Chronic nerve injury-induced Mas receptor expression in dorsal root ganglion neurons alleviates neuropathic pain

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Abstract. Neuropathic pain, which is characterized by hyperalgesia, allodynia and spontaneous pain, is one of the most painful symptoms that can be experienced in the clinic. It often occurs as a result of injury to the peripheral nerves, dorsal root ganglion (DRG), spinal cord or brain. The renin-angiotensin system (RAS) plays an important role in nociception. As an essential component of the RAS, the angiotensin (Ang)-(1-7)/Mas axis may be involved in anti-nociception. The aim of the present study was to explore the expression pattern of Mas in DRG neurons following chronic nerve injury and examine the effects of Mas inhibition and activation on neuropathic pain in a chronic constriction injury (CCI) rat model. The results showed, that compared with the sham group, CCI caused a time-dependent induction of Mas expression at both the mRNA and the protein levels in DRG neurons. Consistent with the results, isolated DRG neurons showed a time-dependent increase in Ang-(1-7) binding on the cell membrane following the CCI surgery, but not the sham surgery. Compared with the sham control groups, CCI significantly decreased the paw withdrawal latency and threshold, and this was markedly improved and aggravated by intrathecal injection of the selective Mas agonist Ang-(1-7) and the selective Mas inhibitor D-Pro7-Ang-(1-7), respectively. In conclusion, this study has provided the first evidence, to the best of our knowledge, that the Mas expression in DRG neurons is time-dependently induced by chronic nerve injury and that the intrathecal activation and inhibition of Mas can improve and aggravate CCI-induced neuropathic pain, respectively. This study has provided novel insights into the pathophysiological process of neuropathic pain and suggests that the Ang-(1-7)/Mas axis could be an effective therapeutic target for neuropathic pain, warranting further study.

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Introduction

Neuropathic pain can arise as a consequence of a lesion or disease affecting the somatosensory system (1). Symptoms may include hypersensitivity to noxious (hyperalgesia) and non-noxious (allodynia) stimuli, as well as spontaneous pain (2). An estimated 7-8% of the general population suffers from mild to moderate forms of neuropathic pain, and 5% may be severely affected by it (3). Such pain is a major health problem that substantially reduces quality of life for the afflicted individuals and poses a significant economic burden to the health system and society (2). Understanding the patho-physiological process of neuropathic pain and the underlying molecular mechanisms will facilitate the development of novel therapies for neuropathic pain. Among the several widely used experimental animal models for neuropathic pain, chronic constriction injury (CCI) of the sciatic nerve is one of the most popular (4).

Angiotensin II (Ang II) is a principle component of the renin-angiotensin system (RAS), which has a pivotal role in the regulation of blood pressure and fluid homeostasis in mammals (5). Numerous studies have demonstrated that Ang II, which interacts with the autonomic nervous system, is involved in the central and peripheral regulation of sensory information (6,7). In several rodent pain models, the intracere-broventricular injection of Ang II has been shown to exert antinociceptive effects (6,8). These findings indicate that the Ang II/Ang II type 1 (AT1) receptor axis has a key function in nociception.

In addition to the angiotensin-converting enzyme (ACE)/Ang II/AT1 receptor axis, the RAS involves a counter-regulatory axis, in which ACE2, Ang-(1-7) and the Mas receptor play a role (9). Ang-(1-7) performs a wide range of actions, several of which counteract the actions of Ang II, and is recognized as a key component of the RAS (10). The direct generation of Ang-(1-7) can occur with high efficiency through the action of ACE2 on Ang II (10-12), as well as through the action of neutral endopeptidase and prolyl endopeptidase on Ang I (13,14). It is well established that the G protein-coupled receptor Mas has a functional binding site for Ang-(1-7) (15,16), and previous studies have suggested that the actions of Ang-(1-7) may be mediated by an interaction with Mas in the central nervous system (17,18). The findings that Mas is localized in the rat dorsal root ganglion (DRG) and

that Ang-(1-7) produces a peripheral antinociceptive effect in rats through Mas have been previously described (19). Thus, as an essential component of the RAS, the Ang-(1-7)/Mas axis may play an important role in antinociception.

The aim of the present study was to explore for the first time, to the best of our knowledge, the expression pattern of Mas in DRG neurons following chronic nerve injury and to examine the effects of Mas inhibition and activation on neuropathic pain in a CCI rat model.

Materials and methods

Animals and reagents. Male inbred Sprague Dawley rats (weight, 250-300 g) were purchased from Central South University (Changsha, China) and were housed at the Xi'an Jiaotong University School of Medicine BioResources Center (Xi'an, China). Animals were placed in a quiet, temperature- (22±2˚C) and humidity- (60±6%) controlled room with a 12-h light/dark cycle (light beginning at 8:00 a.m.), and all tests were performed during the light phase of the cycle. ^{125}I-Sodium iodide (carrier free, 100 mCl/ml) was purchased from Amersham Biosciences (Piscataway, NJ, USA). Ang-(1-7) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). D-Pro7-Ang-(1-7) was purchased from the American Peptide Company (Sunnyvale, CA, USA). Mouse monoclonal anti-MAS1 (G-1) antibody (cat. no. sc-390453) was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). TRIzol® reagent and the SYBR® Green Master Mix were purchased from Invitrogen (Life Technologies, Carlsbad, CA, USA) and Applied Biosystems (Foster City, CA, USA), respectively.

Establishment of the CCI rat model and treatments. The rat CCI model was established as previously described (20). Briefly, each animal was anesthetized via an intraperitoneal injection of sodium pentobarbital (60 mg/kg). The common sciatic nerve of the animal was exposed and freed from adherent tissue at the mid-thigh level by using blunt dissection to separate the biceps femoris muscles. Four loose ligatures were placed 1 mm apart, using chromic gut suture (4-0 absorbable suture; Jorgensen Laboratories, Inc., Loveland, CO, USA). The animals were randomly assigned to one of six groups (n=20 per group): Sham + Saline (animals subject to sham surgery plus intrathecal injection of 20 μl saline every 3 days from 3 days before surgery), Sham + Mas receptor inhibitor (Mas-I) [animals subject to sham surgery plus intrathecal injection of 20 μl 10 nM Mas-I D-Pro7-Ang-(1-7) every 3 days from 3 days before surgery], Sham + Ang-(1-7) [animals subject to sham surgery plus intrathecal injection of 20 μl 120 pM Mas agonist Ang-(1-7) every 3 days from 3 days before surgery], CCI + Saline (animals subject to CCI surgery plus intrathecal injection of 20 μl saline every 3 days from 3 days before surgery), CCI + Mas-I [animals subject to CCI surgery plus intrathecal injection of 20 μl 10 μM D-Pro7-Ang-(1-7) every 3 days from 3 days before surgery], CCI + Ang-(1-7) [animals subject to CCI surgery plus intrathecal injection of 20 μl 120 pM Ang-(1-7) every 3 days from 3 days before surgery]. All animals were sacrificed within 13 days after the surgery. This study was conducted in accordance with the Xi'an Jiaotong University School of Medicine guidelines on the use of live animals for research, and the experimental protocol was approved by the Laboratory Animal Users Committee at Xi'an Jiaotong University School of Medicine.

Behavior tests. Thermal hyperalgesia was assessed in accordance with a previously described method (21) using a plantar analgesia instrument (Stoelting Co., Wood Dale, IL, USA) every day from 3 days before to 13 days after surgery. The intensity of the radiant infrared heat source stimulus was set to IR50 and the cut-off time was set at 15 sec. Prior to each testing session, the rats were placed on a glass platform and left to habituate to the surroundings for ≥15 min. The thermal stimulus was applied to the plantar surface of the paw. The thermal threshold was defined as the latency (sec) to the first sign of pain behavior. Signs of pain behavior included paw withdrawal, flinching, biting and/or licking of the stimulated paw. To assess mechanical allodynia, von Frey monofilaments (Stoelting Co.) with a range of stiffness levels (2.0-15.0 g) were used every day from 3 days before to 13 days after surgery. Prior to each testing session, the rats were placed on a metallic platform and left to habituate to the surroundings for ≥15 min. The stimulus strength was sequentially increased and/or decreased to determine the paw withdrawal threshold response.

DRG neuron cell isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). On days 1, 3, 7 and 13 after the CCI surgery, 5 randomly selected rats were sacrificed at each time-point, and DRG neurons were isolated from the enlarged section of the lumbar spinal cord in accordance with a previously described method (22). Cells were used for experiments 24-48 h after isolation. RNA was prepared using TRIzol reagent and then purified using the Turbo DNA-free™ system (Ambion, Austin, TX, USA). The cDNAs were synthesized using SuperScript™ II reverse transcriptase (Invitrogen, Life Technologies). qPCR was performed using the LightCycler® thermal cycler system (Roche Diagnostics, Indianapolis, IN, USA) and a SYBR Green I kit, following the manufacturer's instructions. The PCR cycling conditions were as follows: 20 sec at 95˚C, followed by 40 cycles of 3 sec at 95˚C and 30 sec at 60˚C. The results were normalized against those of the reference gene GAPDH in the same sample. The primers used (Baolong Oligos, Beijing, China) were as follows: Rat Mas forward, 5'-GACCAGCCCACAGTTACCAGTT-3' and reverse, 5'-CCAGGGTTCCCCTTCTGACT-3'; rat GAPDH forward, 5'-TGGTCTACATGTTCCAGTATGACT-3' and reverse, 5'-CCATTTGATGTTAGCGGGATCTC-3'. Each experiment was performed in duplicate and repeated three times.

Western blot analysis. DRG neurons were incubated at 95˚C for 10 min following lysis in 250 μl 2X sodium dodecyl sulfate (SDS) loading buffer (62.5 mM TrisHCl, pH 6.8, 2% SDS, 25% glycerol, 0.01% bromphenol blue and 5% 2-mercaptoethanol). Equal quantities of lysates were loaded onto 10% SDS-polyacrylamide gels, and proteins were then blotted onto a microporous polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). The membranes were incubated for 1 h with anti-MAS1 antibody (Santa Cruz Biotechnology, Inc.) at a 1:1,000 dilution and then washed and incubated with a

horseradish peroxidase-conjugated secondary antibody for 1 h (1:5,000; Santa Cruz Biotechnology, Inc.). Peroxidase was revealed with an enhanced chemiluminescence kit from GE Healthcare (Shanghai, China).

[^{125}I]Ang-(1-7) binding assay. Isolated rat DRG neurons were rinsed twice with Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA) and equilibrated on ice with incubation buffer (DMEM containing 0.2% bovine serum albumin and a protease inhibitor cocktail, pH 7.4) for 30 min. The plates were then incubated at 4˚C for 60 min with incubation buffer containing 0.5 nmol/l ^{125}I-Ang-(1-7), labeled as described in a previous study (23). Incubation was terminated by rinsing the cells three times with ice-cold phosphate-buffered saline. Cell solubilization was achieved through incubation with 0.1 mol/l NaOH for 60 min, and the radioactivity was then measured. Nonspecific binding was determined in the presence of 10 μmol/l unlabeled Ang-(1-7) and found to be ≤15%. Specific binding was calculated by subtracting the nonspecific binding from the total binding. The disintegration per minute data were normalized against cell number (per 10,000 cells). Each experiment was performed in duplicate and repeated three times.

Statistical analysis. Statistical analyses were performed using SPSS for Windows 10.0 (SPSS Inc., Chicago, IL, USA). Data values are expressed as the mean ± standard deviation. Comparisons of means among multiple groups were conducted using one-way analysis of variance followed by post hoc pairwise comparisons using Tukey's tests. A two-tailed P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of Mas and Ang-(1-7) binding. As shown in Fig. 1, compared with the sham group, CCI time-dependently increased the Mas mRNA level in the DRG neurons. Western blot analyses confirmed that CCI time-dependently increased the Mas protein level in the DRG neurons compared with the sham group (Fig. 2). Consistent with these results, isolated DRG neurons showed a time-dependent increase in Ang-(1-7) binding on the cell membrane following the CCI surgery, but not the sham surgery (Fig. 3). In combination, the results suggest that CCI can significantly increase the density of Mas-ligand binding on the cell membrane of DRG neurons by inducing the expression of Mas at the mRNA level.

Hyperalgesia and allodynia tests. To explore the functional significance of CCI-induced Mas expression in DRG neurons, thermal hyperalgesia and mechanical allodynia tests were next performed in rats treated with the selective Mas agonist Ang-(1-7) (200 pM) or selective Mas inhibitor D-Pro7-Ang-(1-7) (10 nM). As shown in Fig. 4, no significant group differences were found in the paw withdrawal latency or threshold among the animals receiving sham surgery plus saline, Ang-(1-7) or D-Pro7-Ang-(1-7). Compared with the sham control groups, CCI significantly decreased the paw withdrawal latency and threshold, and this effect was markedly improved and aggravated by intrathecal injection of Ang-(1-7) and D-Pro7-Ang-(1-7), respectively. Intrathecal injection of 20 μl Ang-(1-7) at 200 pM or D-Pro7-Ang-(1-7) at 10 nM did not cause any noticeable side effects in the rats.

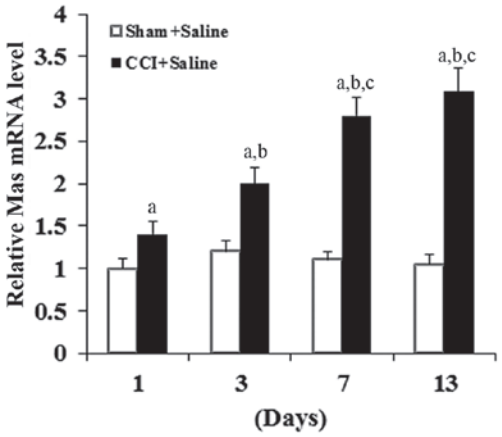

Figure 1. Mas receptor mRNA level in DRG neurons of rats with or without CCI of the sciatic nerve. DRG neurons were isolated from rats with (CCI + Saline) or without (Sham + Saline) CCI of the sciatic nerve. The Mas receptor mRNA level in the DRG neurons was determined using a reverse transcription-quantitative polymerase chain reaction on days 1, 3, 7 and 13 after sham or CCI surgery and expressed as a fold change relative to that of the Sham + Saline group on day 1 (designated as 1). Data values are expressed as the mean + standard deviation; n=5 in each group at each time-point. [a]P<0.05 vs. Sham + Saline; [b]P<0.05 vs. CCI + Saline on day 1; [c]P<0.05 vs. CCI + Saline on day 3. DRG, dorsal root ganglion; CCI, chronic constriction injury.

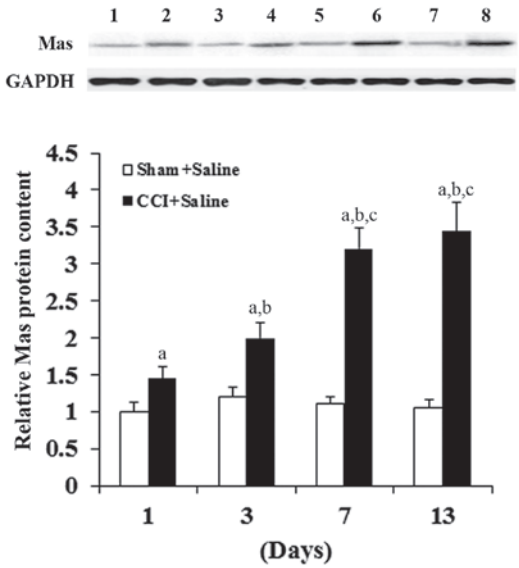

Figure 2. Mas receptor protein level in DRG neurons of rats with or without CCI. The expression of Mas receptor protein in the rat DRG neurons was determined using western blot analyses on days 1, 3, 7 and 13 after sham or CCI surgery. Lanes 1, 3, 5, 7 represent the Sham + Saline group on days 1, 3, 7 and 13, respectively; lanes 2, 4, 6 and 8 represent the CCI + Saline group on days 1, 3, 7 and 13, respectively. GAPDH blotting was used as a loading control. Protein blots were measured densitometrically. The density of the Mas receptor blot was normalized against that of GAPDH to obtain a relative blot density, which was expressed as a fold change relative to that of the Sham + Saline group on day 1 (designated as 1). Three independent experiments were performed for each western blot analysis. Data values are expressed as the mean + standard deviation; n=5 in each group at each time-point. [a]P<0.05 vs. Sham + Saline; [b]P<0.05 vs. CCI + Saline on day 1; [c]P<0.05 vs. CCI + Saline on day 3. DRG, dorsal root ganglion; CCI, chronic constriction injury.

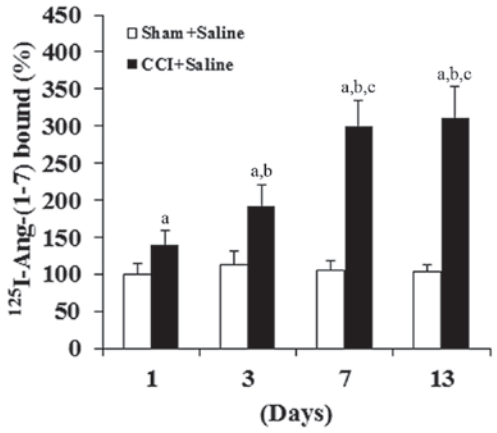

Figure 3. Saturation binding assay of Mas receptor on the cell membrane of DRG neurons of rats with or without CCI. DRG neurons were isolated from rats on days 1, 3, 7 and 13 after sham or CCI surgery. Saturation binding assays were conducted using 0.5 nmol/l ^{125}I-Ang-(1-7) on cell membranes. A single-site receptor binding model provided the best fit for data analysis. The disintegrations per minute data were normalized against cell number (per 10,000 cells) and shown as a percentage of that of the Sham + Saline group on day 1 (designated as 100%). Data values are expressed as the mean + standard deviation; n=5 in each group at each time-point. ^{a}P<0.05 vs. Sham + Saline; ^{b}P<0.05 vs. CCI + Saline on day 1; ^{c}P<0.05 vs. CCI + Saline on day 3. DRG, dorsal root ganglion; CCI, chronic constriction injury; ^{125}I-Ang-(1-7), [^{125}I]-angiotensin-(1-7).

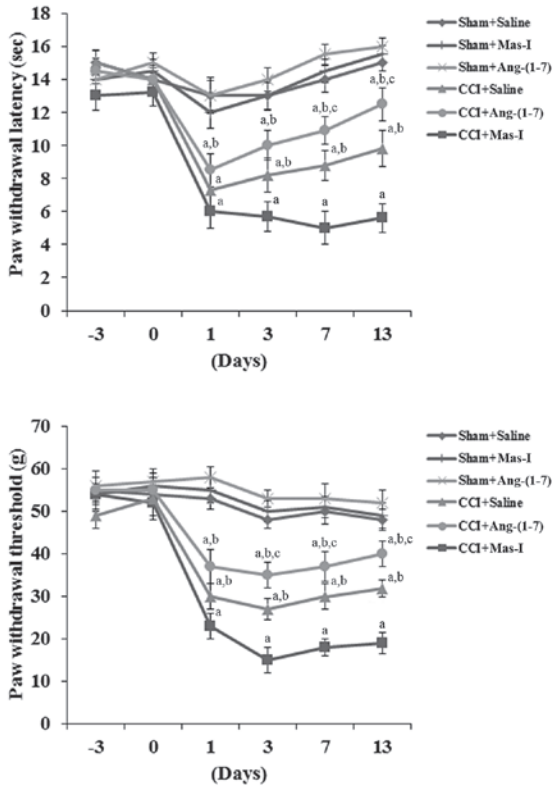

Figure 4. Thermal hyperalgesia and mechanical allodynia in rats with or without CCI. Thermal hyperalgesia and mechanical allodynia were measured in rats 3 days before surgery (day -3) and on days 1, 3, 7 and 13 after sham or CCI surgery. In the Sham + Saline group, rats received sham surgery plus an intrathecal injection of 20 μl saline every 3 days from day -3; in the Sham + Mas-I group, rats received sham surgery plus intrathecal injection of 20 μl 10 nM Mas-I D-Pro7-Ang-(1-7) every 3 days from day -3; in the Sham + Ang-(1-7) group, rats received sham surgery plus intrathecal injection of 20 μl 200 pM Mas agonist Ang-(1-7) every 3 days from day -3; in the CCI + Saline group, rats received CCI surgery plus intrathecal injection of 20 μl saline every 3 days from day -3; in the CCI + Mas-I group, rats received CCI surgery plus intrathecal injection of 20 μl 10 μM D-Pro7-Ang-(1-7) every 3 days from day -3; in the CCI + Ang-(1-7) group, rats received CCI surgery plus intrathecal injection of 20 μl 200 pM Ang-(1-7) every 3 days from day -3. Data values are expressed as the mean + standard deviation; n=5 in each group at each time-point. ^{a}P<0.05 vs. Sham + Saline, Sham + Mas-I and Sham + Ang-(1-7); ^{b}P<0.05 vs. CCI + Mas-I; ^{c}P<0.05 vs. CCI + Saline. CCI, chronic constriction injury; Mas-I, Mas receptor inhibitor; Ang-(1-7), angiotensin-(1-7).

Discussion

Neuropathic pain, characterized by hyperalgesia, allodynia, and spontaneous pain, is one of the most painful symptoms that can be experienced in the clinic (24) and often occurs as a result of injury to the peripheral nerves, DRG, spinal cord or brain (24). The RAS is considered to have an important role in nociception (5-8), and Ang-(1-7) is a biologically active member of the RAS (25). The physiological role of Ang-(1-7) has been firmly established by two discoveries: i) Identification of the ability of ACE2, an enzyme that generates Ang-(1-7) from Ang I or Ang II (25); ii) characterization of the G protein-coupled receptor Mas as a receptor that is associated with several actions of Ang-(1-7) (26). Costa *et al* (19) demonstrated that Mas is expressed in rat DRG neurons and showed, through the subcutaneous injection of prostaglandin E_2 and Ang-(1-7) into the rat's hind paw, that Ang-(1-7) produced a peripheral antinociceptive effect in rats via Mas (19). In the present study, a rat CCI model was employed to provide the first evidence, to the best of our knowledge, that the Mas expression in DRG neurons is time-dependently induced by chronic nerve injury. Furthermore, it was found that intrathecal activation and inhibition of Mas could improve and aggravate CCI-induced neuropathic pain, respectively.

The present results showed that Mas expression at both the mRNA and the protein level was time-dependently increased in DRG neurons following CCI, but not the sham surgery, suggesting that chronic nerve injury can induce Mas expression in DRG neurons at the mRNA level. The increased Mas expression led to an increased density of Mas-ligand binding on the cell membrane of DRG neurons, suggesting the functional significance of this phenomenon. This was confirmed by behavioral tests, which showed that the intrathecal injection of the Mas-I/antagonist D-Pro7-Ang-(1-7) markedly aggravated thermal hyperalgesia and mechanical allodynia in CCI rats, while the intrathecal injection of the Mas agonist Ang-(1-7) significantly improved thermal hyperalgesia and mechanical allodynia in the CCI rats. Taking into account the relatively low concentrations of D-Pro7-Ang-(1-7) (10 nM) and Ang-(1-7) (200 pM) used and the marked effects observed, the Ang-(1-7)/Mas axis could be an effective therapeutic target for neuropathic pain, warranting further study. This is particularly important since neuropathic pain, particularly the nerve-injured neuropathy, is opioid resistant (24).

Notably, the inhibition or activation of Mas in rats with sham surgery did not cause any significant differences in thermal hyperalgesia and mechanical allodynia, which may have been due to the fact that the Ang-(1-7)/Mas axis in the DRG neurons was only activated following pathophysiological changes ensuing from chronic nerve injury. This theory is

supported by the present finding that the Mas expression was time-dependently increased only following the CCI, but not the sham surgery, which also suggests that the increased Mas expression is a compensatory mechanism to reduce chronic nerve injury-induced neuropathic pain. Further studies are required to elucidate the underlying molecular mechanisms. There are several experimental animal models for neuropathic pain (24); since only the CCI model was employed in this study, an examination of the effects of Mas inhibition and activation on neuropathic pain in other neuropathic pain models would be of particular interest in future studies.

In conclusion, the present study has demonstrated that Mas expression in DRG neurons is time-dependently induced by chronic nerve injury; intrathecal activation and inhibition of Mas can improve and aggravate CCI-induced neuropathic pain, respectively. This study provides novel insights into the pathophysiological process of neuropathic pain and suggests that the Ang-(1-7)/Mas axis could be an effective therapeutic target for neuropathic pain.

Acknowledgements

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