acknowledged before drawing the conclusion. The homeostasis of extracellular neurotransmitters is majorly related to the exocytosis of vesicles into cleft and then the subsequent reuptake. Although GAD65 contributes to the pools of glutamate and GABA in the CNS [13], and it is also involved in the control of GABA release [26], it should not be excluded that the changed ratio of spinal glutamate/GABA after SNI is affected by menin through interacting with presynaptic proteins which finally control the exocytosis of vesicles. However, our results still provided us the information that, at least in part, spinal menin affects glutamate/GABA balance after peripheral nerve injury that contributes to the mechanical hypersensitivity. As we hypothesized that

spinal menin causes neuropathic hyper-excitability by elevating synaptic ratio of glutamate/GABA *via* decreasing the level of GAD65, whereas our data showed that nerve injury-induced mechanical threshold only recovered around 40% at the day 35 after SNI, and the levels of spinal glutamate and GABA and their ratio also did not returned to the pre-injury levels, but both expression of menin and *MEN1* mRNA returned to the baseline. For this alteration, one possibility is that nerve injury evoked changes in menin and *MEN1* mRNA expression which played as a short-term trigger of GAD65-associated imbalance of glutamate/GABA, and then some other contributing pathways may be exist to keeping the overbalanced glutamate/GABA ratio long enough as the changes in mechanical hypersensitivity.

In summary, upregulated spinal menin is an important contributor to peripheral nerve injury-induced neuropathic pain, during which an imbalanced glutamate/GABA production in the spinal cord was occurred that is strongly associated with the changed level of GAD65. These are the underlying molecular bases for menin-associated alteration in synaptic plasticity [32] from which nerve injury-induced hypersensitivity developed. Future studies should focus on menin's role in the excytosis of vesicles and subsequent reuptake control of the released transmitters.

Conflict of interests

None.

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