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Original Research

Changes in SLITRK1 Level in the Amygdala Mediate Chronic Neuropathic Pain-Induced Anxio-Depressive Behaviors in Mice

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Abstract

Background: Comorbid chronic neuropathic pain (NPP) and anxio-depressive disorders (ADD) have become a serious global public-health problem. The SLIT and NTRK-like 1 (SLITRK1) protein is important for synaptic remodeling and is highly expressed in the amygdala, an important brain region involved in various emotional behaviors. We examined whether SLITRK1 protein in the amygdala participates in NPP and comorbid ADD. **Methods**: A chronic NPP mouse model was constructed by L5 spinal nerve ligation; changes in chronic pain and ADD-like behaviors were measured in behavioral tests. Changes in SLITRK1 protein and excitatory synaptic functional proteins in the amygdala were measured by immunofluorescence and Western blot. Adeno-associated virus was transfected into excitatory synaptic neurons in the amygdala to up-regulate the expression of SLITRK1. **Results**: Chronic NPP-related ADD-like behavior was successfully produced in mice by L5 ligation. We found that chronic NPP and related ADD decreased amygdalar expression of SLITRK1 and proteins important for excitatory synaptic function, including Homer1, postsynaptic density protein 95 (PSD95), and synaptophysin. Virally-mediated SLITRK1 overexpression in the amygdala produced a significant easing of chronic NPP and ADD, and restored the expression levels of Homer1, PSD95, and synaptophysin. **Conclusion**: Our findings indicated that SLITRK1 in the amygdala plays an important role in chronic pain and related ADD, and may prove to be a potential therapeutic target for chronic NPP-ADD comorbidity.

Keywords: adeno-associated virus; amygdala; anxio-depressive behavior; chronic neuropathic pain; SLITRK1; synaptic functional protein

1. Introduction

In 2020, pain was newly defined as an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage [1]. Neuropathic pain (NPP) is a pathological pain caused by neurological disorders or injuries, and is one of the most common types of chronic pain found in clinical practice [2].

The structural and functional plasticity of synapses in the central nervous system is an important mechanism for chronic NPP [3,4]. Change in synaptic plasticity is also one of the main factors in various psychopathologies such as depression and anxiety [4]. Studies have shown that emotional abnormalities, such as anxiety and depression, are related to abnormal sensitivity to chronic NPP [5,6]. The vicious cycle between the chronic pain and emotional abnormalities makes the condition more severe and complex [5]. Although there is a strong comorbidity between chronic pain and emotional disorders, the underlying molecular mechanism has not been fully elucidated.

The SLIT and NTRK-like (SLITRK) family of proteins consists of six transmembrane proteins known as SLITRK1-6, which are highly expressed in the central nervous system [7,8]. SLITRK proteins are involved in the basic neuronal processes of neurite growth and neuronal survival, maintaining synaptic formation, and coordinating the balance between excitatory and inhibitory synapses [9]. SLITRK1 is enriched in the postsynaptic part of excitatory synapses, participating in synaptogenesis and promoting the differentiation of excitatory synapses [10,11]. Multiple neuropsychiatric disorders have been reported to be closely related to synaptic dysfunction and neuronal dysfunction caused by *SLITRK1* gene mutations [12–14]. In rodent studies, SLITRK1-deficient mice exhibited high levels of anxiety- and depression-like behaviors [15,16], indicating a strong correlation between SLITRK1 and anxiety- and depression-like mental disorders. SLITRK1 protein is highly expressed in multiple brain regions such as the frontal cortex, hippocampus, and amygdala (AMY) [9,11,16]. Among these brain areas, the AMY plays an essential part in emotional perception behaviors such as pain, fear, anxiety, and depression [17–19], and AMY contributes to distinct brain circuits related to the anxio-depressive consequences of NPP, mediating pain-induced emotional dysfunction [17,20]. In 2019, Corder *et al*. [21] reported on

the effect of nociceptive stimulation on excitatory neurons in the AMY during NPP caused by peripheral nerve injury, suggesting that AMY excitatory neurons may be a potential structural basis for chronic pain. However, the role of SLITRK1 in comorbid anxio-depressive symptoms with chronic pain has not been thoroughly investigated, and neither has the role of SLITRK1 in the AMY, which is an important brain region that regulates various emotional behaviors.

Based on the above research, we hypothesized that SLITRK1 protein in the AMY may be involved in chronic NPP and comorbid anxio-depressive disorders (ADD) consequences.

In the present study, using an NPP model based on a lumbar spinal nerve ligation (SNL) to induce the occurrence of ADD-like behavior, we investigated changes in SLITRK1 protein and excitatory synaptic function-related proteins in the contralateral AMY. We also investigated whether SLITRK1 overexpression can produce an easing of chronic NPP and comorbid ADD-like behavior.

2. Methods

2.1 Animals

Eight-week-old c57BL/6 male mice (Shanghai Jihui Experimental Animal Breeding Co., Ltd., Shanghai, China) were raised in standard cages with 5/cage. The mouse room was on a 12/12 day/night cycle. The mice were allowed to habituate to the room for one week before initiation of the experiments. The mouse experiments complied with the Animal Management Regulations and Ethical Requirements of the Second Affiliated Hospital of Naval Medical University Animal Management Committee (Shanghai, China) (No. 2022DW37, Shanghai, China), and followed the principles of the American Physiological Association in animal care and use.

2.2 Antibodies

The antibodies used in our experiments were: Anti-MAP2 (microtubule-associated protein 2; ab254144, Abcam, Cambridge, UK), Anti-SLITRK1 (ab67238, Abcam, Cambridge, UK), Anti-Homer1 (ab184955, Abcam, Cambridge, UK), Anti-synaptophysin (Anti-SYN) (ab32127, Abcam, Cambridge, UK), Anti-PSD95 (postsynaptic density protein 95; ab18258, Abcam, Cambridge, UK), Cy3-AffiniPure Goat Anti-Rabbit IgG (H+L) (GB21303, Servicebio, Wuhan, Hubei, China), Alexa Fluor 488-labeled Goat Anti-Mouse IgG (H+L) (GB25301, Servicebio, Wuhan, Hubei, China), beta-Actin (GB15001, Servicebio, Wuhan, Hubei, China), Horseradish Peroxidase (HRP) conjugated Goat Anti-Mouse IgG (H+L) (GB23301, Servicebio, Wuhan, Hubei, China), and HRP conjugated Goat Anti-Rabbit IgG (H+L) (GB23303, Servicebio, Wuhan, Hubei, China) and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (10494-1-ap, Proteintech Group, Chicago, IL, USA) .

2.3 The Spinal Nerve Ligation (SNL) Model

The mice were anesthetized with 1.5% pentobarbital sodium (P3761, Sigma Aldrich, Shanghai, China) at a dose of 40–50 mg/kg (i.p.). The skin was disinfected with iodine and cut along the median aspect of the back. The left paraspinal muscle from L4 to L5 was passively separated, and the area between the L6 transverse process and the sacrum was exposed. The L6 transverse process was removed, and spinal nerves L4 and L5 were exposed. Then, the left L5 was separated and tightly tied with silk thread. Special attention was paid to sterilizing the surgical field throughout the process. The Sham-op mice were subjected to the same surgical procedure without ligation of the L5 spinal nerve.

2.4 Virus Injection in the AMY

Two weeks before the SNL surgery, the eight-week-old c57BL/6 male mice were placed in a stereotaxic device (RWD, Shenzhen, Guangdong, China) after anesthetization. Stereotaxic surgery was conducted as described previously [22]. The virus was injected into the right AMY by stereotaxic injection using a microinjection pump (Harvard Apparatus, Holliston, MA, USA) and a 5-µL micro-syringe (HAMILTON, Franklin, MA, USA) at 50 nL/min. The total injection volume was 500 nL. The coordinates were –4.5 mm ventral to the brain surface, –1.5 mm posterior to bregma and +2.5 mm lateral to the midline. The promoter of the virus was CaMKIIα and the titer of the viral vectors was 10^{12}v. g/mL. After injection, the syringe was kept in place for 15 min before removal.

For viral injection, the adeno-associated virus (AAV) serotype 2/9 viral vectors (AAV2/9) were used: rAAV2/9-CaMKIIα-SLITRK1-EGFP-3Flag (experimental group) and rAAV2/9-CaMKIIα-EGFP-3Flag (control group). They were purchased from Shanghai Genechem Co., Ltd. (Shanghai, China).

2.5 Measurement of Mechanical Pain Threshold

For this test, the mouse was individually placed on a high hanging-wire mesh and a bottomless, clean, organic glass box (8-cm wide, 8-cm long, with 7-cm-high walls) was used to limit its range of movement. The environment was kept quiet to allow the mouse to habituate for approximately 30 min. A positive reaction was recorded if the mouse lifting its foot to avoid the stimuli or licked its foot. An electronic Von Frey device (BW-EVF2393, IITC Life Science, Los Angeles, CA, USA) was used to stimulate the left hind paw from below to observe whether the mouse showed a positive response to the stimulus. If so, the reading on the sensor was recorded. Measurement was repeated 3 times at 5-min intervals, and the mean of the positive reactions was calculated.

2.6 Measurement of Thermal Pain Threshold

The mouse was placed in a clean glass box with a 3-mm-thick glass-plate bottom, for habituation, for 1 h. A thermal radiation stimulator (37370, IITC Life Science, Los Angeles, CA, USA) was used to heat the left hind paw of the mouse to measure thermal nociception and assess to heat hyperalgesia. The latency for the thermal foot-contraction reflex was the time from the application of the stimulus to the occurrence of foot flinching, licking, or withdrawing. Throughout the entire experiment, the intensity of the stimulation remained constant, with 5-min intervals between stimuli at the same site. The experiment was repeated 3 times and the mean value was calculated for analysis.

2.7 Open Field Test (OFT)

The mouse was placed in the corner in a square open area (40 × 40 × 40 cm) and allowed to explore the environment freely for 20 min. A camera was used to track the activity of the mouse and its time in the central area was analyzed using SMART video tracking software (Version 3.0, Harvard Apparatus , Holliston, MA, USA) to assess the anxiety level of the mouse. Locomotor activity was evaluated as the total distance traveled in 20 min. Anxiety-like behavior was assessed by the time spent in the central zone; less time spent in the central area was interpreted as a higher level of anxiety. Mice were tested in a random order, and the surface was wiped clean with 75% ethanol to dilute and remove olfactory cues after each trial.

2.8 Elevated Plus-Maze (EPM)

The anxiety-like behavior was also measured by the elevated plus-maze (EPM) test. The maze was composed of two open arms (5-cm wide, 30-cm long, without walls) and two closed arms (5 × 30 × 15 cm) that were interconnected by a 5 × 5-cm central area. It was positioned 50 cm above the ground and had no roof. The mouse was placed in the central area facing one of the two open arms and allowed to freely explore for 5 min. Its path was recorded by a video camera and analyzed with SMART video tracking software (Version 3.0, Harvard Apparatus, Holliston, MA, USA). Mice were tested in random order, and after each test, the test site was wiped clean with 75% ethanol.

2.9 Tail Suspension Test (TST)

Depression-like behavior was measured by tail suspension test (TST). The mouse was placed in the testing room for 1-h habituation, and then suspended by taping its tail (1 cm from the tail tip) to a bar for 6 min. Its behaviors were recorded using a video camera, and the immobility duration (presumably indicative of depression) of each mouse within the last 5 min was recorded and analyzed with SMART video tracking software (Version 3.0, Harvard Apparatus, Holliston, MA, USA).

2.10 Forced Swimming Test (FST)

The mouse was placed in the testing room for a 1-h habituation period. Forced swimming test (FST) was conducted by gently lowering each mouse into a glass cylinder (height 30 cm, diameter 12 cm) containing 20 cm water (23–25 °C). The test duration was 6 min. The mouse was considered immobile when it floated in the water, in an upright position, and made only small movements to keep its head above water. The duration of immobility was quantified over the last 5 min of the 6-min test. Its behaviors were recorded and analyzed with SMART video tracking software (Version 3.0, Harvard Apparatus, Holliston, MA, USA). Depression-like behavior was assumed to be indicated by the duration of immobility (the more immobility, the greater the depression). After the test, each mouse was removed from the water, dried, and returned to its home cage.

2.11 Immunofluorescence Staining

Brains were removed and placed in 4% paraformaldehyde at 4 °C for 6 h, then dehydrated in 10%, then 20% and then 30% sucrose solution. After embedding in Optical Coherence Tomography (OCT) solution (4583, Thermo Fisher Scientific, Waltham, MA, USA) for slicing, the tissue was sliced into 10-µm sections on a freezing microtome (CMl950, Leica, Nussloch, Germany), then washed 3 times (5 min/wash) to remove OCT. After being blocked with 10% goat serum (ab7481, Abcam, Cambridge, UK) containing 0.1% Triton X-100 (85111, Thermo Fisher Scientific, Waltham, MA, USA) for 1 h at room temperature, the sections were incubated with the diluted primary antibodies, including anti-SLITRK1 (1:200), anti-Homer1 (1:500), anti-SYN (1:400), anti-PSD95 (1:200), and anti-MAP2 (1:5000), at 4 °C overnight. After washing three times (10 min/wash) with phosphate buffered saline (PBS), the sections were incubated with diluted secondary antibodies including Alexa Fluor 488 Goat Anti-Mouse IgG (1:400) and CY3 Goat Anti-Rabbit IgG (1:300), at room temperature for 1 h in the dark. After washing, 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI) (62248, Thermo Fisher Scientific, Waltham, MA, USA) was added for incubation for 15 min. The fluorescence signals were visualized using confocal laser scanning microscopy (LSM 900, Zeiss, Jena, Germany), and the quantification of the fluorescence intensity was the arithmetic mean intensity of target-protein fluorescence, analyzed by ZEN 3.4 software(Zeiss, Jena, Germany). One slice from each of 6 mice were used for immunofluorescence quantification.

2.12 Western Blot Assay

Mice were anesthetized by i.p. injection of sodium pentobarbital (40 mg/kg) (P3761, Sigma Aldrich, Shanghai, China) and perfused through the left ventricle of the heart with 0.9% normal saline. Then the brain tissue was

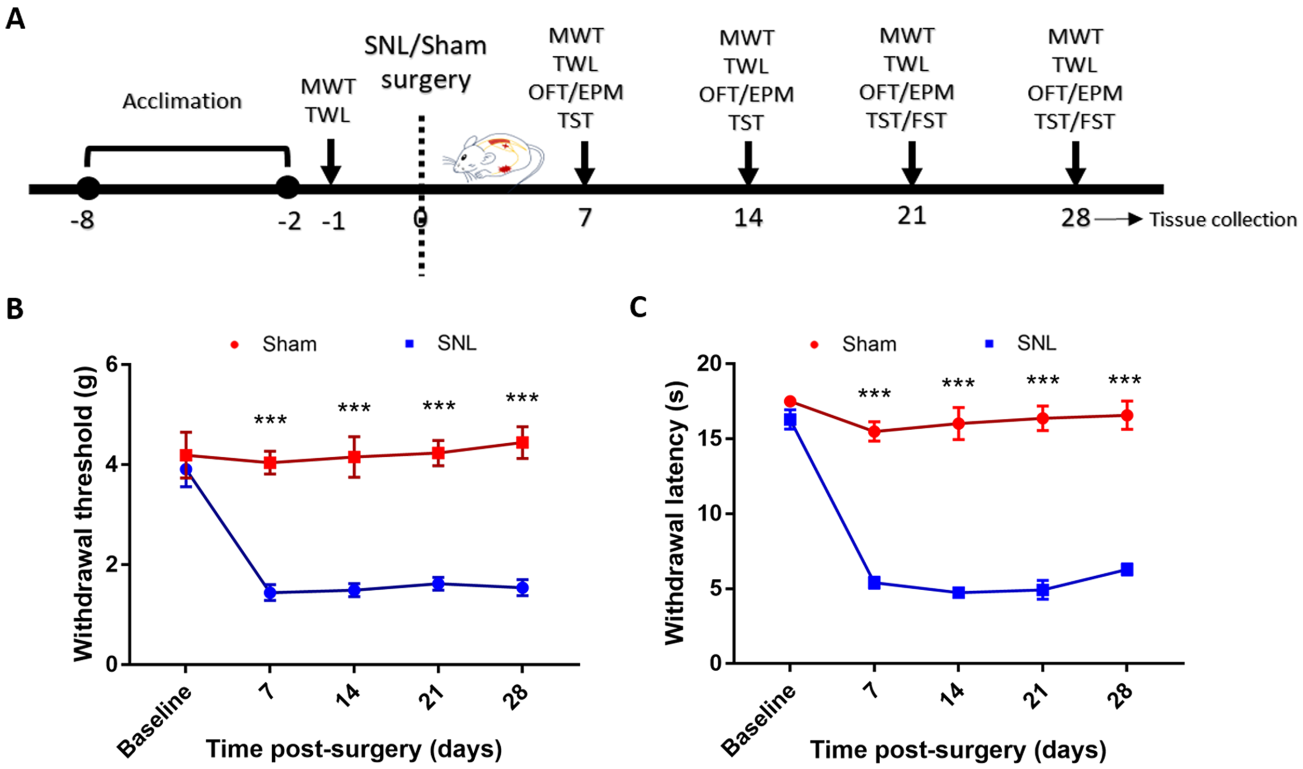

Fig. 1. SNL-induced mechanical allodynia and thermal hyperalgesia. (A) Experimental paradigm. (B) Mechanical withdrawal threshold (MWT) of mechanical allodynia was determined using Von Frey filaments (F = 6.283, $p = 0.006$). (C) Thermal hyperalgesia was assessed as thermal withdrawal latency (TWL) (F = 64.293, $p < 0.001$). Sham, sham-operated group; SNL, spinal nerve ligation group; OFT, Open Field Test; EPM, Elevated Plus-Maze; TST, Tail Suspension Test; FST, Forced Swimming Test. $n = 10$/gp. ***$p <$ 0.001.

rapidly extracted, and then fresh AMY tissue was homogenized in ice-old Radio Immunoprecipitation Assay (RIPA) buffer (Radio Immunoprecipitation Assay; P0013, Beyotime, Shanghai, China) containing protein inhibitors. The protein concentration was determined by BCA kit (P0012, Beyotime, Shanghai, China). Equivalent protein samples were loaded onto 10% gradient sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) gels and transferred to polyvinylidene difluoride (PVDF) membranes (IEVH85R, Millipore, Darmstadt, Germany). After blocking in 5% Bovine Serum Albumin (BSA) (37520, Thermo Fisher Scientific, Waltham, MA, USA), the membranes were incubated with the indicated primary antibodies, including anti-GAPDH (1:1000), anti-SLITRK1 (1:1000), anti-Homer1 (1:1000), anti-SYN (1:1000), anti-PSD95 (1:1000), and anti-beta Actin (1:500), at 4 °C overnight, followed by addition of the corresponding HRP-conjugated secondary antibodies. Target-protein content was measured using the enhanced chemical-light-detection system, and all films were analyzed using Image J software (version 1.8.0, National Institutes of Health, Bethesda, MD, USA).

2.13 Statistical Analysis

The animal sizes were deemed suitable for statistics and were similar to other studies in the field. The results of behavioral tests were evaluated by two-way analysis of variance (ANOVA) (Days X Behavioral test) using SPSS Statistics, version 21.0 (IBM, Armonk, NY, USA). Least significant difference (LSD) post hoc tests were performed only if the F value achieved statistical significance ($p <$ 0.1). The parameters (F, p) for the interaction and the post hoc test (p) were added in the figure legends. The results of grayscale, and immunofluorescence-intensity tests were evaluated by statistical analysis using GraphPad Prism 8.0 plotting software (GraphPad, Boston, MA, USA) and comparisons between two groups were performed by two tailed Student's t test (for two-group comparisons). Data are expressed as the mean (SD, standard deviation); and $p \leq 0.05$ was considered statistically significant.

3. Results

3.1 Successful Construction of the NPP Model by SNL

SNL is one of the most widely used methods for establishing NPP [3,4]. There were no differences in the baseline values of the left hind paw contraction reflex under mechanical and thermal stimulation between the SNL group and

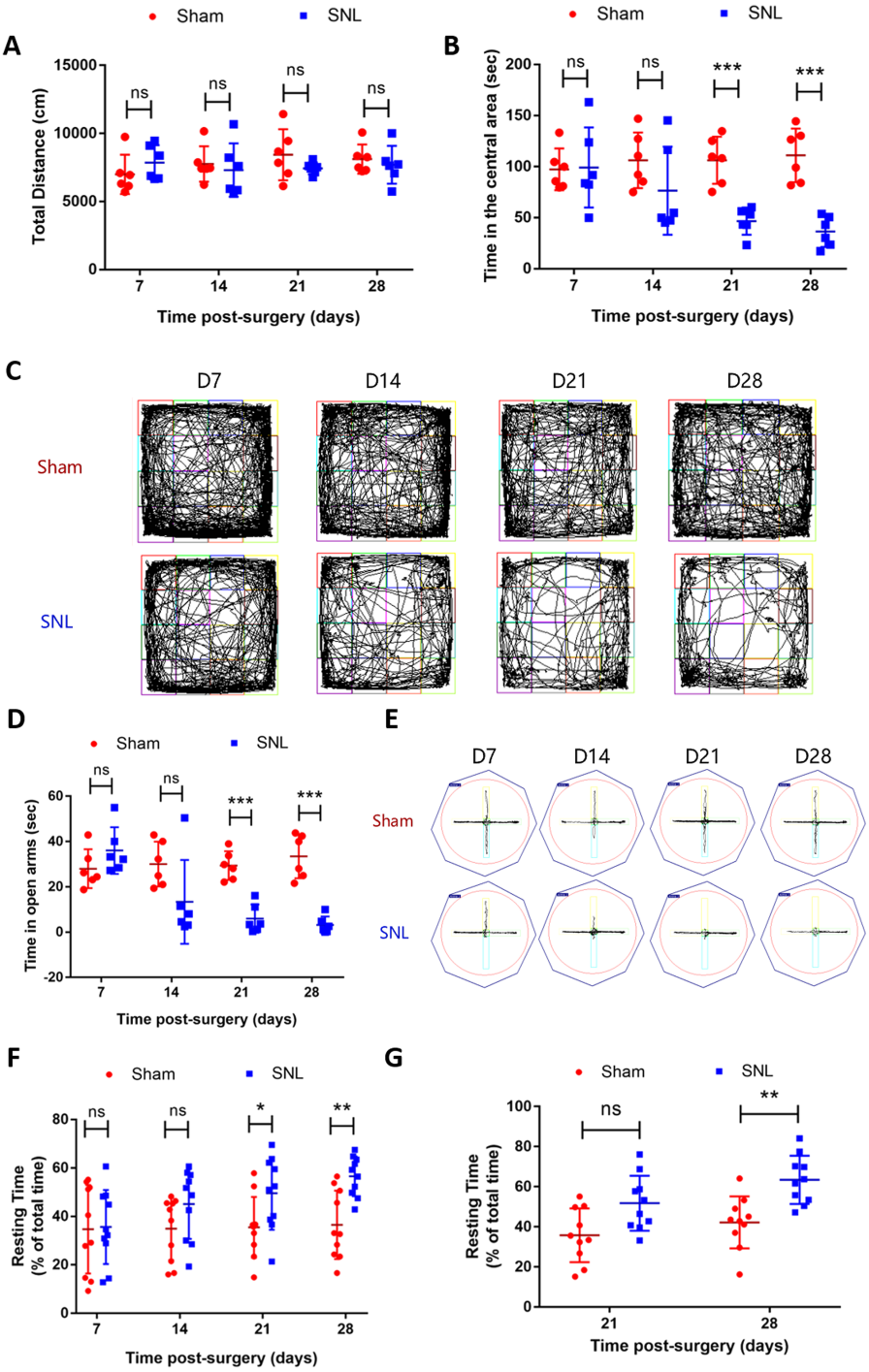

Fig. 2. Detection of anxio-depression-like behaviors induced by chronic pain in SNL mice. (A) SNL caused no changes in total distance traveled in the open field test at 7 ($p = 0.293$), 14 ($p = 0.649$), 21 ($p = 0.231$) and 28 ($p = 0.567$) days after surgery ($F = 0.745$, $p = 0.532$), $n = 6$/gp. (B) The residence time of the SNL group in the central area was significantly lower than of the Sham group on Day 21 ($p < 0.001$) and Day 28 ($p < 0.001$) after surgery ($F = 5.231$, $p = 0.005$), $n = 6$/gp. (C) Typical trajectory in OFT at different times after surgery. (D) The residence time in the open arm in the EPM test was lower in the SNL group at 21 ($p < 0.0001$) and 28 ($p < 0.001$) days ($F = 8$, $p < 0.001$), $n = 6$/gp. (E) Representative trajectory in EPM. (F) The difference in immobility duration in the tail-suspension test was evident at 21 ($p = 0.036$) and 28 ($p = 0.001$) days ($F = 2.194$, $p = 0.099$), $n = 10$/gp. (G) The immobility duration in the forced-swim test was significantly different at 28 days ($p = 0.001$) ($F = 6.239$, $p = 0.022$), $n = 10$/gp. EPM, Elevated Plus-Maze. ns, no significant difference, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

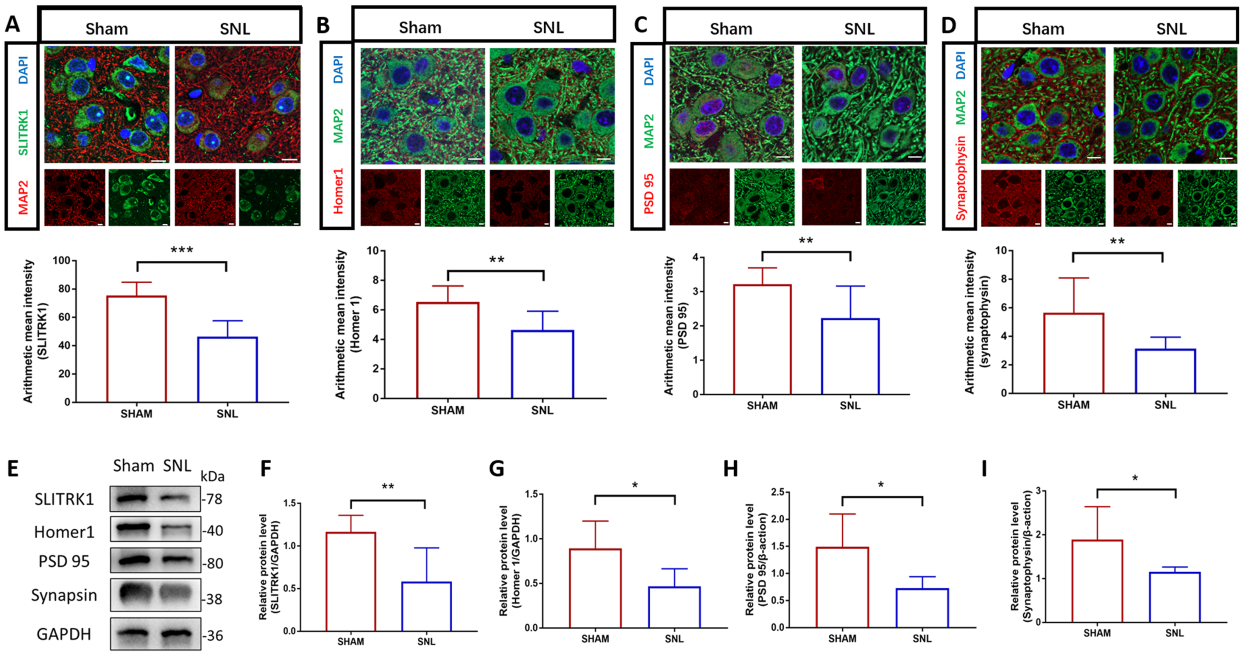

Fig. 3. Changes in SLITRK1 protein and three synaptic functional proteins in amygdala (AMY) at 28 days after surgery. (A–D) Upper: Representative images of immunofluorescence staining for (A) SLITRK1; (B) Homer1; (C) PSD95; and (D) synaptophysin. Scale bar = 5 µm. Lower: The fluorescence intensity analysis of SLITRK1 ($t = 5.514$, $p < 0.001$), Homer1 ($t = 3.769$, $p = 0.001$), PSD95 ($t = 3.451$, $p = 0.002$), and synaptophysin ($t = 3.087$, $p = 0.008$). (E) Representative Western blot protein bands. (F) The protein levels of SLITRK1 ($t = 3.24$, $p = 0.009$), (G) Homer1 ($t = 2.869$, $p = 0.016$), (H) PSD95 ($t = 2.913$, $p = 0.016$), and (I) synaptophysin ($t = 2.386$, $p = 0.038$) were assayed by Western blot analysis. $n = 6$/gp. PSD95, postsynaptic density protein 95; SLITRK1, The SLIT and NTRK-like 1; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; DAPI, 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

the Sham group. The mechanical and thermal pain thresholds were tested at 7, 14, 21, and 28 days after surgery to determine whether the NPP model had been successfully constructed (Fig. 1A).

The experiments showed that the mechanical and thermal pain thresholds in SNL group were significantly lower at all test times after surgery than were those of the Sham group. The symptoms remained stable for 4 weeks, indicating that the SNL-elicited chronic NPP model was constructed successfully (Fig. 1B,C).

3.2 Successful Construction of SNL-Induced ADD-Like Behavior

After successful construction of the NPP model, the anxiety- and depression-like behaviors between SNL group and Sham group were examined at 7, 14, 21, and 28 days after surgery.

Anxiety-like behavior in mice was assessed with the open field test (OFT) and the EPM. OFT showed no difference in locomotor activity between the two groups, but the time in the central zone was significantly lower in the SNL group at 21 and 28 days after surgery, indicating successful induction of the anxiety-like behavior during this period (Fig. 2A–C). The EPM test showed that the time spent in the open arms was significantly lower in the SNL group than in the Sham group at 21, and 28 days after surgery (Fig. 2D,E). The above tests indicated that anxiety-like behavior was successfully displayed after SNL.

Depression-like behavior in mice was measured by the TST and the FST. TST showed that the immobility duration in the SNL group was significantly longer than that of the Sham group on days 21 and 28 after surgery (Fig. 2F). FST showed that immobility duration was significantly longer in the SNL group than that in the Sham group on day 28 after surgery (Fig. 2G). The above behavioral tests indicated that SNL successfully induced depression-like behaviors on day 28 after SNL.

3.3 SNL-Induced Chronic NPP and ADD Suppress SLITRK1 Protein in the AMY

After testing at 28 days, the mice were euthanized and the expression of SLITRK1 protein in the right AMY was assayed by immunofluorescence staining and Western blot analysis. Homer1, postsynaptic density protein 95 (PSD95), and SYN are known to play crucial roles in synaptic plasticity and function [23–26]. Among them, Homer1 and PSD95 are important scaffold proteins for excitatory synapses. The results of our immunofluorescence staining (Fig. 3A–D) and Western blot (Fig. 3E–I) demonstrated that the expression level of SLITRK1 protein in the right

AMY of the SNL group mice was markedly lower than that in the Sham group mice, as were the expression levels of Homer1, PSD-95 and SYN. These results suggest that the expression level of SLITRK1 protein in the AMY was significantly lower in the SNL-induced chronic NPP and ADD mice, accompanied with the lower expression of some excitatory synaptic proteins.

3.4 Over-Expression of SLITRK1 in Excitatory Neurons of the AMY

The AMY is divided into two parts, namely the basolateral amygdala (BLA) and the central nucleus amygdala (CeA). Calcium-calmodulin (CaM)-dependent protein kinase IIα (CaMKIIα) is a useful marker for pyramidal neurons, which are glutamatergic neurons dominantly locating in the BLA [27].

We examined whether SLITRK1 over-expression in the AMY alleviated SNL-induced pain and reduced comorbid ADD-like behaviors. Eight-week-old wild-type mice in a SLITRK1 over-expression group and a control group received intra-AMY injection of pre-prepared recombination adeno-associated virus (rAAV) 2/9-Calmodulin dependent protein kinase II-alpha (CaMKIIα)-the SLIT and NTRK-like (SLITRK)-enhanced green fluorescent protein (EGFP)-3 fluorescence activating and absorbing Tag (Flag) (over-expression group) or rAAV2/9-CaMKIIα-EGFP-3Flag (control group) two weeks before SNL. The timeline of the procedure is shown in Fig. 4A.

As shown in Fig. 4B,C, the successful expression of enhanced green fluorescent protein (EGFP) indicates successful transfection, mainly in the region of BLA. Furthermore, a 5× magnification of fluorescence image of the BLA area showed the expression of EGFP protein was co labeled with CaMKII red immunofluorescence (Fig. 4D,E), which suggested a high degree of specificity of virus transfection into excitatory neurons. In order to identify the differences in SLITRK1 overexpression between BLA and CeA, we performed immunofluorescence staining analysis on the AMY and CeA after virus transfection, separately (Fig. 4F,G). The results indicated that the SLITRK1 protein increased in both CeA and BLA, while the increase in BLA was more pronounced than CeA ($p < 0.001$) (Fig. 4H).

3.5 Over-Expression of SLITRK1 Alleviates Chronic NPP and ADD-Like Behaviors

SNL was introduced two weeks after injection of the virus.

Compared with the control group, the SLITRK1 over-expression group showed higher mechanical pain thresholds at 21 and 28 days after SNL (Fig. 5A). Thermal pain thresholds were significantly elevated at 14, 21, and 28 days after SNL (Fig. 5B), indicating the effective recovery from SNL-induced NPP in the SLITRK1 overexpression group.

The OFT results showed that there was no significant difference in activity levels between the SLITRK1 over-expression group and the control group (Fig. 5C), whereas the SLITRK1 overexpression group showed effectively reduced anxiety-like behavior 14, 21 and 28 days after SNL (Fig. 5D,E). The EPM test results showed that SLITRK1 overexpression could also effectively alleviate anxiety-like behavior at 7, 14, 21, and 28 days after SNL (Fig. 5F,G). The TST showed that SLITRK1 overexpression significantly reduced depression-like behavior 21 and 28 days after SNL, and the FST results showed that the depression-like behavior was significantly lower in the SLITRK1 over-expression group 28 days after SNL (Fig. 5H,I). These results indicate that the upregulation of SLITRK1 in the CaMKIIα positive neurons could apparently relieve SNL-induced chronic pain and ADD symptoms.

On day 28, up-regulation of the expression level of Homer1, PSD-95, and SYN proteins in the right AMY was seen in immunofluorescence staining and Western blot analysis, suggesting the recovery in the general expression levels of some excitatory synaptic proteins (Fig. 6).

4. Discussion

NPP is one of the most common types of chronic pain found in clinical practice, and the patients often experience emotional disorders such as anxiety and depression [2–4]. The remodeling of synaptic function is an important mechanism involved in chronic pain, and is also one of the important influencing factors for various neuropathological diseases such as depression and anxiety [3,4]. The study by Becker *et al*. [20] indicated that the AMY plays a significant role in the process of chronic pain-induced depression. SLITRK1 protein is involved in synaptic remodeling and highly expressed in the AMY, but it is unclear whether SLITRK1 protein is involved in the response to NPP and in comorbid emotional conditions such as anxiety and depression. In the present study, we constructed an SNL-induced chronic NPP mouse model and found that it could effectively induce anxiety- and depression-like behavior at 28 days after surgery. We found that the expression of SLITRK1 protein in the AMY was reduced as a result of chronic NPP-ADD comorbidity exposure, accompanied with the lower expression of some excitatory synaptic proteins. Furthermore, we used unilateral microinjection to up-regulate the expression level of SLITRK1 in excitatory, CaMKIIα positive neurons, which are dominantly located in the BLA, and found that overexpression of SLITRK1 protein seemed to simultaneously alleviate chronic NPP and ADD-like behavior, and accompanied with the recovery in the general expression levels of some excitatory synaptic proteins. This indicated to us that SLITRK1 protein in the AMY plays an important role in chronic NPP-induced ADD-like behavior.

The recent development of animal models has greatly accelerated the study of potential mechanisms and consequences of NPP and depression/anxiety comorbidity [3]. Three main animal models, including NPP, inflammatory

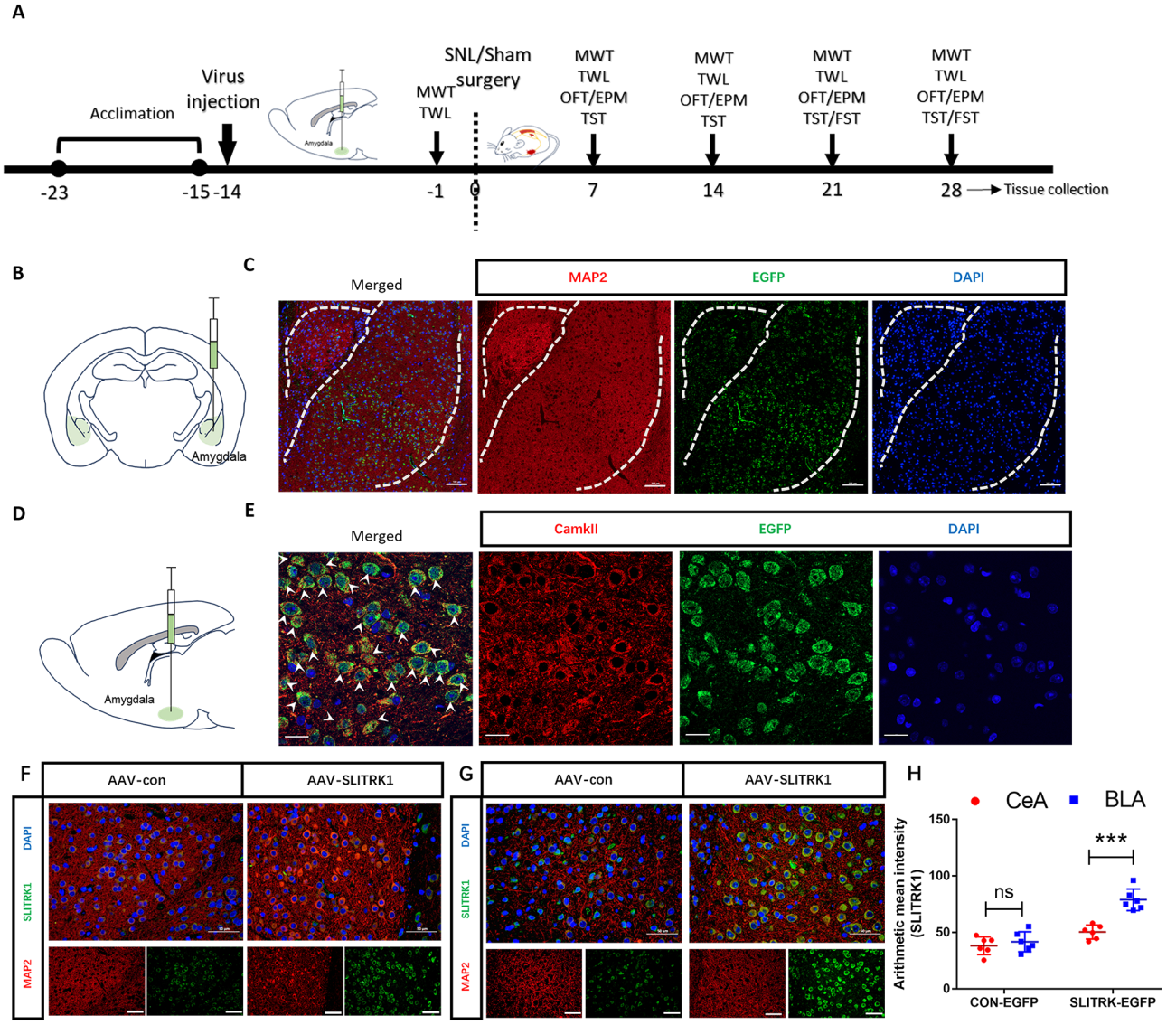

Fig. 4. AAV-mediated SLITRK1 overexpression in the excitatory neurons of the AMY. (A) Schematic diagram of the experimental process. (B) Coronal brain section showing virus injection into the right AMY. (C) Representative images of immunofluorescence staining for EGFP green fluorescence in the right AMY. Scale bar = 100 µm. (D) Sagittal brain section showing virus injection into the right AMY. (E) Representative co-labelling of EGFP green immunofluorescence and CaMKII red fluorescence in excitatory neurons in the BLA. Scale bar = 20 µm. (F) Representative immunofluorescence staining of CeA. Scale bar = 50 µm. (G) Representative immunofluorescence staining of BLA. Scale bar = 50 µm. (H) The increase of SLITRK1 protein in BLA was more pronounced than CeA ($t = 6.131$, $p < 0.001$). AMY, amygdala; BLA, basolateral amygdala; AAV, adeno-associated virus; EGFP, enhanced green fluorescent protein; CaMKII, Calcium-calmodulin (CaM)-dependent protein kinase II; CeA, central nucleus amygdala; MAP2, Microtubule associated protein 2; DAPI, 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride. ns, no significant difference, *** $p < 0.001$.

pain, and fibromyalgia, have been reported to produce chronic pain [3]. Typical NPP usually develops from peripheral nerve injury; chronic constriction injury (CCI) and SNL are typical NPP-induced pain models [3]. In our preliminary experiments, the CCI mouse model may have affected motor function of the lower extremities and was easily influenced by operator factors. In comparison, the SNL mouse model is more stable and less likely to affect the motor activity of experimental animals, and has greater reliability, as in previous reports [6,23,28]. Therefore, the left L5 SNL model was used to produce chronic NPP in the present study. Our results indicate that the chronic NPP accompanied by ADD-like comorbidity, with this mouse model, could be elicited reliably on day 28 after SNL.

As an excitatory postsynaptic marker, PSD-95 is an important scaffold protein abundantly expressed in the postsynaptic density of excitatory synapses [24]. Homer1 is an important component of the postsynaptic density, regulat-

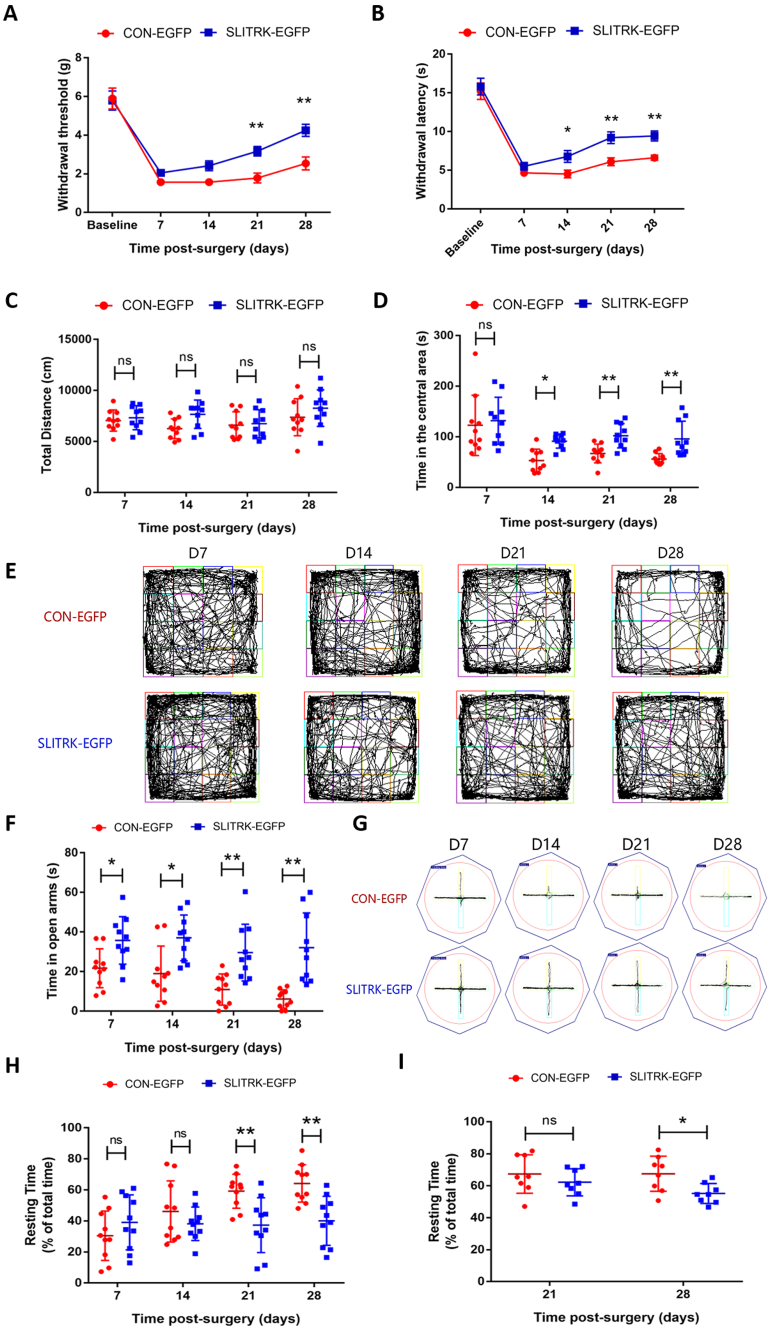

Fig. 5. Effects of SLITRK1 overexpression on chronic pain and anxio-depressive disorders (ADD) comorbidities in spinal nerve ligation (SNL) mice. (A) MWT was higher in SLITRK-EGFP group than the contral group at 21 ($p = 0.013$) and 28 days ($p = 0.001$) days ($F = 2.438$, $p = 0.092$). (B) TWL was higher in SLITRK-EGFP group than the contral group at 14 ($p = 0.028$), 21 ($p = 0.004$) and 28 days ($p = 0.001$) days ($F = 2.349$, $p = 0.098$). (C) No changes in total distance traveled in the OFT at 7 ($p = 0.579$), 14 ($p = 0.171$), 21 ($p = 0.811$) and 28 ($p = 0.285$) days after surgery ($F = 0.868$, $p = 0.463$). (D) The duration of the SLITRK-EGFP group in the central area (OFT) was higher than the that of the control group at 14 ($p = 0.03$), 21 ($p = 0.004$) and 28 ($p = 0.002$) days after surgery ($F = 2.484$, $p = 0.098$). (E) Representative trajectory in OFT. (F) The duration in the open arm in the EPM was higher in the SLITRK-EGFP group than in the control group at 7 ($p = 0.03$), 14 ($p = 0.01$), 21 ($p = 0.006$), and 28 ($p = 0.002$) days after surgery ($F = 2.803$, $p = 0.073$). (G) Representative trajectory in EPM. (H) The immobility duration of the SLITRK-EGFP group in the TST was lower than that of the control group at 21 ($p = 0.004$) and 28 ($p = 0.001$) days after surgery ($F = 5.032$, $p = 0.004$). (I) The difference in immobility duration in the FST between the two groups was evident at 28 days ($p = 0.015$) ($F = 14.948$, $p = 0.002$) after the surgery. $n = 10$/gp. ns, no significant difference, * $p < 0.05$, ** $p < 0.01$

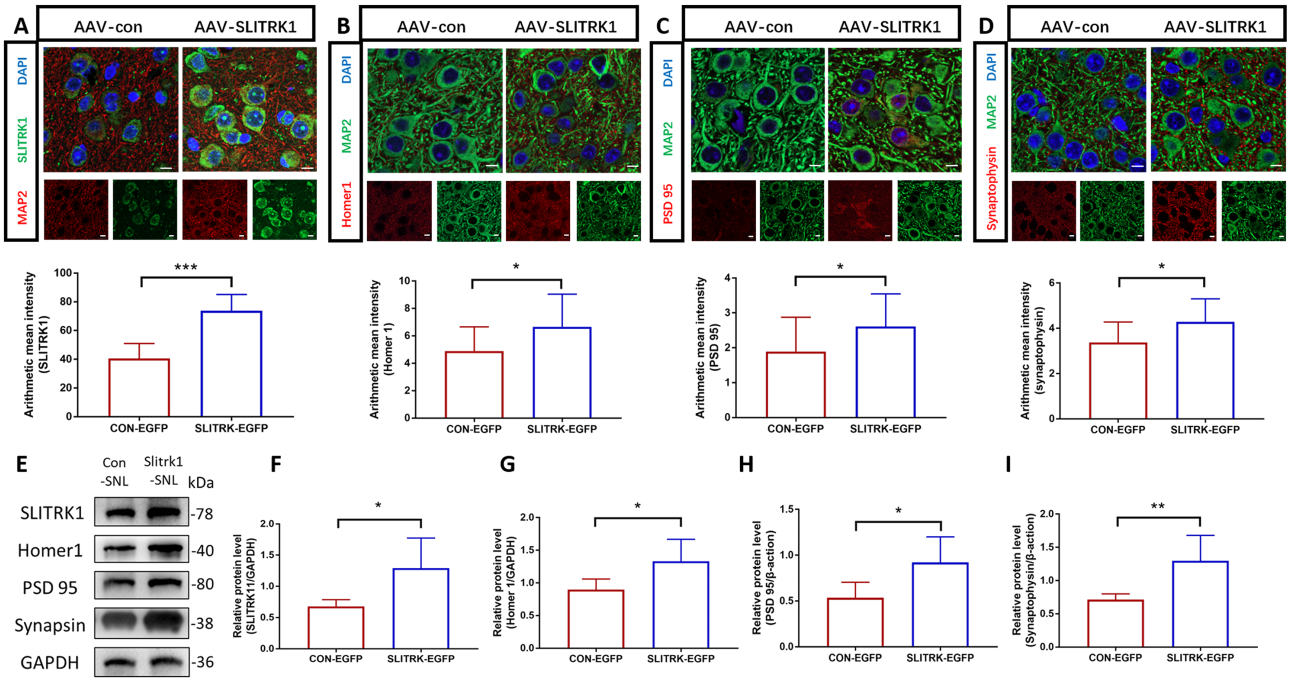

Fig. 6. Over expression of SLITRK1, Homer1, PSD95 and synaptophysin in AMY at 28 days after SNL surgery. (A–D) Upper: Representative images of immunohistochemical staining for of SLITRK1 (t = 5.701, $p < 0.001$), Homer1 (t = 2.702, $p = 0.01$), PSD95 (t = 2.561, $p = 0.014$), and Synaptophysin (t = 2.592, $p = 0.015$). Scale bars = 5 μm. Lower, the fluorescence intensity analysis. (E) Representative Western blot protein bands. (F–I) The protein level of SLITRK1(t = 3.051, $p = 0.012$), Homer1 (t = 2.839, $p = 0.019$), PSD95 (t = 2.918, $p = 0.015$), and Synaptophysin (t = 3.669, $p = 0.004$) was detected by a Western blot analysis. n = 6 for each group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

ing synaptic metabotropic glutamate function and the structural and functional integrity of dendritic spines [25,26]. SYN plays a crucial role in the formation, maintenance, and plasticity of synapses and is primarily located in the presynaptic terminals of neurons regulating the fusion of the synaptic vesicles and modulating neurotransmitter release [29]. The correlation between the expression changes of these proteins (Homer1, PSD95, SYN) and functional changes of excitatory synapse has been confirmed in previous studies [30–32]. In the present study, the general expression levels of SYN, PSD95, and Homer1 in the right AMY were assessed, by immunofluorescence staining and Western blot, in order to suggest the possible functional changes of excitatory synapses. However, the fact that fluorescence signals were measured from all the tissue, and not specifically from proteins located at the synapses, could preclude any certain conclusion that this reflects changes in synaptic function, which needs future investigations.

The CaMKIIα promoter of the virus is capable of recognizing excitatory, pyramidal neurons, which are dominantly located in the BLA [23,32]. As unilateral modulation of AMY activity could effectively influence anxio-depressive symptoms in chronic pain [33,34], we specifically upregulated the expression of SLITRK1 protein in CaMKIIα positive neurons through virus transfection in unilateral AMY. The latency of gene expression often takes about 2–3 weeks, which means that modulation starts to take effect after 2 weeks, and the expression tends to peak after 3 weeks [34]. Therefore, 2 weeks after the virus injection, we prepared the SNL-induced-pain mouse model, and completed relevant behavioral tests at the specified time. The results showed that SLITRK1 protein was successfully over-expressed in the AMY, especially the region of BLA. The overexpression of SLITRK1 protein could successfully relieve the chronic NPP and ADD-like behavior behavior in the SNL-treated mice after 28 days, which could show at least some indication that SLITRK1 in the BLA is more important than the CeA for chronic NPP and ADD-like behavior.

Overall, our study explored the potential mechanism of anxio-depressive disorders caused by chronic NPP and found that SLITRK1 protein in the AMY was significant in mediating the process of SNL-induced emotional dysfunction. However, there are several limitations to the study: (1) we did not address the functional impact of SLITRK1 downregulation at the synaptic level (whether it leads to increased or decreased synaptic plasticity, or enhanced or reduced excitability) or at the level of the amygdala; (2) we did not examine how excitatory and inhibitory neurotransmitters change during this process; and (3) the mechanism by which SLITRK1 overexpression alleviated chronic NPP and ADD-like behavior needs further investigation;

(4) further characterization of the affective component of pain (supraspinal-mediated behaviors, such as licking or biting the injured area, prolonged lifting of the affected paw, increased locomotion indicative of escape behavior, and jumping) is required in future studies.

5. Conclusion

The present study demonstrated that, in a mouse model, SNL induced anxio-depression-like behavior associated with chronic NPP after 28 days, which was accompanied by reduced expression levels of SLITRK1 protein and excitatory synaptic associated proteins SYN, PSD95, and Homer1 in the amygdala. Overexpression of SLITRK1 protein in excitatory neurons effectively alleviated chronic NPP and anxio-depression-like comorbidity, and improved excitatory synaptic function, indicating that SLITRK1 protein in the amygdala is an important protein in the process of chronic NPP and its induced emotional dysfunction.

Availability of Data and Materials

All data points generated or analyzed during this study are included in this article and there are no further underlying data necessary to reproduce the results.

Author Contributions

MLT, HBY, CFH and ZHX conceived the study and designed all of the biological experiments. RTC, YL and XYF performed the animal experiments with assistance from CYL, JL and RY. RTC performed the immunofluorescence and Western blot analysis. RTC and YL performed stereotactic surgery. RTC and HBY analyzed the data from biological experiments. RTC, MLT and HBY wrote the manuscript, with inputs from all authors. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

The animal experiments complied with the Animal Management Regulations and Ethical Requirements of the Second Affiliated Hospital of Naval Medical University Animal Management Committee (No. 2022DW37, Shanghai, China).

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Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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