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Original Research

Adrenomedullin is an Important Pathological Mediator in Progression of Chronic Neuropathic Pain

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Abstract

Background: The characterization of neuropathic pain is maladaptive plasticity within the nociceptive system. Multiple alterations contribute to complex pain phenotypes. Adrenomedullin (AM) has been documented to be a pain mediator. However, its involvement in pathological pain is poorly understood. We studied the contribution of AM to chronic neuropathic pain in the spinal nerve ligation (SNL) model. **Methods**: Daily injection of the AM receptor antagonist AM_{22-52} (10 nmol) via an intrathecal (i.t.) route after SNL inhibited mechanical allodynia starting on day 6. Single administration of AM_{22-52} produced an immediate attenuation on pain hypersensitivity on day 2 or 10 post-SNL. Protein and mRNA levels were assayed by immunofluorescent staining and qRT-PCR, respectively, on days 1, 3, 7 and 15 post-SNL. **Results**: The results showed that AM at both protein and mRNA levels was increased in both injured (L5) and adjacent uninjured (L4) nerves starting on day 3 post-SNL. In dorsal root ganglion (DRG) at L5, AM was increase on days 1–7 at mRNA level but only on day 7 at protein level. However, AM was increase at mRNA level on days 1–7 and at protein level on days 3–15 in L4 DRG. AM mRNA expression was upregulated on days 1–7 in the spinal cord. Expression of receptor activity-modifying protein 2 (RAMP2), an essential AM1 receptor component, was upregulated in small and medium-diameter neurons on days 1–15 in both L5 and L4 DRG. Furthermore, single administration of AM_{22-52} suppressed the increase of nNOS in DRG induced by SNL and daily injection of AM_{22-52} for 7 days inhibited SNL-induced increase of CGRP mRNA in the spinal dorsal horn. **Conclusions**: This study indicates that the increased AM bioactivity in injured and uninjured peripheral nerves, uninjured adjacent DRG and the spinal dorsal horn play a critical role mainly in the late-phase development of neuropathic pain. The mechanism may involve the recruitment of nNOS and CGRP.

Keywords: adrenomedullin (AM); dorsal root ganglion (DRG); neuropathic pain; spinal nerve; spinal cord

1. Introduction

Neuropathic pain is often caused by damage within the nervous system arising from physical injury, diabetes mellitus, infection or autoimmune disease [1]. Pronociceptive mediators are released at the lesion site in response to nerve injury [2] and increase the excitability of both the damaged and adjacent undamaged nerve fibres [3] and primary afferent neurons. As a result, peripheral sensitization is produced [4]. Ectopic spontaneous activity is generated in dorsal root ganglion (DRG) neurons by ongoing activity originating in injured nerves and increased expression of voltage-gated sodium channels [5]. Neurotransmitters, such as glutamate and substance P, are therefore released at the presynaptic sites on terminals of primary afferent nociceptors [1]. These molecules cause rapid-onset homo- and heterosynaptic facilitation in the spinal dorsal horn, resulting in hyperexcitability of secondary sensory neurons, called central sensitization [6]. However, mechanisms underlying neuropathic pain is not fully understood. As neuropathic pain is refractory to most available analgesics including opioids [7], investigating mechanisms of neuropathic pain is essential for the development of new therapies.

Adrenomedullin (AM) is a 52-amino acid peptide. It belongs to calcitonin family with a partial sequence homology with calcitonin gene-related peptide (CGRP). AM is broadly expressed in the peripheral and central nervous systems [8,9], including the spinal dorsal horn and small-diameter neurons in DRG [10], the important tissues of pain processing. AM receptors that mediate AM activity are the obligatory association of the calcitonin-like receptor (CLR) with the receptor activity-modifying protein (RAMP) 2 or 3 [11]. CLR and RAMP2/3 are distributed in superficial laminae of the spinal cord [10] and DRG neurons [12]. AM_{22-52} is a synthetic selective antagonist for AM receptors, only low or no affinity for CGRP [13], with a preferred affinity for AM1 receptor (CLR with RAMP2) over AM2 receptor (CLR with RAMP3). AM is released in DRG and the spinal cord in pathological states, contributing to inflammatory pain [14] and morphine-associated hyperalgesia [12]. Increased AM activity can also upregulate CGRP [12], nNOS [15] and interlukin-1β (IL-1β) [16,17] in the spinal cord or/and DRG. Particularly, this molecule is involved in neuron-glia communication as evidenced by the studies demonstrating that AM can activate spinal microglial and astrocytes [17] and DRG satellite glial cells [16]. The current study investigated the role of AM receptor signaling in the pathogenesis of neuropathic pain in the spinal nerve ligation (SNL) model.

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2. Materials and Methods

2.1 Animals

Male Sprague-Dawley rats (Fuzhou Animal Center, Fuzhou) weighing 220–260 g at the time of surgery were used. Animals were housed in a quiet room kept at 22 $\pm$ 0.5 °C under 12 h light/dark cycles (lights on at 6:30 h and off at 18:30 h) and had free access to food and water ad libitum. Experiments were performed following the guidelines for investigations of experimental pain in conscious animals [18]. Experimental protocols were approved by Fujian Normal University Institutional Laboratory Animal Care and Use Committee. After implantation of intrathecal catheter, animals were housed individually. Surgeries and experimental procedures were done between 9:30 and 16:30 h. Every effort was made to minimize animal numbers and their discomfort.

2.2 Ligation of L5 Spinal Nerve

Nerve lesion in the periphery was produced by ligation of unilateral spinal nerve at L5 [19]. Rat was anesthetized with pentobarbital (50 mg/kg, i.p.). Ligation of L5 spinal nerve was carried out as described before [19]. The dorsal vertebral was exposed at lumbar level (L4 to L6). The paraspinal muscles were bluntly separated and transverse spinal processes at L5 was removed. L5 nerve at the right side was exposed and ligated tightly with 5-0 suture. The muscle layer was closed with nylon threads. Ceftiofur sodium (Shenggong, Shanghai, China) was given in the wound to prevent possible infection. Sham surgery was performed with same procedure without removing spinal process and disturbance of the spinal nerves. The same procedure was followed without ligation of the spinal nerve in the sham operated animals. The rats were allowed to fully recover.

2.3 Implantation of Catheter

Animals were implanted with indwelling catheters in the subarachnoid space. Briefly, rat was injected with pentobarbital (50 mg/kg, i.p., Shenggong, Shanghai, China). The dura mater overlying the atlanto-occipital membrane of rats was exposed and a small slit was cut in the membrane. A PE-10 catheter (Stoelting, Wood Dale, IL, USA) was inserted into the subarachnoid space with its tip being positioned at the L4 segment. The catheter was flushed with saline (10 μL) and closed. Then the rats were allowed to recover for 1 week. The location of intrathecal catheter tip was checked by i.t. administration of lidocaine (200 μg/10 μL, Shenggong, Shanghai, China), which caused a motor paralysis of the lower limbs. Vehicle (saline) or drag (AM_{22-52}) (Angtuolaisi, Zhejiang, China) was given i.t. in a volume of 10 μL.

2.4 Nociceptive Test for Pain Evaluation

Nociceptive test responding to mechanical stimulation was used to evaluate the effect of drugs on pain threshold in rats. Mechanical pain threshold was tested in the right hindpaw (n = 7–9 each) using an electrodynamic von Frey apparatus (Dynamic Plantar Anesthesiometer, Ugo Basile, Italy). Animals were placed in plastic cages with a metal net floor. They were acclimatized to the environment for 40 min for five days and again habituated for 15 min prior to testing to minimize variability of behavioral measurements. The von Frey filament (0.5 mm diameter) was applied to the mid-plantar surface of hindpaw. Paw withdrawal threshold (PWT) was tested by application of a force. The actuator automatically lifted the filament and applied an increasing force. The force was stopped once rat moved its hindpaw. Forty g for 30 s was preset as a cut-off force in the apparatus to avoid possible injury in the tissue. PWT was measured three times at 2 min interval to generate a mean value. Investigator was blind to the tested drug conditions.

2.5 Immunofluorescence

Animals were anesthetized with pentobarbital (50 mg/kg i.p.). Rats were perfused intracardially with cold 0.01 M PBS (phosphate buffered saline) followed by 4% paraformaldehyde in 0.1 M PB (phosphate buffer). L4/L5 spinal nerves and L4/L5 DRG (n = 5 or 6 each) were cut out. After being fixed in 4% paraformaldehyde overnight, the tissues were transferred to sucrose (30% in PB). The nerve or DRG was sectioned at a thickness of 10 μm. Immunostaining procedures were done at room temperature. Nonspecific proteins in nerve or DRG sections were blocked with H_2O_2 (0.3%) and goat serum (10%, Shenggong) for 1 hour. The tissue sections were incubated with rabbit AM (1:100, Santa Cruz Biotechnology Inc., California, USA) or RAMP2 (1:200, Bioss Biotechnology Inc., Beijing, China) antibody overnight at 4 °C. After being thoroughly washed in PBS, the sections were incubated with donkey anti-goat or anti-rabbit IgG conjugated to FITC (fluorescent marker, 1:1000, Abcam, Cambridge, UK) for 2 hours. The sections were dehydrated and coverslipped. The AM or RAMP2 antibody was omitted in the immunostaining protocol for controls. Negative staining was resulted in this procedure. Stained sections were examined with a fluorescence digital microscope (BX51, Olympus, Tokyo, Japan). Images were captured with a Q-Fire cooled camera (DP70, Olympus, Tokyo, Japan). AM-positive staining in nerves was quantified as pixel number above a predefined threshold using software Image-Pro Plus 6.0 (Media Cybernetics, Silver Spring, MD, USA) as described previously [12,17]. The AM staining intensity in the nerve was analyzed in 870 $\times$ 640 μm^2 area at 40$\times$ magnification with 6 sections per animal. The total pixel from 6 tissue sections was averaged to give a mean number of pixel for each rat [20,21].

AM and RAMP2 immunoreactivity-stained DRG neurons was quantified using software Image-Pro Plus 6.0. A field of 870 $\times$ 640 μm^2 at 20$\times$ magnification was taken randomly from each of 10 DRG sections each rat. To analyze staining neurons, a threshold of cytoplasmic optical inten-

sity of AM or RAMP2 immunofluorescence was preset by the software. The threshold was applied to whole DRG section. The neurons with optical intensity over the threshold were taken as AM or RAMP2-positive. Other cells were counted as negative staining neurons. Neuronal soma area was measured by imaging software to determine neuron size (small neurons $<600\ \mu m^2$, medium neurons 600–1200 μm^2 and large neurons $>1200\ \mu m^2$ [22]).

2.6 Double Immunostaining

The preparation of stained sections was consistent with the method described above by immunofluorescence. The tissue sections were incubated with rabbit AM (1:500, Bioss Biotechnology Inc., Beijing, China) or RAMP2 (1:200, Bioss Biotechnology Inc., Beijing, China) or CLR (1:500, Bioss Biotechnology Inc., Beijing, China) antibody and mouse nNOS antibody (1:100, Santa Cruz Biotechnology Inc., California, USA) together overnight at 4 °C. After being thoroughly washed in PBS, the sections were incubated with donkey anti-rabbit or anti-mouse IgG conjugated to FITC (1:1000, Abcam, Cambridge, UK) or Rhodamine (1:800, Abcam, Cambridge, UK) for 2 hours at room temperature. AM, CLR and RAMP2 immunoreactivity were labeled as green, whereas nNOS were labeled as red.

2.7 Western Blot

The rats were decapitated, L4–L6 dorsal root ganglia were removed and collected. A mixture of cell lysate and protease inhibitor (10:1, Beyotime, Shanghai, China) was added to the tissue, and then all samples were centrifuged (15,000 r/min, 30 min, 4 °C). The extracted protein was quantified by BCA kit (Beyotime, Shanghai, China). Proteins were denatured with SDS loading buffer (95 °C,10 min) and processed to SDS polyacrylamide gels. After transferring the protein, nitrocellulose filter membrane was blocked with 10% skimmed milk in TBST at room temperature for 2 h. Then the membrane was incubated with rabbit anti-nNOS (1:500, Bioss Biotechnology Inc., Beijing, China) and β-actin (1:2000, Proteintech, Wuhan, China) overnight at 4 °C in 5% milk. The Membrane was then incubated with Donkey anti-Rabbit IgG and Donkey anti-Mouse IgG (1:1000, LI-COR, Lincoln, USA) for 2 h at room temperature in 5% milk. The strip band was analyzed with Odyssey CLX laser imager (LI-COR, Lincoln, USA) and was normalized to β-actin loading control. The final result was obtained by the change multiple of the control group.

2.8 Quantitative Real-Time-PCR

The dorsal half of the spinal cord at L4/5 was dissected. To isolate RNA, samples were homogenized in 1 ml of Trizol reagent using RNA prep pure kit (Transgen Biotech, Beijing, China). Deoxyribonuclease I treatment was done to prevent DNA contamination. Concentration of RNA was measured using an ultraviolet–visible spectrometer (NanoDrop ND-2000, Thermo Scientific, Waltham, USA). The quality and quantity of the RNA were assessed at 260/280 A. All samples showed absorbency ratios ranging from 1.8 to 2.0. A total of 1 μg of RNA was reverse-transcribed using TransScript one-step gDNA removal and cDNA synthesis supermix (Transgen Biotech, Beijing, China).

Forward (5'-3') and reverse (5'-3') primer sequences used were: GTTTCCATCGCCCTGATGTTATT and GTAGTTCCCTCTTCCCACGACTTAG for the gene encoding AM; AACCTTAGAAAGCAGCCCAGGCATG and GTGGGCACAAAGTTGTCCTTCACCA for the gene encoding CGRP; GTTTGTGATGGGTGTGAAC and TCTTCTGAGTGGCAGTGA for the gene encoding the gene encoding GAPDH. Twenty ng of cDNA from the same cDNA batch was subjected to real-time PCR to amplify all genes in triplicate in a total reaction volume of 20 μL using Platinum SYBR Green Master mix (Invitrogen, Carlsbad, USA). Reactions were conducted on an Mx300P thermocycler (Stratagene, La Jolla, USA). one cycle at 28 °C for 15 min, 42 °C for 30 min, 99 °C for 5 min, and 4 °C for 5 min. Each cycle consisted of 4 steps: 28 °C (15 min), 42 °C (30 min), 99 °C (5 min) and 4 °C (5 min). A non-template reaction served as negative control. Melting curve analysis of products as well as amplicon size verification on a 3% agarose gel confirmed the specificity of the PCR. The raw expression level for each gene was calculated using the same external standard curve made with a mixture of cDNA samples. GAPDH was used as internal reference for normalization of target gene expression.

2.9 Statistical Analysis

All data were presented as the mean $\pm$ SEM (standard error of mean). Statistical evaluation of AM or RAMP between groups was performed by one-way repeated measure analysis of variance. Statistical comparisons of changes over time between groups (group $\times$ time) were performed by a two-way ANOVA. A value of $p < 0.05$ was accepted to be statistically significant.

3. Results

3.1 Intrathecal Injection of AM_{22-52} Inhibited SNL-Induced Mechanical Allodynia

PWT in the hindpaw on the surgical side significantly shortened one day following nerve ligation operation. Behavioral examination showed that basic mechanical threshold (the value measured before i.t. administration) was remarkably decreased by SNL, but not by sham, surgery, indicating mechanical pain hypersensitivity or allodynia. This state was maintained thereafter throughout the experiment (15 days) and was not changed by i.t. administration of saline (once per day). Daily administration of AM_{22-52} (10 nmol) did not change mechanical allodynia on the first 5 days. However, the basic mechanical threshold elevated on day 6 and this elevation was statistically significant com-

pared to the pre-drug administration (Fig. 1A, $p < 0.05$). Moreover, the elevated mechanical threshold still remained 24 hours after the last AM_{22-52} administration on day 7. On the other hand, AM_{22-52} administration (10 nmol) immediately increased mechanical threshold. Fig. 1B,C illustrated that mechanical threshold was significantly increased by AM_{22-52}, but not by saline, administration on day 2 or 10 compared to the pre-drug level ($p < 0.05$). The increase started at 40 min and 20 min after the drug administration on day 2 or 10, respectively, and maintained for 20 min.

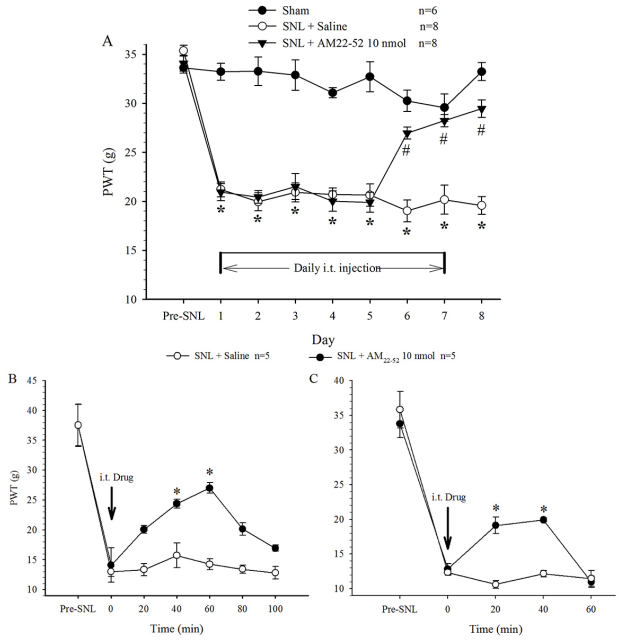

Fig. 1. Effect of i.t. administration of AM_{22-52} on SNL-induced mechanical hypersensitivity in the hindpaw. Spinal nerve ligation (L5) surgery was performed on day 0. (A) AM_{22-52} (10 nmol) was administered i.t. once per day for 7 days. PWT responding to mechanical stimuli was tested before the drug administration. (B) AM_{22-52} (10 nmol) or saline was administered i.t. on day 2. (C) AM_{22-52} (10 nmol) or saline was administered i.t. on day 10. PWT was measured before and after the i.t. administration. The data are presented as mean ± SEM. *$p <$ 0.05 compared to pre-SNL surgery. #$p < 0.05$ compared to saline group.

3.2 AM Expression in Peripheral Nerves, DRG and Spinal Cord was Increased

To characterize the association of AM receptor activity with neuropathic pain, nerve ligation was performed on day 0. L4/5 spinal nerves, DRG and the dorsal half of the lumbar spinal cord were taken on naive rats (day 0 or D0) and 1, 3, 7 and 15 days post-SNL. The samples were proceeded by qRT-PCR assay or immunofluorescence staining to examine the changes of AM mRNA or protein with respect to the development of neuropathic pain. The experiment in each assay was repeated five times. AM immunoreactivity was shown in nerve fibers. The density of AM-immunoreactive fibers was remarkably increased in L5 nerve (damaged nerve) on day 3 and remained at a high level throughout the experiment (up to 15 days, Fig. 2A–F, $p < 0.05$ *vs* D0). Similarly, the expression of AM mRNA was also increased on days 3–15 (Fig. 2G, $p < 0.05$ *vs* D0). In L4 nerve (undamaged nerve), the increased level of AM protein was observed on day 3 and maintained until day 15 (Fig. 3A–F, $p < 0.05$ *vs* D0). However, the expression of AM mRNA was increased on days 1–5 but significantly declined to the level on day 15 that was even lower than that on day 0 (Fig. 3G, $p < 0.05$ *vs* D0).

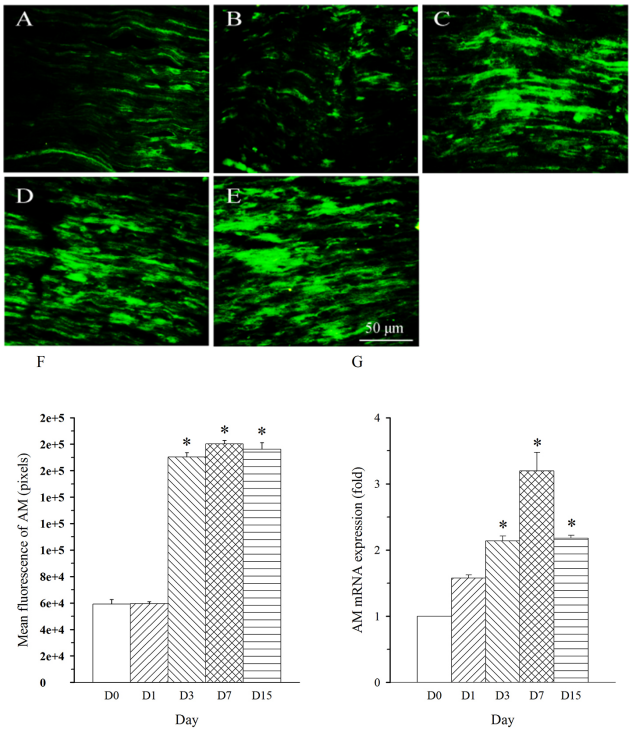

Fig. 2. Expression pattern of AM protein and mRNA in L5 spinal nerve in SNL rats. Spinal nerve ligation (L5) or sham surgery was performed on day 0. The L5 spinal nerve on the operate side was harvested at various times. The samples were assayed using immunofluorescence staining and RT-PCR techniques. Representative immunofluorescence images are presented as following: A-day 0, B-day 1, C-day 3, D-day 7, E-day 15. The AM staining intensity was quantified as pixels in F. AM mRNA levels at various times were shown in G. *$p < 0.05$ compared to day 0. N = 5 each. Scale bar = 50 μm.

AM neurons were mainly observed in medium- and small-diameter DRG neurons. In DRG at L5 (containing damaged neurons), SNL induced an increase in the expression of AM in both medium- and small-diameter neurons only on day 7 (Fig. 4A–F). The expression of AM mRNA in L5 DRG exhibited a different profile. It was increased on

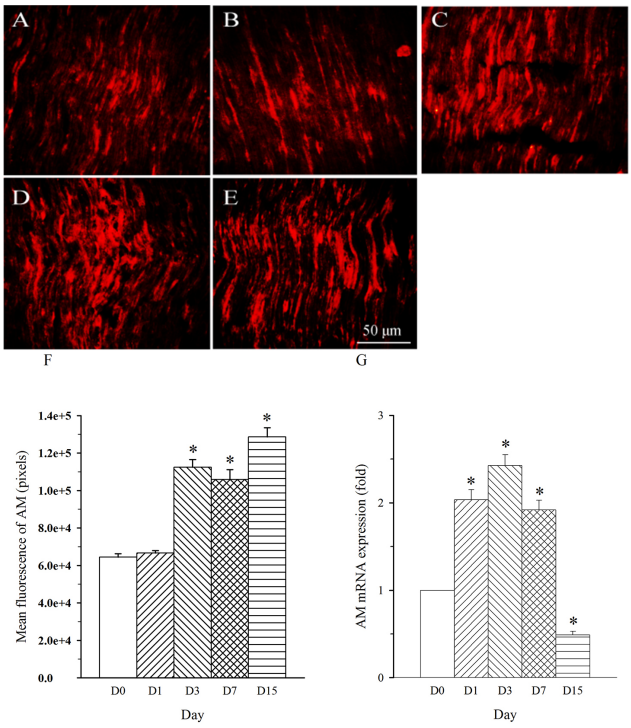

Fig. 3. Expression pattern of AM protein and mRNA in L4 spinal nerve in SNL rats. Spinal nerve ligation (L5) or sham surgery was performed on day 0. The L4 spinal nerve on the operate side was harvested at various times. The samples were assayed using immunofluorescence staining and RT-PCR techniques. Representative immunofluorescence images are presented as following: A-day 0, B-day 1, C-day 3, D-day 7, E-day 15. The AM staining intensity was quantified as pixels in F. AM mRNA levels at various times were shown in G. *$p < 0.05$ compared to day 0. N = 5 each. Scale bar = 50 μm.

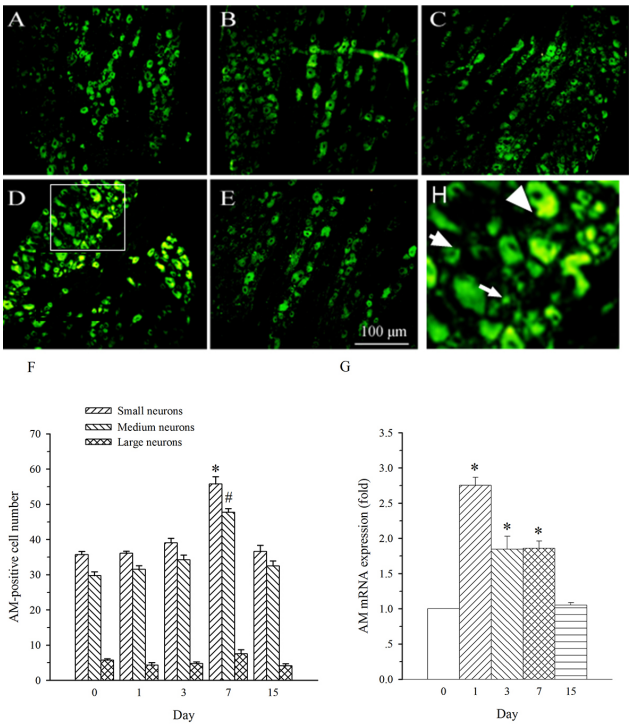

Fig. 4. Expression of AM protein and mRNA in DRG at L5 in SNL rats. Spinal nerve ligation (L5) or sham surgery was performed on day 0. The L5 DRG on the operate side was harvested at various times. The samples were assayed using immunofluorescence staining and RT-PCR techniques. Representative immunofluorescence images are presented as following: A-day 0, B-day 1, C-day 3, D-day 7, E-day 15. Arrows of different sizes indicate small-, medium- or large-sized cells (H). Quantification of AM expression is represented as a number of AM-positive cells in small- or medium-sized subpopulation (F). AM mRNA levels at various times were shown in G. *$p < 0.05$ compared to day 0. N = 5 each. Scale bar = 100 μm.

days 1–7 post-SNL (Fig. 4G, $p < 0.05$ *vs* D0) and returned to the normal level on day 15. In L4 DRG containing undamaged neurons, AM-positive neurons were increased in small and medium populations throughout the experiment starting on day 3. AM expression in large-sized neurons was also slightly but significantly increased on days 7–15 (Fig. 5A–F, $p < 0.05$ *vs* D0). The expression of AM mRNA was increased on days 1–7 post-SNL (Fig. 5G, $p < 0.05$ *vs* D0). In the spinal dorsal horn, AM mRNA level was remarkably increased on days 1–7 (Fig. 6, $p < 0.05$ *vs* D0). The increase disappeared on day 15 ($p > 0.05$ *vs* D0).

3.3 Expression of RAMP2 in DRG was Increased

AM mainly activates AM1 receptor which is a complex of CLR with RAMP2 [13]. This study examined characteristics of the temporal expression of RAMP2 in DRG. In consistence with previous report [12], RAMP2 immunofluorescence staining was seen in medium- and small-diameter DRG neurons (Fig. 7). The expression of RAMP2 in both sized neurons was remarkably increased on day 1 post-SNL in L5 DRG. This increase maintained throughout the experiment (Fig. 7A–F, $p < 0.05$ *vs* D0). Particularly, expression of RAMP2 was increased in large-sized neurons on days 3 and 7 ($p < 0.05$ *vs* D0). As illustrated in Fig. 7G, RAMP2 mRNA in L5 DRG was increased only on day 3 compared with day 0 ($p < 0.05$) and did not change on days 1, 7 and 15 ($p > 0.05$).

RAMP2 expression in medium- and small-diameter neurons in L4 DRG was also increased on day 1 post-SNL (Fig. 8A,B, $p < 0.05$ *vs* D0). This increase maintained throughout the experiment including day 15 (Fig. 8C–F, $p < 0.05$ *vs* D0). The expression of RAMP2 was also increased in large-sized neurons on days 3 and 7 ($p < 0.05$ *vs* D0). RAMP2 mRNA was increased on day 1 ($p < 0.05$ *vs* D0). The increase maintained for a couple days ($p < 0.05$ *vs* D0) and was completely abolished on day 15 (Fig. 8G, $p > 0.05$).

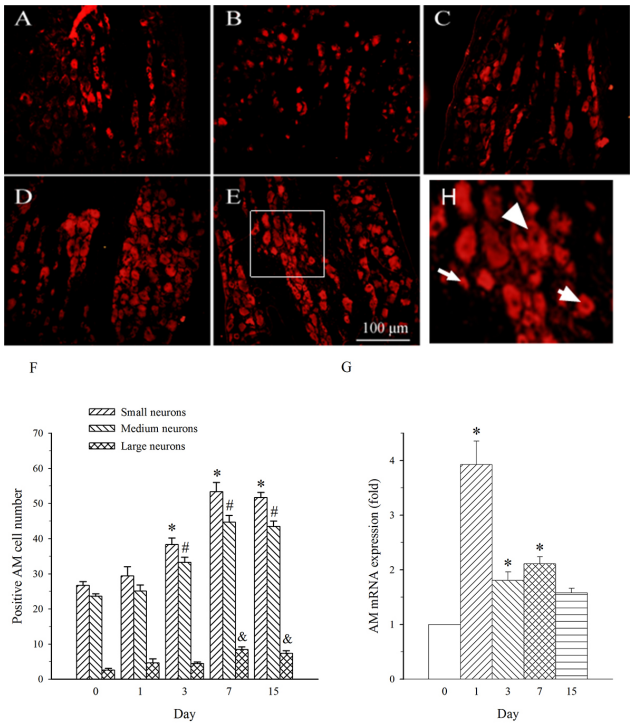

Fig. 5. Expression of AM protein and mRNA in DRG at L4 in SNL rats. Spinal nerve ligation (L5) or sham surgery was performed on day 0. The L4 DRG on the operate side was harvested at various times. The samples were assayed using immunofluorescence staining and RT-PCR techniques. Representative immunofluorescence images are presented as following: A-day 0, B-day 1, C-day 3, D-day 7, E-day 15. Arrows of different sizes indicate small-, medium- or large-sized cells (H). Quantification of AM expression is represented as a number of AM-positive cells in small- or medium-sized subpopulation (F). AM mRNA levels at various times were shown in G. *$p < 0.05$ compared to day 0. N = 5 each. Scale bar = 100 μm.

3.4 Intrathecal Injection of AM_{22-52} Inhibited SNL-Induced Increase of nNOS Protein in DRG

To explore cellular mechanism of AM-induced activity, nNOS protein in DRG was assayed. AM_{22-52} (10 nmol) or saline was given immediately on day 2 or day 10, which time point is the early and late stage of neuropathic pain. The DRG was taken at 40 min. Protein analysis showed that SNL remarkably elevated the expression of nNOS protein compared to sham surgery on day 2 (Fig. 9A, $p < 0.05$). However, in the group in which AM_{22-52} was given immediately on day 2, the level of nNOS was back to the level that was identical to the sham group ($p > 0.05$). Similarly, nNOS protein increased significantly after nerve injury on day 10 (Fig. 9B, $p < 0.05$), the expression of nNOS returned to near control group level when AM_{22-52} was given immediately on day 10 ($p > 0.05$). Moreover, the double immunostaining confocal results show that nNOS immunoreactivity (nNOS-IR) colocalizes with AM, CLR and RAMP2 (AM/CLR/RAMP2-IR) immunoreactivity in DRG neurons (Fig. 9C).

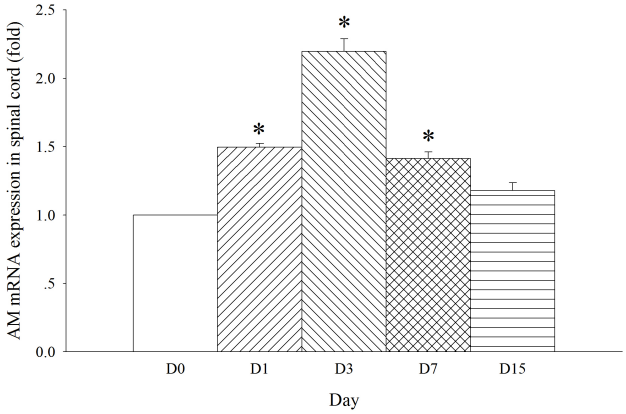

Fig. 6. Expression of AM mRNA in the spinal dorsal horn in SNL rats. Spinal nerve ligation (L5) or sham surgery was performed on day 0. The dorsal part of the lumbar spinal cord on the operate side was harvested at various times. The samples were assayed using RT-PCR techniques. Histograms indicate the mean ± SEM of AM mRNA. *$p < 0.05$ compared to day 0. N = 5 each.

3.5 Intrathecal Injection of AM_{22-52} Inhibited SNL-Induced Increase of CGRP mRNA in Spinal Dorsal Horn

To further explore cellular mechanism of AM-induced activity, CGRP mRNA in the spinal dorsal horn was assayed. AM_{22-52} (10 nmol) or saline was given once per day for 7 days. The dorsal half of the lumbar spinal cord was taken on day 8. mRNA analysis showed that SNL remarkably elevated the expression of CGRP mRNA compared to sham surgery (Fig. 10, $p < 0.05$). However, in the group in which daily administration of AM_{22-52} was given for 7 days, the level of CGRP mRNA was back to the level that was identical to the sham group ($p > 0.05$).

4. Discussion

The current study investigated the role of AM receptor signaling in neuropathic pain. Our results showed that blockade of AM receptor inhibited nerve injury-induced mechanical allodynia. AM mRNA expression was increased in the damaged (L5) and adjacent undamaged (L4) peripheral nerves following SNL surgery. The levels of AM mRNA were also elevated in DRG (L5) containing damaged neurons and adjacent DRG (L4) containing complete neurons as well as the spinal dorsal horn. AM protein was increased in L4/L5 peripheral nerves and L4 DRG. SNL also increased the protein level of RAMP2 in both L5 and L4 DRG but enhanced mRNA level mainly in L4 DRG. Furthermore, chronic injection of AM_{22-52}, an AM receptor antagonist, attenuated SNL-induced expression of nNOS in DRG and CGRP in the spinal dorsal horn on the lesion side. These results suggest that AM plays a critical role

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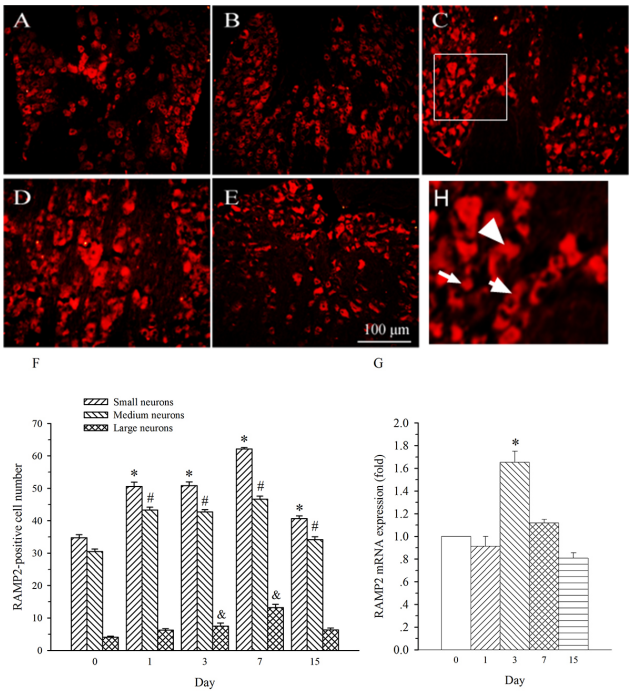

Fig. 7. Expression of RAMP2 protein and mRNA in DRG at L5 in SNL rats. Spinal nerve ligation (L5) or sham surgery was performed on day 0. The L5 DRG on the operate side was harvested at various times. The samples were assayed using immunofluorescence staining and RT-PCR techniques. Representative immunofluorescence images are presented as following: A-day 0, B-day 1, C-day 3, D-day 7, E-day 15. Arrows of different sizes indicate small-, medium- or large-sized cells (H). Quantification of AM expression is represented as a number of AM-positive cells in small- or medium-sized subpopulation (F). AM mRNA levels at various times were shown in G. *$p < 0.05$ compared to day 0. N = 5 each. Scale bar = 100 μm.

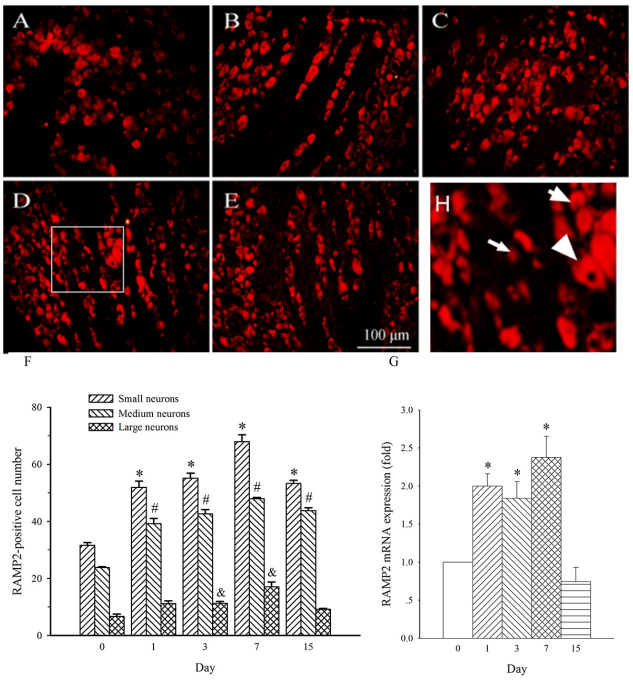

Fig. 8. Expression of RAMP2 protein and mRNA in DRG at L4 in SNL rats. Spinal nerve ligation (L5) or sham surgery was performed on day 0. The L4 DRG on the operate side was harvested at various times. The samples were assayed using immunofluorescence staining and RT-PCR techniques. Representative immunofluorescence images are presented as following: A-day 0, B-day 1, C-day 3, D-day 7, E-day 15. Arrows of different sizes indicate small-, medium- or large-sized cells (H). Subpopulation Quantification of AM expression is represented as a number of AM-positive cells in small- or medium-sized subpopulation (F). AM mRNA levels at various times were shown in G. *$p < 0.05$ compared to day 0. N = 5 each. Scale bar = 100 μm.

in the pathogenesis of neuropathic pain and the increased AM activity was mediated by enhanced CGRP synthesis in the spinal dorsal horn.

Bolus injection of AM_{22-52}, a specific antagonist of AM receptor (AMR) with a preferential affinity for AM1 receptor (AM1R) over AM2 receptor (AM2R) [13], in the spinal cord for 7 days abolished SNL-induced mechanical allodynia. Interestingly, mechanical allodynia did not change until the sixth day following the administration. The inhibition of mechanical allodynia even remained 24 hours after discontinuing of the drug administration. On the other hand, the intrathecal AM_{22-52} administration also induced an immediate inhibition on mechanical allodynia on both day 2 and 10 which are the early- and late-phase developments of neuropathic pain, respectively [23]. These results suggest that AM causes or exacerbates neuropathic pain and mainly being involved in the maintenance of neuropathic pain. The contribution of AM receptor signaling to inflammatory pain [14] and morphine-associated pain hypersensitivity [12,24] has been demonstrated.

The spatio-temporal expression profile was examined to explore functional characterizations of AM in neuropathic pain. It has been documented that sensory signals transported in adjacent uninjured afferents, but not injured afferents, contribute to the sensitization of primary and the secondary sensory neurons, producing hypersensitivity response [25–27]. Moreover, some molecules, such as CGRP [28], Nav1.3 sodium channel [29], MrgC [30] and $GABA_A$ receptor [31], are not identically expressed in injured and adjacent uninjured DRG. Therefore, we examined the alterations in damaged and undamaged spinal nerves and DRG. The results showed that the expression of AM mRNA and protein was increased not only in damaged L5 nerve but also in undamaged L4 nerve. The increase of AM mRNA in L4 nerve occurred slightly earlier (on day 1) than L5 nerve (on day 3). However, AM at a protein level was elevated in both nerves during the similar duration (on days 3–15), indicating that the alteration of AM protein exhibited the same profile in the injured and adjacent uninjured

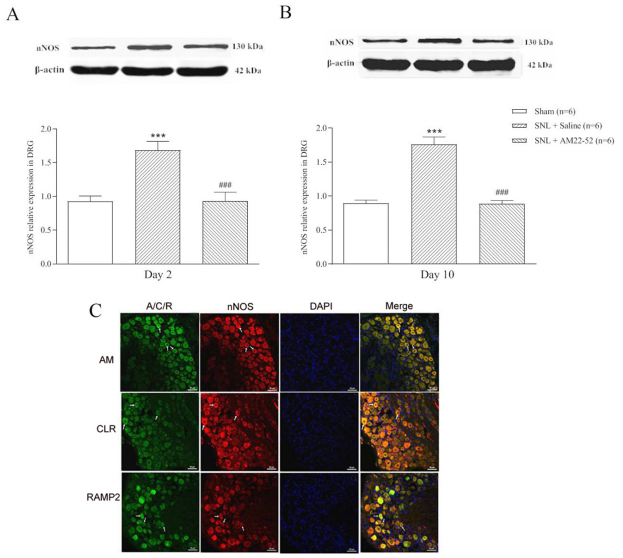

Fig. 9. Effect of i.t. AM_{22-52} on SNL-induced level of nNOS protein in DRG. Spinal nerve ligation (L5) or sham surgery was performed on day 0. AM_{22-52} (10 nmol) was administered i.t. on day 2 (A) or day 10 (B) post-SNL. The DRG on the operate side was harvested at 40 min after injection. Histograms indicate the mean ± SEM of nNOS protein. *$p < 0.05$ compared to sham group. #$p < 0.05$ compared to SNL + Saline group N = 6 each. Confocal images showing localization of nNOS-IR, AM-IR and its receptor components in DRG neurons (C). AM-IR, CLR-IR and RAMP2-IR neurons are identified by Alexa Fluor 488 (Leftmost pictures; arrows; green) fluorescence, whereas nNOS-IR-positive neurons are labeled by Alexa Fluor 568 (Second column pictures; arrows; red) fluorescence. DAPI is the staining of nucleus (blue). nNOS-IR is extensively colocalized with AM-IR, CLR-IR, RAMP2-IR in DRG neurons (Rightmost pictures; arrows; yellow). Scale bar = 50 μm.

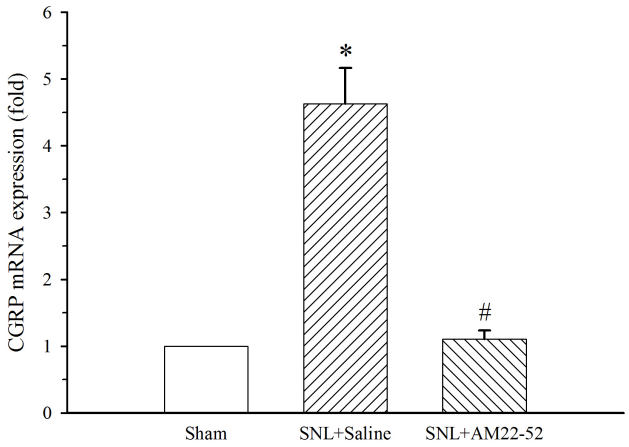

Fig. 10. Effect of i.t. AM_{22-52} on SNL-induced level of CGRP mRNA in the spinal dorsal horn. Spinal nerve ligation (L5) or sham surgery was performed on day 0. AM_{22-52} (10 nmol) was administered i.t. once per day for 7 days. The dorsal part of the lumbar spinal cord on the operate side was harvested on day 8. Histograms indicate the mean ± SEM of CGRP mRNA. *$p < 0.05$ compared to sham group. #$p < 0.05$ compared to SNL + Saline group N = 5 each.

nerves. Similar to what was observed in inflammatory pain [14], AM expression was increased in medium- and small-diameter neurons in DRG post-SNL surgery. In L5 DRG, AM mRNA expression was increased on days 1–7 post-SNL but AM protein exhibited an increase only on day 7, suggesting that SNL mainly promoted transcriptional activation of the *AM* gene in DRG containing damaged neurons. It is a possibility that as a neuropeptide transmitter, AM protein usually stored in vesicles be beneficial to reach the cell membrane at any time. There may also be abnormal protein synthesis such as the translation or the protein renewal speed is fast and quickly used under pathological conditions. In L4 DRG, the increase of AM mRNA and protein levels prolonged (on days 1–7 or 3–15). These results suggest that SNL promoted AM synthesis at both transcriptional and ongoing post-translational levels in the adjacent DRG containing complete neurons. Time-lag between AM mRNA and protein expression in L5 not in L4 DRG may be due to abnormal neurons with nerve injury. Increase of AM protein uninjured adjacent DRG plays the role of compensation. As terminating unmyelinated fibres of injured and adjacent uninjured nerves interact at level of the spinal cord [32], we examined expression of AM mRNA in the lumbar spinal dorsal horm. The results showed that SNL also induced an increase in AM mRNA in the spinal dorsal horn. Taken together, the current study indicates that increase of AM bioactivity in injured and uninjured peripheral nerves, uninjured adjacent DRG and the spinal cord was closely associated with the development of neuropathic pain.

Physiological relevance of AM bioactivity depends on AM receptor. AM receptor is the association of CLR with RAMP [11]. CLR actually forms the basis of the receptors for CGRP and AM. CGRP and AM bind to the same receptor CLR with receptor specificity being determined by RAMP. Three different RAMP that have been found in human tissue are RAMP1, RAMP2 and RAMP3. Coexpression of RAMP1 and CLR reveals CGRP receptor whereas coexpression of RAMP2 or RAMP3 and CLR forms AM1 or AM2 receptor [33]. As RAMP2 is a component of AM1 receptor and is not expressed in AM2 or CGRP receptor, we examined the expression of RAMP2. RAMP2 has been found to be expressed in DRG [12]. The current study demonstrated that RAMP2 expression was increased in both medium- and small-diameter neurons on days 1–15 in L5 DRG. Interestingly, RAMP2 was also increased even in large-sized neurons on days 3 and 7. However, RAMP2 mRNA was increased only on day 3. For RAMP2, different expression patterns from AM was happened in L5 DRG which is possible that maybe no matter increases in AM or its receptors, it will produce enhanced cytological

effect. These alternating changes act together to cause neuropathic pain. In L4 DRG, RAMP2 protein was increased in medium- and small-diameter neurons on days 1–15 and in large-sized neurons on days 3 and 7. RAMP2 mRNA was increased on days 1–7 which was longer than that in L5 DRG. These results suggest that SNL promoted ongoing post-translational changes of RAMP2 in DRG containing injured and uninjured neurons but transcriptional activation of the RAMP2 gene mainly in the adjacent DRG containing complete neurons. The changes of RAMP2 exhibited a similar profile with AM. Based on the notion that the increase impulses are ascribed to neurons in the adjacent uninjured DRG [26], studies to determine the involvement of the increased AM/RAMP2 expressions in adjacent uninjured DRG in hyperexcitability of neurons are warranted.

Peripheral nerve injury increases the excitability of DRG neurons, which AM must be involved in this process. Our results showed that nNOS protein was increased in DRG whether in the early or late stage of pathological pain following SNL. This is similar to previous reports [34–36]. Innovatively, single administration of AM_{22-52} produced an immediate attenuation the expression of nNOS protein, suggesting that AM receptor signaling can recruit nNOS. Nitric oxide which is produced by L-arginine catalyzed by nitric oxide synthase, is a small, highly soluble and diffusible free radical, which is an important signal molecule between nerve cells. As a neuronal synthase, nNOS has been proved to play a role in neuropathic pain by generous studies [37,38]. In peripheral nerve injury, NMDAR [39,40] is activated in advance to promote the opening of Ca^{2+} channel [41], and then activate nNOS to synthesize NO. The increase of NO synthesis and release stimulates guanylate cyclase to form cyclic guanosine monophosphate (cGMP) [42], which further activates inflammatory factors, cytokines [43,44], PKG signal pathway [45] and ion channels in cells, induces the enhancement of excitability of DRG neurons and participates in peripheral sensitization. AM induces the upregulation of nNOS via the activation of the cAMP/PKA signaling pathway directly [15]. It's also possible that AM recruit nNOS through increasing the expression of transient receptor potential vanilloid 1 (TRPV1) [46] or BDNF [47] or activating satellite glial cells to release inflammatory factors such as IL-1β or by ERK signaling pathway [16].

The increase of AM and RAMP2 may be a cause or consequence of neuropathic pain. In consistence with previous reports [24,48,49], our results showed that CGRP mRNA levels were upregulated in the spinal dorsal horn following SNL. Importantly, the chronic administration of AM_{22-52} completely inhibited the expression of CGRP mRNA, suggesting that AM receptor signaling can recruit CGRP. AM can stimulate CGRP synthesis through an autocrine or paracrine mechanism [10,14]. Recruiting of CGRP by increased AM activity has been found in inflammatory pain [14,50] and morphine-induced pain hypersensitivity [12,50]. The increased CGRP activity in the spinal cord plays a pivotal role in pain hypersensitivity induced by peripheral [51,52] or central nerve damage [53]. These results suggest that increased AM bioactivity played a role in maintenance of neuropathic pain and the underlying mechanisms involved the mediation of CGRP.

5. Conclusions

Collectively, our results clearly demonstrate that the expression of AM dynamical changes with the progression of neuropathic pain and that blockade of AM receptor is an effective pharmacological intervention to inhibit mechanical allodynia in late-phase development of neuropathic pain. Furthermore, targeting AM can even abolish the increase of the important nociceptive mediator nNOS and CGRP. The present study suggests that AM receptor should be considered as a new therapeutic target for relieving chronic neuropathic pain.

Author Contributions

DW designed the study; CW, YX and QL performed the research; YX and QL analyzed the data; YS and WT provided technical assistance and produced graphs; CW and DW wrote and revised the manuscript. All authors read and approved the final manuscrip.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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