

## Original Article

# Kalirin-7 contributes to type 2 diabetic neuropathic pain via the postsynaptic density-95/N-methyl-D-aspartate receptor 2B-dependent N-methyl-D-aspartate receptor 2B phosphorylation in the spinal cord in rats

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**Abstract:** Objective: Diabetic neuropathic pain (DNP) is one of the common complications in type 2 Diabetes Mellitus (DM) patients. However, molecular mechanisms in underlying diabetic neuropathic pain are still poorly understood. Kalirin-7, a multifunctional Rho GDP/GTP exchange factor, located at the excitatory synapses, was reported to modulate the neuronal cytoskeleton. Therefore, in this study, we explored the effects of Kalirin-7 on type 2 diabetic neuropathic pain and the mechanisms in spinal cord in rats. Methods: The type 2 diabetic neuropathic pain model was established in rats by feeding them with a high-sugar and high-fat diet for 8 weeks, and then fasting them for 12 hours, followed by a single intraperitoneal injection of STZ. Kalirin-7 was knocked down in the spinal cord by an intrathecal administration of Kalirin-7 siRNA. Results: The levels of Kalirin-7, p-NR2B and PSD-95 as well as the PSD-95-NR2B coupling were significantly increased in the spinal cord of type 2 DM rats. The knockdown of Kalirin-7 expression in the spinal cord by the intrathecal administration of Kalirin-7 siRNA not only reduced the levels of p-NR2B and the PSD-95-NR2B coupling in the spinal cord, but also relieved mechanical allodynia and thermal hyperalgesia in type 2 DM rats. Conclusions: Our findings suggest that spinally expressed Kalirin-7 likely contributes to type 2 diabetic neuropathic pain through regulating the PSD-95/NR2B interaction-dependent NR2B phosphorylation in the spinal cord.

**Keywords:** Type 2 diabetes mellitus, neuropathic pain, Kalirin-7, PSD-95-NR2B, p-NR2B

## Introduction

Type 2 Diabetes Mellitus is a common disease with various symptoms with a high morbidity in modern society [1]. Diabetic neuropathic pain (DNP) is one of the major symptoms in type 2 Diabetes Mellitus. It occurs in approximately 10-20% of patients with diabetes, or roughly 40-50% patients with diabetic neuropathy. It is usually characterized by spontaneous pain, hyperalgesia, and allodynia [2]. The mechanisms underlying DNP are extremely complex and thus the pathogenesis of diabetic neuropathic pain is still largely unknown. Hyperglycemia mediated metabolic pathways have long been associated in the pathogenesis of DNP, but their role in those with neuropathic pain

is less clearly defined. It's been found that DNP is related to numerous metabolic pathways, including the polyol pathway, protein kinase C activity, and increased advanced glycation end-products (AGEs). However, there is limited evidence to support glycemic control or lifestyle modifications to improve painful neuropathic symptoms. Moreover, the evidence to support pathogenic treatments for neuropathic pain in DNP has generally been disappointing, and only a few pharmacotherapeutic agents are available in selected countries. Neuropathic pain plagues many T2DM patients and is often an uncomfortable experience. However, little effective therapy without side effects exist that could be used to treat this type of pain [3].

Central sensitization represents the augmented neuronal excitability and enhanced synaptic efficacy in nociceptive pathways in the spinal cord and the brain [4-6]. The increasing evidence indicates that central sensitization within the central nervous system is considered as a key mechanism in underlying chronic pain including DNP [7, 8]. Glutamatergic neurotransmission was enhanced in the spinal cord during development and persistence of chronic neuropathic pain [9]. Previous studies have revealed that the N-methyl-D-aspartate receptor 2B subunit (NR2B), especially phosphorylated NR2B (p-NR2B), participated in central sensitization during chronic neuropathic pain [10] and inflammatory pain [11]. Interaction between NR2B and postsynaptic density-95 (PSD-95) contributes to NR2B phosphorylation. Knockout of NR2B abolished NMDA receptor-dependent long-term potentiation (LTP) and reduced dendritic spine density [12].

Kalirin-7, a multifunctional guanine nucleotide exchange factor (GEF) of Rho GTPase, is localized to dendritic spines and participates in structural and functional plasticity at excitatory synapses. Knockdown of Kalirin-7 led to spine loss [13]. Kalirin-7-dependent dendritic spine formation and enhancement were essential for the modulation of the synaptic strength [14]. Recent evidence shows that Kalirin-7 might contribute to the development of chronic pain. Incisional rats with remifentanyl-induced hyperalgesia displayed increases in spinal Kalirin-7 expression and spine density [15]. Moreover, knockdown of spinal Kalirin-7 impaired remifentanyl-induced hyperalgesia and spine plasticity [15]. Kalirin-7 has a C-terminal PDZ-binding motif, which may regulate the expression of PSD-95 and NR2B and their bindings. Rats with spinal Kalirin-7 knockdown exhibited a decrease in expression of spinal p-NR2B submit [16]. These studies suggest that Kalirin-7 is a key player in spinal central sensitization, possibly through affecting p-NR2B.

In the present study, we hypothesized that spinal cord Kalirin-7 might be involved in type 2 DNP. The expression of Kalirin-7, PSD-95, total NR2B and p-NR2B, as well as the PSD-95-NR2B coupling, were first examined in spinal cords from type 2 DNP rats. The effect of spinal Kalirin-7 knockdown through an intrathecal injection of Kalirin-7 siRNA on the NR2B-

PSD-95 coupling and NR2B phosphorylation in spinal cord and pain behaviors in type 2 DNP rats was also investigated.

### Methods

#### *Animals*

Male Sprague Dawley (SD) rats (4-6-week-old, bodyweight 110 g-150 g) were fed ad libitum and housed in a 12/12 h light/dark cycle with 4 per cage at 23-25°C. All experimental operations were approved by the Animal Use Committee of Wenzhou Medical University.

#### *Establishment of type 2 diabetic neuropathic pain model in rats*

The type 2 diabetic neuropathic pain model was carried out as described previously [17]. Rats were divided randomly into two groups (Control group and DM group). DM group (N = 24) were fed with a high-sugar and high-fat diet (67% normal feed, 10% oil, 20% sucrose, 1% sodium cholate and 2% cholesterol), whereas the Control group (N = 6) were fed with normal feed. After 8 weeks of feeding, rats were made to fast for 12 hours with free access to water. Rats in DM group received a single intraperitoneal injection of STZ (Sigma Aldrich, St. Louis, MO, USA) at a dose of 35 mg/kg [17]. Simultaneously, the rats from Control group were given the intraperitoneal injection of the same volume of citrate buffer as a control. Bodyweight, fasting blood glucose, insulin levels, and insulin sensitivity index were measured before the diet, 8 weeks after the high-sugar and high-fat diet, and two weeks after the STZ/citrate buffer injection.

#### *Measurement of serum insulin and insulin sensitivity index*

Before collecting tail vein blood, rats were made to fast for 12 hours with free access to water. 0.75 ml tail vein blood was collected and kept at 25°C for 1 hour. After the samples were centrifuged at  $3,000 \times g$  at 4°C for 25 minutes, the supernatants were harvested for the measurement of insulin concentration by using ELISA kits (Haixi Tang Biotechnology Co., Ltd., Shanghai, China). The insulin sensitivity index was calculated according to the following formula: insulin sensitivity index =  $1/(\text{fasting glucose} \times \text{fasting insulin})$  [18].

### *Pain behavior test*

To identify whether the type 2 diabetic neuropathic pain rat models were established successfully, we performed mechanical and thermal tests in rats before STZ injection and on days 14, 17, 21 and 28 after STZ injections, which were evaluated by a blinded experimenter.

*Thermal test:* An analgesia meter (IITC 336, Woodland Hills, CA, USA) was used to measure the thermal withdrawal latency (TWL). In brief, each rat was placed in a transparent plexiglass compartment containing a smooth 3 mm thick glass floor, through which the thermal radiation was focused on the hind paw. The time from heat stimulation initiation to paw lift was recorded as the TWL. Each trial was repeated five times with 5 min intervals between trials. The mean of the values from latter three trials was considered as TWL.

*Mechanical test:* The rats were placed in transparent organic glass cages (22 cm × 22 cm × 22 cm) on a metal mesh with 1 × 1 cm hole at the bottom. After 15-minute habitation, each rat's plantar surface of their rear toes was vertically stimulated with the IITC 2390 series electronic von Frey tactile pain measurement instrument, with a single-stimulus duration of ≤1 second and a stimulus interval of 10 seconds. The intensity of the stimulus was measured when the rats lifted or licked their feet during the test. The test was repeated five times, and the mechanical withdrawal threshold (MWT) was calculated as the average intensity that caused the rats to lift or lick their feet.

Diabetic rats with neuropathic pain (DNP group, N = 18) were defined as both MWT and TWL ≤85% base value [19]. Meanwhile, in the type 2 DM rats, neither TWL nor MWT > 85% base value was defined as DL group (N = 6).

### *Intrathecal injection*

In the article of Peng HY, et al [16], researchers used dose of 1, 5, 10 µg/µl, and 5 and 10 µg/µl both had satisfactory results. So we first chosen 10 µg/µl as our curative dose, we found after siRNA injection, pain hypersensitivity was obviously relieved and the expression of Kalirin-7 was significantly influenced, and had no influence on motor function, so we used 10 µg/µl.

Under anesthesia of 10% chloral hydrate (0.3 g/kg), a PE-10 catheter was implanted intrathecally between the L5 and L6 vertebrae, reaching the lumbar enlargement of the spinal cord. After a 3-day recovery, 2% lidocaine was intrathecally injected to identify the catheter placement. Only rats with transient hind paw paralysis were selected for the drug administration that followed. After the rats were allowed to recover for a further 3 days, Kalirin-7 siRNA (5-GCAACAAAGCCUCUGUUA-3 (sense); 5-UU-AACAGAGGCUUUGUUGC-3 (antisense)), and missense nucleotides (5-UUCUCCGAACGUGU-CACGUTT-3 (sense); 5-ACGUGACACGUUCGGA-GAATT-3 (antisense)) [16] were administered through the intrathecal catheter for fourteen consecutive days at a dose of 10 µg. siRNA was bought from Genepharma, and its dsDNA, so anti-sense plus sense is 10 µg, incubating with 10 µl Lipofectamine 2000 (Invitrogen). On days 3, 7, and 14 after injection, behavioral tests as described above were carried out.

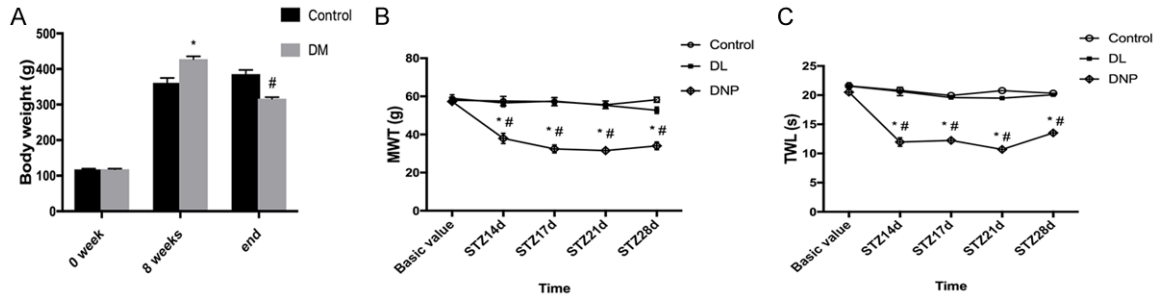
### *Western blotting*

Spinal lumbar enlargement segments in L4-L6 vertebrae were collected under deep anesthesia and rapidly stored in liquid nitrogen. After homogenization in Radio Immuno Precipitation Lysis buffer, the samples were centrifuged at 12,000 rpm at 4°C. The supernatant was collected as the cytosolic proteins for western blotting analysis. A BCA Protein Assay Kit was used to measure protein concentration. An equal amount of protein (30 µg/sample) was loaded onto 10% SDS-PAGE gels and then transferred to PVDF membranes. After being blocked with 10% non-fat milk for 2 hours at room temperature, the membranes were incubated with the primary antibodies, including mouse anti-Kalirin antibody (Millipore), mouse anti-NR2B antibody (Abcam), rabbit anti-p-NR2B (phospho Y1472) antibody (Abcam), mouse anti-PSD-95 antibody (Abcam), and mouse anti-Rac1 antibody (Abcam), at 4°C for 14 hours. After being washed sufficiently, the membranes were then incubated into a secondary antibody for 2 hours. Enhanced chemiluminescent (ECL) was used to detect the protein.

### *Double immunofluorescent staining*

After being anesthetized, rats were perfused with normal saline (NS) followed by 4% paraformaldehyde which dissolved in 0.1 M phos-

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**Figure 1.** The development of neuropathic pain in type 2 diabetes mellitus (T2DM) rats. A. Body weight on 0 weeks, 8 weeks and before execution. B, C. Mechanical allodynia and thermal hyperalgesia were measured by MWT and TWL before injection (basal values) and on days 14, 17, 21, and 28 after STZ injection. N = 6 rats. \* $P < 0.05$  compared to the control group at the corresponding time point; # $P < 0.05$  compared to the DL group at the corresponding time point.

phate-buffered saline to fix tissue. Then lumbar enlargements were collected and post-fixed at 4°C for 8 hours. The tissues were dehydrated in 20% and 30% sucrose dissolved in PBS for 3 days, respectively, at 4°C. The spinal cords were cut transversely at a thickness of 10 mm. After the sections were blocked in PBS containing 10% donkey serum and 0.2% TritonX-100 for 1 hour at a temperature of 37°C, they were incubated for 16 hours at 4°C with goat anti-Kalirin antibody (1:50; Abcam) plus mouse anti-NeuN antibody (1:200; Millipore), rabbit anti-Iba-1 antibody (1:50; Proteintech Group), or mouse anti-GFAP antibody (1:200; Santa Cruz Biotechnology). The sections were finally incubated with a mixture of donkey anti-goat IgG (1:500; Abcam, UK) conjugated with Alexa Fluor 488 and donkey anti-mouse IgG conjugated with Alexa Fluor 594 (1:500; Abcam, UK) or donkey anti-rabbit IgG conjugated with Alexa Fluor 594 (1:500; Abcam, UK) for 1 h at 37°C. The staining was examined using a fluorescence microscope (OLYMPUS, BX53 Micro-Publisher™ 5.0 RTV, Japan).

### Co-immunoprecipitation

Mice monoclonal PSD-95 antibodies were incubated with the supernatants extracted from lumbar enlargement segments obtained from different treated groups for 2 hours. The immuno-complex solutions were then incubated overnight at 4°C after adding 20  $\mu$ l of Protein A/G PLUS-Agarose (Santa Cruz Biotechnology). Pellets were collected after the centrifugation. After being washed 4 times using PBS, the pellets were re-suspended in 40  $\mu$ l of Electrophoresis Sample Buffer and boiled for 5 min before extracting supernatant. Protein sampl-

es were separated by SDS-PAGE, transferred to PVDF membranes, and detected using rabbit polyclonal anti-NR2B as described above.

### Statistical analysis

Sample size was calculated based on previous studies of TWL and MWL in DM rats. The data from the Western blot analysis were measured with quantity one. The band densities were calculated by the ratio of each protein signal to  $\beta$ -actin signal. All data were presented as mean  $\pm$  SEM and analyzed by SPSS 19.0 (SPSS Inc., Chicago, IL, USA). The results were analyzed statistically with one-way ANOVA (when comparisons were made among three or more groups), or unpaired Student's t-test (when comparisons were made between two groups), or Mann-Whitney test (when data were not in Gaussian distribution). After ANOVA showed a significant difference, pairwise comparisons between means were tested by the post hoc Tukey method. Statistical comparison was set at  $P < 0.05$ .

## Results

### Validation of type 2 diabetic neuropathic pain rat models

As shown in **Figure 1**, the weights between normal-diet fed (Control) rats and high-fat/high-sugar diet-fed (DM) rats did not show a significant difference on week 0, but, on week 8 post-diet feeding, the weight of high-fat/high-sugar diet-fed rats was significantly higher than normal diet rats ( $P < 0.05$ ). In addition, the level of fasting blood glucose ( $P < 0.05$ ) and insulin ( $P < 0.05$ ) in the DM group was strikingly

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**Table 1.** Changes in the levels of blood glucose and insulin and insulin sensitivity index (ISI) 8 weeks after normal diet (Control) or high-sugar/high-fat diet (DM)

Group	Blood glucose (mmol/L)			Insulin levels (mIU/L)		ISI	
	0 week	8 week	3 d after STZ	0 week	8 week	0 week	8 week
Control	4.42±0.14	4.37±0.16	4.85±0.17	16.40±0.38	17.18±1.00	-4.28±0.01	-4.31±0.04
DM	4.67±0.11	5.43±0.20	31.10±0.98*	16.67±0.45	56.25±3.08*	-4.35±0.03	-5.71±0.08*

Note: All data are shown as mean ± SEM, n = 6. Compared with Control, \*P < 0.05.

increased compared to the Control group on week 8 post-diet feeding. In contrast, the insulin sensitivity index ( $P < 0.05$ ) was decreased (**Table 1**) 3 days after STZ injection. The average level of fasting blood glucose in the diabetic group (DM) was remarkably higher than those in the Control group ( $P < 0.05$ ). All data are shown as mean ± SEM. The evidence indicates that our type 2 diabetic rat models were well established.

Furthermore, the rats from the diabetic neuropathic pain group also showed a dramatic decline in both mechanical withdrawal threshold (MWT) and thermal withdrawal latency (TWL) compared to the Control group ( $P < 0.05$ ), but there was no significant difference between the DL group and Control group ( $P > 0.05$ ) from day 14 to 28 after STZ injection (**Figure 1B, 1C**).

### *Upregulation of spinal Kalirin-7 expression in diabetic neuropathic pain rats*

To determine the role of Kalirin-7 in DNP, we analyzed the expression of Kalirin-7 in rat spinal cords from the different treated groups (Control group, DL group, DNP group). The level of Kalirin-7 expression was significantly increased in rats in DNP group compared to those in Control group and DL group on days 14, 17 and 28 after the STZ injection (**Figure 2A, 2B**). Moreover, immunofluorescence staining showed an increase in the Kalirin-7-like immunoreactivity in the spinal cord in DNP group on day 17 after the STZ injection (**Figure 2C, 2D**). These results confirmed that Kalirin-7 was increased in the spinal cord of diabetic neuropathic pain rats.

### *Kalirin-7 expression in spinal neurons in DNP rats*

We next examined the cellular localization of Kalirin-7 in DNP rats by using double-labeled immunofluorescent staining. As shown in **Figure 2E-G**, Kalirin-7 was predominantly co-

expressed with NeuN (a neuronal marker) and few with OX-42 (a microglial marker) or GFAP (an astrocytic marker) in the spinal cord, which suggests that Kalirin-7 may affect neuronal activation in the development of diabetic neuropathic pain.

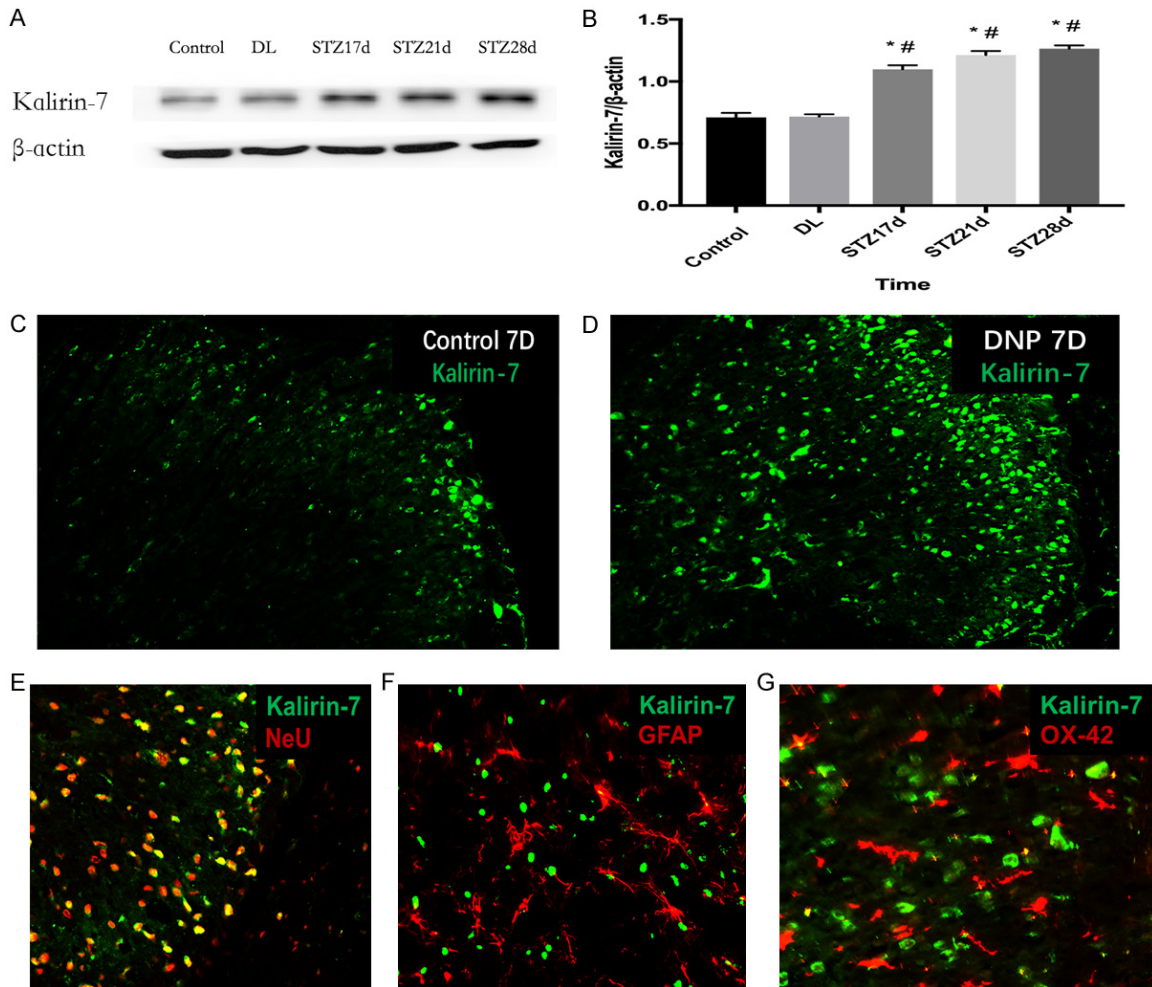
### *Knockdown Kalirin-7 expression reverses pain behavior in DNP rats*

To further verify the role of Kalirin-7 in the development of pain behavior in DNP rats, we carried out daily administration of small-interfering RNA targeted to spinal Kalirin-7 mRNA via an intrathecal catheter. Missense siRNA was used as a control. Intrathecally injection of siRNA didn't change body weight. As expected, Kalirin-7 siRNA, but missense siRNA, significantly decreased spinal Kalirin-7 expression (**Figure 3A, 3B**). Moreover, the intrathecal injection of Kalirin-7 siRNA reduced the level of Tyr1472-NR2B in the spinal cord and alleviated pain behaviors (**Figure 3C, 3D**) without altering the level of blood glucose in the DNP rats (**Figure 3E**). Missense siRNA administration had no influence on pain behaviors in the DNP rats.

### *Knockdown of spinal cord Kalirin-7 decreases Tyr1472-NR2B via the coupling of NR2B to PSD-95 in DNP rats*

We further examined whether Kalirin-7 regulated Tyr1472-NR2B expression via the coupling of NR2B to PSD-95. As shown in **Figure 4**, the abundance of PSD-95-bound NR2B was remarkably enhanced in the DNP group rats compared to the DL group rats and the Control group rats. Kalirin-7 siRNA could block the enhanced coupling of PSD-95 to NR2B along with reduction of p-NR2B (**Figure 4E**). The findings suggest that Kalirin-7 regulates p-NR2B expression via the coupling of PSD-95 to NR2B

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**Figure 2.** Expression of Kalirin-7 in the spinal cord of type 2 diabetic neuropathic pain rats. A, B. Time course for the expression of Kalirin-7 in spinal cord demonstrated by Western blot analysis.  $n = 6$  rats.  $*P < 0.05$  compared to the Control group at the corresponding time point;  $\#P < 0.05$  compared to the DL group at the corresponding time point. C, D. Immunofluorescence staining of Kalirin-7 in the spinal cord from the Control group and DNP group. E-G. Co-localization of Kalirin-7 with NeuN, GFAP, and Iba (40  $\times$ ).

in the spinal cord in type 2 diabetic neuropathic pain.

### *Spinal Kalirin-7 knockdown decreases Rac1 expression in DNP rats*

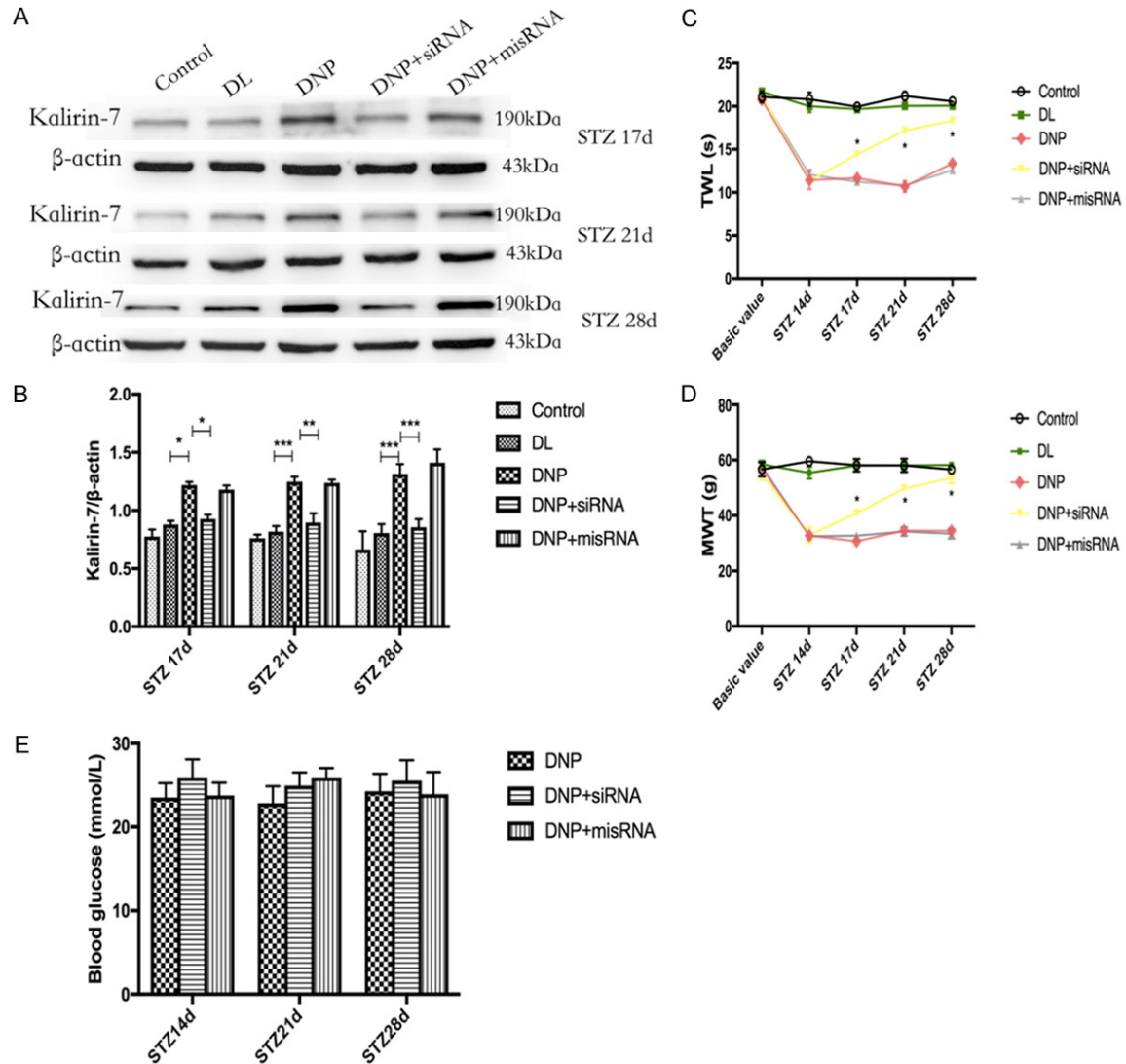
Kalirin-7, a Rac-GEF, activates Rac1 and plays a significant role in the modulation of synaptic plasticity [20]. Therefore, we finally observed whether spinal Rac1 expression was changed, and the role of spinal Kalirin-7 in this change in the DNP group rats. Western blot analysis revealed that Rac1 expression was increased in the spinal cord of the DNP group compared to the Control group and DL group. The intrathecal injection of Kalirin-7 siRNA decreased spi-

nal Rac1 expression. The findings indicate that Kalirin-7 participates in spinal Rac1 expression in the DNP group rats (Figure 5).

### Discussion

PSD-95 exists in postsynaptic membrane. It can interact with NR2B via its structural domain and transfer phosphate molecules to NR2B, causing NR2B phosphorylation. Knockout of NR2B abolished NMDA receptor-dependent long-term potentiation (LTP) and reduced dendritic spine density [12]. The present study demonstrates that spinal expression of Kalirin-7 may participate in the development of diabetic neuropathic pain through regulating PSD-95-NR2B-dependent NR2B phosphorylation.

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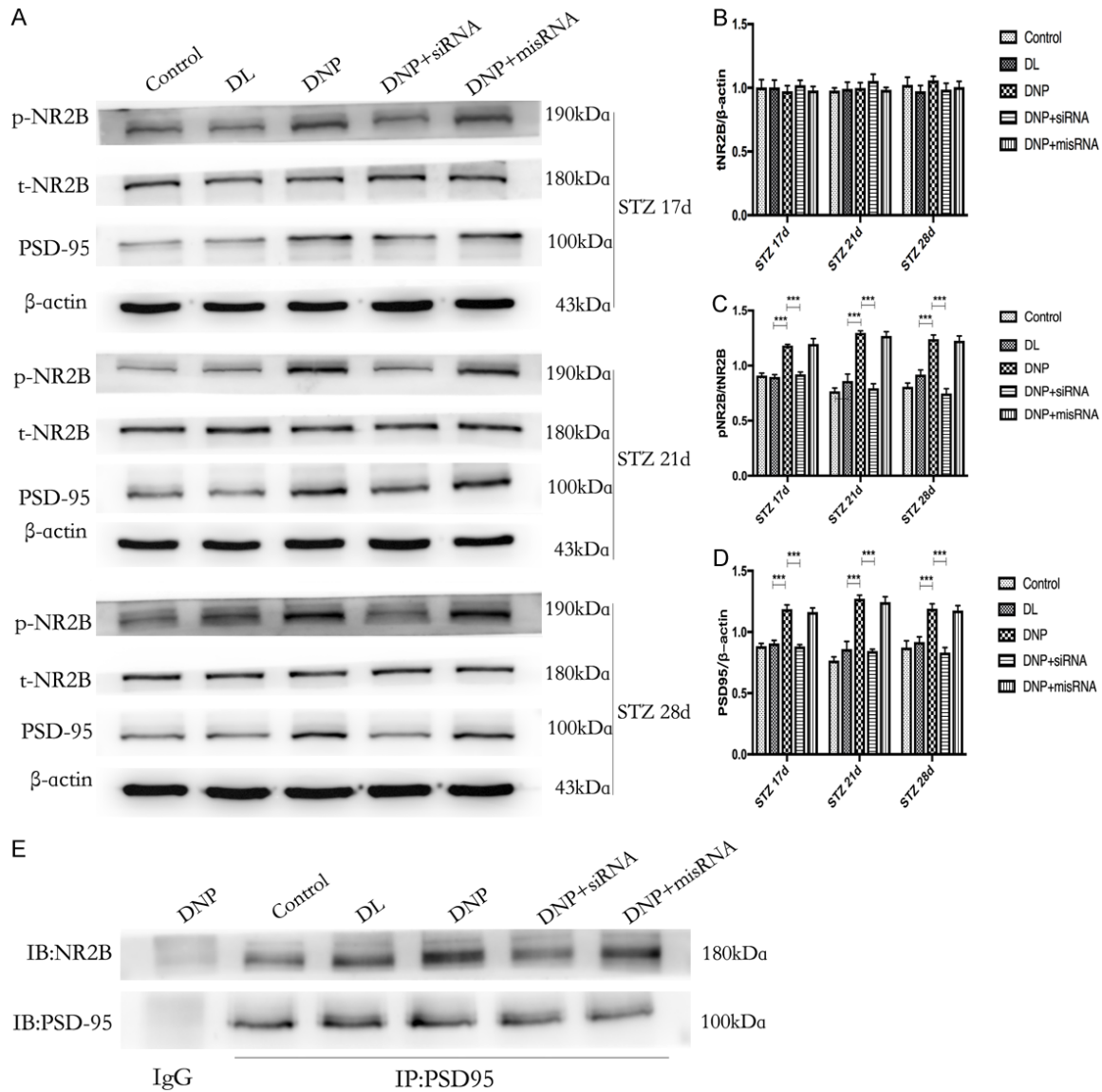
**Figure 3.** The effect of intrathecal Kalirin-7 siRNA on pain behaviors in type 2 diabetic neuropathic pain rats. Kalirin-7 siRNA was injected intrathecally for 14 consecutive days from days 14 to 28 after the STZ injection, and the pain behavior was measured on day 14, 17, 21, 28 after the STZ injection. (A, B) Western blot analysis showed the expression of Kalirin-7 in each group 17, 21 and 28 days after STZ injection. All data are shown as mean  $\pm$  SEM,  $n = 4$  rats. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . (C, D) Intrathecal administration of Kalirin-7 siRNA significantly reversed type 2 diabetes-induced thermal hyperalgesia (C) and mechanical allodynia (D) ( $n = 4$ ). \* $P < 0.05$  compared to the DNP group. (E) Intrathecal administration of Kalirin-7 siRNA or missense siRNA didn't influence blood glucose level.

Previous studies suggested that Kalirin-7 participates in behavior-related neural plasticity. It is essential for synapse remodeling in mature cortical neurons and is thought to play a vital role in the pathogenesis of schizophrenia. Similarly, the present study revealed that Kalirin-7 expression in spinal lumbar enlargement segments increased for 2 weeks after a single dose of STZ injection, which is parallel to the development of mechanical allodynia and thermal hyperalgesia in the DNP rats. Intrathecal

treatment with Kalirin-7 siRNA ameliorated T2DM-induced pain hypersensitivity. Given that Kalirin-7 is expressed in spinal cord neurons, our findings suggest that spinal Kalirin-7 plays a role in development of T2DM-induced pain hypersensitivity.

T2DM produced inflammation and also damaged the peripheral nerve [21], which caused NMDAR-mediated central sensitization in the spinal cord neurons [22] and was essential in

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**Figure 4.** The effect of intrathecal Kalirin-7 siRNA on the interaction between PSD-95 and NR2B in type 2 diabetic neuropathic pain rats. A-D. Western blot analysis showed that daily intrathecal injection of Kalirin-7 siRNA changed the expression of p-NR2B and PSD-95 without affecting total NR2B on days 17, 21 and 28 after STZ injection. All data were shown as mean  $\pm$  SEM,  $n = 4$  rats. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . E. The effect of intrathecal injection of Kalirin-7 siRNA on spinal coupling of PSD-95 to NR2B measured by co-immunoprecipitation.

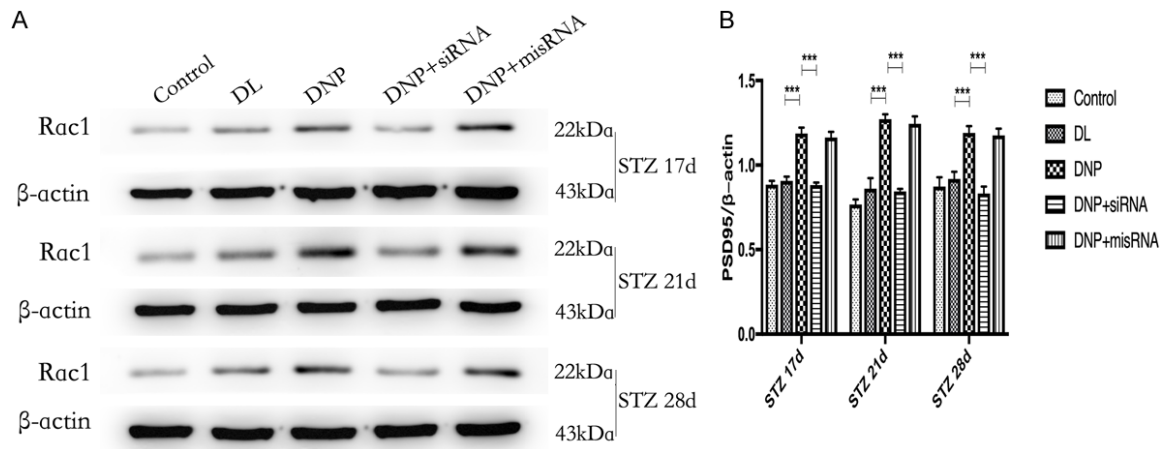
the development and maintenance of neuropathic pain. PSD-95 is almost enriched at excitatory synapses and interacts with synaptic receptors, such as NMDA and AMPA receptors [23]. PSD-95 also play a critical role in recruiting Fyn, which has been shown to be the predominant kinase responsible for the phosphorylation of Y1472 of the NR2B subunit [33]. Previous studies have revealed that the coupling of NR2B-PSD-95 and subsequent NR2B phosphorylation were an essential component in pain pathology [16, 24, 25]. Consistent with these studies [16, 24, 25], we demonstrated

that spinal p-NR2B (Tyr1472) was increased in the T2DM rats. Given that intrathecal injection of Ro-25-6891, an antagonist of NR2B receptor, or Tat-NR2B9c, a peptide disrupts NR2B-PSD-95 coupling, could attenuate pain behavior in our T2DM model [34], the coupling of NR2B-PSD-95 and p-NR2B in the spinal cord are required for the development of T2DM-induced pain hypersensitivity.

Research has proven that Kalirin-7 co-localizes with PSD-95 and NR2B in the dendritic spines of excitatory synapses in hippocampal neurons



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**Figure 5.** Role of Kalirin-7 in expression of Rac1 in spinal cord on days 17, 21 and 28 after STZ injection. All data were shown as mean  $\pm$  SEM.  $n = 4$  rats. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

[12]. Additionally, animals lacking Kalirin-7 showed a decrease in NR2B-dependent current and displayed a distinctly reduced conditioned place preference for cocaine [12]. Kalirin-7 appears to be an important molecular regulator for NR2B expression [25-28]. Our present results demonstrated the increased PSD-95-NR2B coupling and enhanced p-NR2B in the spinal cord of the diabetic rats, suggesting that interaction between NR2B and PSD-95 and subsequent phosphorylation of NR2B in the spinal cord are associated with T2DM-induced neuropathic pain. Intrathecal Kalirin-7 siRNA not only decreased the level of p-NR2B and the interaction between NR2B and PSD-95, but also relieved pain behaviors in the diabetic rats. The evidence indicates that spinal Kalirin-7 participates in T2DM-neuropathic pain likely through regulation of the NR2B-PSD-95 coupling and subsequent NR2B phosphorylation in the spinal cord.

Kalirin-7 mediates the formation and maintenance of dendrites and spines. Its activation can lead to the formation of mature dendritic spines by binding to the ephrin of the EphB2 receptor, which in turn activates Rac1. Rac1, a small G-protein, was reported to be involved in glucose-stimulated insulin secretion [29]. Exposure to hyperglycemic conditions led to Rac1 activation, resulting in the dysfunction of pancreatic  $\beta$ -cells [29]. Rac1 also regulates dendritic growth and spine maturation [30]. Interestingly, a previous study revealed that Rac1 was increased in the STZ-induced type 1 diabetic neuropathic pain with morphologic

changes in the dendritic spines in the spinal cord [31]. Treatment with NSC23766, an inhibitor of Rac1, relieved the change of spine structure and pain behaviors in this model [31]. But the role of Kalirin-7 that mediates neuroprotection through Rac1 is still not fully clear. A study found that Kalirin-7 and Rac1 protected against apoptosis through up-regulation of Bcl-2 and down-regulation of Bax in injured Neuro-2A cells, indicating that Rac1 signaling is probably involved in Kalirin-7 mediated neuroprotection by regulating Bax and Bcl-2. Rac1 was also considered as a novel binding partner of Bcl-2 and stabilized its anti-apoptotic activity. In our present study, persistent hyperglycemia induced a significant increase in spinal Rac1 expression 2 weeks after STZ-injection. This increase paralleled with the development of T2DM-induced pain hypersensitivity. More importantly, daily intrathecal injection of Kalirin-7 siRNA reduced Rac1 expression on days 17, 21 and 28 after STZ injection. Therefore, it is very likely that spinal Kalirin-7 participates in T2DM-neuropathic pain by targeting additional spinal Rac1. It should be noted that Kalirin-7 has also been shown to be involved in multiple signaling pathways, including ephrins and neuregulins, thus it may regulate synaptic function in many other ways [32], which should be verified in future studies.

### Conclusions

The present study demonstrated that the increased spinal Kalirin-7 might participate in the development of DNP through the regulating interaction between PSD-95 and NR2B as well

as Rac1 in the rat spinal cord. Given that knock-down spinal expression of Kalirin-7 significantly relieved pain-related behaviors, without affecting basal acute pain, targeting Kalirin-7 may be a potential therapeutic avenue for neuropathic pain induced by T2DM.

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### Disclosure of conflict of interest

None.

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