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Persistent Nociception Induces Anxiety-like Behavior in Rodents: Role of Endogenous Neuropeptide S

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Abstract

Anxiety disorder is a comorbid condition of chronic pain. Analgesics and anxiolytics, subject to addiction and abuse, are currently used to manage pain and anxiety symptoms. However, the cellular mechanism underlying chronic pain and anxiety interaction remains to be elucidated. We report that persistent nociception following peripheral nerve injury induced anxiety-like behavior in rodents. Brain expression and release of neuropeptide S (NPS), a proposed endogenous anxiolytic peptide, was diminished in rodents with co-existing nociceptive and anxiety-like behaviors. Intracerebroventricular administration of exogenous NPS concurrently improved both nociceptive and anxiety-like behaviors. At the cellular level, NPS enhanced intra-amygdaloidal inhibitory transmission by increasing presynaptic GABA release from interneurons. These findings indicate that the interaction between nociceptive and anxiety-like behaviors in rodents may be regulated by the altered NPS-mediated intra-amygdaloidal GABAergic inhibition. The data suggest that enhancing the brain NPS function may be a new strategy to manage comorbid pain and anxiety.

Keywords

Chronic pain; Anxiety; Neuropeptide S; NPS; Amygdala; GABA

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1. Introduction

Epidemiological studies have shown that pain and anxiety disorders are closely linked in the clinical setting [4; 19; 54], showing a reciprocal, comorbid relationship in longitudinal (> 3 years) studies [2]. The prevalence of anxiety disorders in patients with chronic pain ranges from 20 to 51% [19; 28; 32; 48], which is much higher than that of 10 to 15% in the general population [20; 26]. Compared to patients with acute pain (< 3 months), those with chronic pain (>1 year) have significantly higher anxiety scores and less pain reduction at a one-year follow-up [40]. Moreover, improving clinical anxiety is accompanied by a parallel decrease in pain intensity [51]. Despite this well-documented clinical association between pain and anxiety, the underlying mechanism of their comorbid interaction remains to be elucidated. Currently, analgesics and anxiolytics, subject to addiction and abuse, remain the mainstay in managing symptoms of both conditions.

Recently, 20-amino acid neuropeptide S (NPS) has been shown to be a prominent endogenous anxiolytic in rodents [24; 60] without affecting depressive behavior [58]. A study in naive rodents suggests that NPS mediates anxiolytic effects through a GABAergic mechanism in the amygdala [25]. NPS binds to its cognate receptor NPS receptor (NPSR) [46] to modulate anxiety, arousal, memory and immune response. Abnormal NPS-NPSR signaling caused by NPSR gene mutation or polymorphism has been associated with pathological conditions including panic disorder [14; 41], catastrophizing [45], asthma [6], and rheumatoid arthritis [1] in human subjects. Another study in mice revealed the antinociceptive effect of NPS on inflammatory pain [44]. The amygdala has been implicated in the integration of pain and mood disorders [3; 8; 12; 61]. NPS-responsive neurons express NPSR and are concentrated in the amygdala [11; 59]. Moreover, GABAergic transmission regulates both anxiety [17; 34; 39] and pain [36].

We hypothesize that NPS-mediated GABA transmission in amygdala may play a role in the interaction between pain and anxiety. Here, we tested the hypotheses that 1) persistent nociception secondary to chronic constriction sciatic nerve injury (CCI) would induce anxiety-like behavior as well as a diminished endogenous brain NPS level in rodents; 2) exogenous NPS would concurrently improve nociceptive and anxiety-like behaviors; and 3) exogenous NPS would enhance GABAergic inhibitory transmission in the amygdala.

2. Materials and Methods

2.1. Experimental animals

Adult male Sprague – Dawley rats (18–28 days old; for patch-clamp recording) weighing 280–300 g were purchased from Charles River Laboratories. Animal rooms were artificially lit (7 AM to 7 PM) and water and food pellets were available *ad libitum*. The experimental protocols were approved by the Massachusetts General Hospital Institutional Animal Care and Use Committee.

2.2. Surgical procedures

CCI—Rats were anesthetized with pentobarbital (50 mg/kg, i.p.). CCI was produced by placing four ligatures around the left side of the sciatic nerve [5; 7; 30]

Sham operation—Animals in sham groups underwent the same procedure with the exception of nerve ligation.

Implantation of a guide cannula for microinjection—A guide cannula (Plastics One, Roanoke, VA) was implanted in rats for intracerebroventricular microinjection. The coordinates was -1.5 mm lateral and 1.0 mm posterior from Bregma with 4.0 mm depth. The cannula location was confirmed histologically at the end of experiment. NPS was purchased from Sigma (Cat. N2412, St Louis, MO, USA) and dissolved in sterile PBS. Intracerebroventricular injection ($20\ \mu\text{L}$, over 3–5 min) was made using a microsyringe.

2.3. Behavioral tests

All behavioral experiments were carried out with the investigators blind to treatment conditions. Animals were habituated to the test environment for two consecutive days (30 minutes per day) before baseline testing.

Mechanical allodynia—A von Frey filament was perpendicularly applied to the plantar surface of each hind paw using an up-and-down approach. A threshold force of response (in grams) was defined as the first filament that evoked at least 40% of response (two withdrawals out of five applications) [52].

Thermal hyperalgesia—Response to radiant heat was assessed using the foot-withdrawal test [21]. Each animal underwent three trials and their results were averaged to yield mean withdrawal latencies. The cutoff was set at 20 seconds to avoid tissue damage.

Elevated plus maze test—Rats were placed at the junction of four arms in the maze (Stoeling). The initial position of a rat was always facing an open arm. The number of entries, the duration of stay in each arm, and the locomotor activity were recorded by both a video-tracking system [62] and an observer for 5 min [60]. The test was only performed once on each rat.

Open field test—This was carried out using a Plexiglas square box (57×57 cm) with 50-cm high walls. Behaviors were observed for six minutes under dim lighting, including a habituation period of one minute. The number of squares crossed and the time spent in the centre area by a rat were recorded for five minutes.

Marble burying test—The bottom of each cage was covered in a 5-cm layer of sawdust bedding containing evenly spaced 30 glass marbles. An animal was then placed in each cage and left for 30 min. The number of buried marbles after this 30-min period was counted [60].

2.4. Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed according to our published protocol [57]. NPS ELISA kit was purchased from CEDARLANE Laboratories USA Inc (All assays were made in triplicates). *In vivo* microdialysates from the contralateral amygdala were harvested on sham and day 14

after CCI according to a previously reported protocol, including collection of microdialysates (one hour) after a basal perfusion of artificial CSF for two hours [16].

2.5. Patch-clamp recording

Rats (18 to 28 days old) were anesthetized with pentobarbital (50 mg/kg, i.p.) and decapitated. The brain was quickly removed and the amygdala was transferred into a chilled oxygenated cutting solution containing (in mM) 234 sucrose, 11 glucose, 24 NaHCO₃, 2.5 KCl, 1.25 NaH₂PO₄, 10 MgSO₄, and 0.5 CaCl₂, and gassed with 95% O₂/5% CO₂ at 5°C. Amygdala slices (300 µm) were cut using a vibratome (Model 1000, Vibratome Company, St. Louis, USA) and then incubated in standard artificial cerebrospinal fluid (ACSF) bubbled with 95% O₂/5% CO₂ to a final pH of 7.3. Brain slices were placed into warm (34 ± 0.5°C) bubbled cutting solution for 30 min, then transferred to room temperature (22°C) for an additional 30 min before being placed into a submersion recording chamber containing ACSF. Recordings were performed in a whole-cell mode using a patch-clamp amplifier (MultiClamp700B) under an infrared video-microscopy (Olympus BX51W1).

To record sIPSCs or mIPSCs, patch-clamp electrodes (tip resistance = 3–5 MΩ) were filled with an intracellular solution containing (in mM) Cs-gluconate, 117; CsCl, 13; MgCl₂, 1; CaCl₂, 0.07; EGTA, 1; HEPES, 10; MgATP, 3, NaGTP, 0.5 (pH 7.2). mIPSCs were recorded with 1 µM TTX in the bath solution. When holding the cell at –70 mV to measure IPSCs, 20 µM NBQX and 50 µM L-AP5 were added into the bath solution.

NPS stock solution (4 mM) was made in PBS with 0.1% bovine serum albumin. SHA68 was dissolved in DMSO and applied prior to NPS (DMSO < 0.07 %). Bicuculline or TTX was dissolved in DMSO or 0.1% acetic acid, respectively. All agents and reagents were purchased from Tocris or Sigma-Aldrich.

The patch-clamp data was calculated using Clampfit 10.2 software. To fit an activation curve, currents from each cell at each holding potential were normalized to the peak current at the most hyperpolarized holding potential. The normalized data were averaged across cells and fit to Boltzmann functions using Origin 8.0. The effects of a drug treatment were analyzed using Student's t test. P<0.05 was considered to be statistically significant. Bar graphs were expressed as Mean ± SEM.

2.6. Real-time qRT-PCR

Total RNA was isolated from brain tissue using *TRIzol*®. cDNA was synthesized using Superscript III 1st Strand from Invitrogen. Real time qPCR was performed using SYBR Green (Invitrogen) on AB Biosystems 7500. The PCR protocol consisted of the following cycle steps: initial denaturation at 98°C 30 sec, 40 cycles including denaturation at 98°C 5 sec, 58°C 10 sec, 72°C 15 sec, final extension at 72°C 5 min. PCR primers were designed based on gene sequences from PubMed: rNPSf (3'-TGTCCGTGGTACACGTGATT-5'), rNPSr (3'-CTGGGCAGGTAAGTCAAGAA-5'); rNPSRf (3'-AAACTCGGTGCTGCTGTTCT-5'), rNPSRr (3'-CAGGCCTGTGAAGGAGTCTG-5'). GAPDH was used as internal control: GAPDHf (3'-AAGCTGGTCATCAATGGCAAAC-5') and GAPDhr (3'-

GAAGACGCCAGTAGACTCCACG-5'). The qPCR data was analyzed using AB Biosystems 7500 Software v.2.0.6.

2.9. Immunohistochemistry and Western blot

Immunostaining and Western blot were carried out as previously reported [31; 57] using the following primary and secondary antibodies: rabbit anti-NPSR antibody 1:500 (ab92425, Abcam), mouse anti-GAD67 antibody 1:1000 (ab26116, Abcam), Alexa 488-conjugated donkey anti-rabbit antibody or Cy3-conjugated donkey anti-mouse antibody (1:300; Jackson ImmunoResearch Laboratories Inc.), and HRP conjugated goat-anti rabbit antibody (Santa Cruz). Primary antibody omission was used as a control. Sections were examined and captured with an Olympus fluorescence microscope and digital camera.

2.8 Statistical analyses

Behavioral data were analyzed using two-way analysis of variance (ANOVA) repeated across time points and groups [31; 57]. *Post-hoc* Waller-Duncan K-ratio *t* test was performed to determine the source(s) of differences. Non-parametric Mann-Whitney U test was used to examine additional behavior test data including those from von Frey filament test, marble burying test, EMP, and open field test [31; 57]. One-way ANOVA was used to analyze the data from various assays (qRT-PCR, ELISA, Western blot). SPSS 12.0 software was used for statistical analyses. All data were expressed as Mean $\pm$ SEM and the statistically significant level was set at $P < 0.05$.

3. Results

3.1. Persistent nociception enhances anxiety-like behavior

Unilateral constriction sciatic nerve injury (CCI) in rats produced thermal hyperalgesia and mechanical allodynia in the ipsilateral hindpaw as compared to sham controls, which lasted during the entire experimental period. These CCI rats exhibited enhanced anxiety-like behavior as revealed by both elevated plus maze (EPM) test and open field test (OFT). As compared to sham controls, CCI rats made fewer entries to (Fig 1A–C, $P < 0.05$) and spent less time in (Fig 1D, $P < 0.05$) the open arms of an EMP apparatus. CCI did not affect locomotor activity of these rats in the EMP test (Fig 1E). In open field test, CCI rats were less explorative and spent less time in the center area as compared with sham controls (Fig. 2A–D $P < 0.05$). By day 14 of CCI, the injured rats showed distinctly enhanced anxiety-like behaviors in both EMP and open field tests. These results demonstrate that persistent nociception enhanced anxiety-like behavior in CCI rats.

3.2. Brain NPS expression is reduced in CCI rats

Neuropeptide S exerts anxiolytic effects by binding to its receptor NPSR. Recent studies have shown that NPS may be involved in both pain and anxiety [44]. Therefore, we examined whether the expression of NPS would be altered in the brain following CCI. In CCI rats with co-existing nociceptive and anxiety-like behaviors, their NPS mRNA level (qRT-PCR) in bilateral locus coeruleus, a primary source of endogenous NPS [10, 49], was reduced as compared to that of sham controls (Fig 3A, $P < 0.05$). Moreover, the NPS content (ELISA) in microdialysates collected from the contralateral amygdala, a primary site of NPS

function [10, 49], was reduced on day 14 as compared to sham rats (Fig 3B, $P < 0.05$), indicating a decrease in the intra-amygdaloidal NPS release. No significant changes in NPSR expression in the amygdala after CCI were observed by qRT-PCR, immunohistochemistry and western blot analysis (Fig 4A–D). Collectively, these results demonstrate a down-regulation of endogenous NPS in rats with co-existent nociceptive and anxiety-like behaviors.

3.3. Exogenous NPS improves both nociceptive and anxiety-like behaviors

Since endogenous NPS was down-regulated in CCI rats, we asked whether both conditions could be improved by intracerebroventricular administration of exogenous NPS (0.1, 1.0 nmol, or vehicle; once daily $\times$ 14 days, first dose on day 1). This NPS treatment (1.0 nmol $>$ 0.1 nmol=vehicle; $P < 0.05$) improved nociceptive (Fig 5A, B) as well as anxiety-like behavior (Fig 5C–F) in CCI rats when examined on day 14. NPS-treated rats (1.0 nmol) made more entries to the open arms in EMP test, exhibited more explorative activities in open field test. There was no statistical difference in marble burying test between NPS-treated and vehicle-treated rats ($P = 0.13$). The same NPS treatment (1.0 nmol) did not alter baseline nociceptive response (Fig 6A, B, $P > 0.05$) but improved baseline anxiety-like behavior in sham rats (Fig 6C, D). These results indicate that exogenous NPS currently improved both nociceptive and anxiety-like behaviors in the same CCI rats.

3.4. NPS enhances GABAergic transmission in the amygdala

The amygdala has been critically implicated in the integration of pain and mood disorders [12]. Therefore, it is possible that NPS may regulate intra-amygdaloidal GABAergic inhibitory transmission contributing to the interaction between nociceptive and anxiety-like behaviors. To test this hypothesis, synaptic responses to exogenous NPS were examined in both interneurons and pyramidal neurons located in the medial portion of amygdala (MeA) where NPSR mRNA was extensively expressed [59]. MeA interneurons were visualized as round multipolar cell bodies lacking an apical dendrite and with a characteristic adapting firing pattern (Fig 7A). We recorded both spontaneous and miniature inhibitory postsynaptic currents (sIPSCs and mIPSCs).

In the first experiment, intrinsic GABA-mediated currents (sIPSCs) were isolated when a recorded cell was held at -70 mV voltage clamp in the presence of NBQX (20 μ M) and L-AP5 (50 μ M) that blocked AMPA and NMDA receptors, respectively. Application of NPS (500 or 1,000 nM, but not 10, 50, or 100 nM or vehicle) increased both the frequency and amplitude of sIPSCs (Fig 7B–F, each $P < 0.05$, $n = 15$). This NPS effect was abolished by adding the NPSR antagonist SHA68 (Fig 7E, F, 100 μ M; $P < 0.05$, $n = 10$). These results indicate that NPS increased intra-amygdaloidal GABAergic transmission. To rule out a direct NPS effect on GABA receptor activity, we used 100 μ M GABA [9] to saturate GABA receptors in a brain slice. Under this condition, application of exogenous NPS showed no effect on exogenous GABA-induced post-synaptic currents ($P > 0.05$; $n = 6$) in MeA neurons (Fig 7G). Moreover, we found that NPS increased the frequency ($P < 0.05$, $n = 10$) but not amplitude of mIPSCs in MeA interneurons (at -70 mV) (Fig 8A–E).

In the second experiment, we measured intrinsic sIPSCs by holding a recorded cell at 0 mV (TTX in bath solution) that minimized glutamate-evoked currents in order to further confirm the NPS effect on presynaptic GABA release. Under this condition, application of NPS (500 nM) also increased sIPSCs (Fig 9A–E, each $P < 0.05$, $n = 15$). This NPS effect was abolished by adding the NPSR antagonist SHA68 (Fig 9D, E, 100 μM ; $P < 0.05$, $n = 10$). sIPSCs were also blocked by the GABA_A receptor antagonist bicuculline (Fig 9F). NPS (500 nM) also enhanced the frequency ($P < 0.05$; $n = 10$) and amplitude ($P < 0.001$, $n = 10$) of sIPSCs in MeA pyramidal neuron (Fig 10A–F). This NPS effect was also abolished by adding 100 μM SHA68 (Fig 10E, F). Consistent with the recording when a cell was held at -70 mV, NPS also increased the frequency ($P < 0.05$, $n = 10$) but not amplitude of mIPSCs in MeA interneurons when held at 0 mV (Fig 11A–E), indicating that NPS did not directly modulate GABA receptor activity. Finally, immunostaining revealed that NPSR and GAD67 (glutamate decarboxylase, a marker for GABAergic neuron) were co-localized in MeA neurons from which the patch-clamp recording was conducted (Fig 11F).

Collectively, these findings indicate that NPS enhanced intrinsic GABA-mediated sIPSCs, which is likely to be a presynaptic event induced by the increased GABA release.

4. Discussion

A recent clinical study found that 45% of chronic pain patients exhibited one or more anxiety disorder [28]. While the impact of depression on chronic pain is well recognized, much less is known regarding the comorbid interaction between anxiety and chronic pain [28]. Clinical studies have also shown that pre-operative anxiety can increase post-operative pain experiences [10; 35]. Despite the fact that anxiety disorder is a common comorbid condition of chronic pain, the underlying mechanism of their interaction remains unclear. Utilizing a rodent CCI model with nociception-induced anxiety-like behavior, we demonstrated that there was an interaction between nociceptive and anxiety-like behaviors in rodents, which was regulated by the endogenous NPS system possibly through NPS-mediated intra-amygdaloidal GABAergic inhibition (Fig 12).

There is an ongoing debate about the relationship between nerve injury and anxiety-like behaviors in mice. A recent study suggests that nerve injury induced persistent pain does not significantly alter “quality of life” in mice using a battery of tests including tests for anxiety behaviors [55]. However, several other studies have demonstrated that rats with persistent nociception also exhibit anxiety-like behavior [13; 22; 27; 49]. The data from this study also indicates that CCI rats with persistent nociception exhibited anxiety-like behaviors.

NPS, an endogenous anxiolytic neuropeptide, has been shown to play a unique role in regulating anxiety disorders [16; 24; 25; 37; 42; 60], although its tonic effect on anxiety-like behavior in rodents remains unclear [15; 18; 50]. We also found that NPS expression in the locus coeruleus and its release in the amygdala were reduced in rats with co-existing nociceptive and anxiety-like behaviors. Intracerebroventricular administration of NPS restored the endogenous NPS function and concurrently attenuated both nociceptive and anxiety-like behaviors. Although a transient effect of NPS on acute nociception has been reported [44], we did not find a tonic effect of NPS on baseline nociceptive threshold in

control rats without CCI although repeated administration of exogenous NPS appears to reduce anxiety-like behavior in sham rats. Collectively, our data indicate that a diminished NPS signaling in the brain critically regulated anxiety-like behavior, which may promote the transition to a chronic state of nociceptive behavior in rodents. Future studies need to examine whether NPS could modulate nociceptive behaviors when its administration is initiated after neuropathic pain is established. In agreement with studies by others [24; 60] our data show that NPS attenuates anxiety-like behavior in sham animals subjected to open field and elevated plus maze tests. We speculate that NPS may also alleviate anxiety-like behavior induced by other factors in addition to neuropathic pain. Further studies using animal models other than CCI, including chronic inflammatory pain and/or stress that could also induce anxiety behavior in rodents, may shed light on this issue.

NPS is an excitatory neuromodulator and its binding to NPSR produces a transient increase in the intracellular Ca^{2+} and cAMP level possibly via Gs and Gq proteins [47]. NPS and NPSR mRNA have distinctive brain expression profiles in rodents [11; 59]. NPS mRNA is highly expressed in the brainstem, whereas NPSR mRNA is widely expressed in many brain regions including the amygdala. Furthermore, there are distinct expression patterns for the precursor NPS and NPSR mRNA in the mouse and rat [11; 59]. For example, prominent NPSR mRNA expression is found in the basolateral amygdala (BLA) of mice, whereas scattered neurons in the medial anterior region of the amygdala displays only moderate levels of NPSR expression [59]. In the rat amygdala, however, the NPSR mRNA expression level is low in BLA but high in MeA [11]. An increasing number of studies have shown that MeA is involved in generalized anxiety disorder [23] and fear conditioning [56].

Consistent with the previous studies suggesting that the amygdala is a critical region involved in the integration of pain and anxiety [12; 43; 53], we found that NPS directly enhanced GABAergic activities in MeA interneurons resulting in the increased intra-amygdaloidal inhibitory transmission in rats. A possible mechanism of this NPS action is that NPS can enhance GABA release from interneurons. This notion is supported by both patch-clamp and cell culture data and would be consistent with an excitatory effect of NPS on GABAergic interneurons in the amygdala [25]. Thus, down-regulation of endogenous NPS in rodents with nociception-induced anxiety behavior could prolong the time course of nociceptive behavior, a possible pathway leading to the transition to chronic pain. Future studies need to understand intra-amygdaloidal connections between NPS-expressing GABAergic interneurons and projection neurons that participate in the limbic regulation of affective and emotional aspects of pain experiences.

NPS precursor mRNA is expressed primarily by neurons in the locus coeruleus and principal sensory trigeminal nuclei, whereas NPS-responsive neurons express NPSR and are concentrated in the limbic system such as the amygdala [11; 59]. Expression of NPS in peripheral neurons such as neurons in the dorsal root ganglion was not detected in the current study (unpublished data). Our findings of the decreased NPS expression and release in the brain of rats with chronic nociception support the notion that NPS is likely to modulate pain and anxiety at the supraspinal level. NPS, through modulation of synaptic activities in the amygdala, may serve as a molecular link between pain and anxiety.

Previous studies have shown that anxiolytics such as diazepam and midazolam decreased nociceptive behavior in rats [38; 49]. However, anxiolytic medications have the liability of physical dependence, addiction and abuse in clinical patients. Therefore, a new strategy based on enhancing the intrinsic NPS-mediated inhibitory mechanism could be advantageous over existing medications to treat certain comorbid chronic pain conditions. This may be accomplished by using NPS, small molecule NPSR agonist, and agents that may prevent down-regulation of endogenous NPS and its subsequent cellular functions. In this regard, intra-nasal NPS has been shown to reduce anxiety-like behavior in rodents [33] and intra-nasal delivery of neuropeptide-loaded nanoparticles appears to be a viable method at least in small animals [29]. These options may offer a new therapeutic approach to alleviating comorbid chronic pain and anxiety without resulting in significant adverse consequences.

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Summary

Persistent nociception following nerve injury induces anxiety-like behavior possibly mediated by the alteration of endogenous neuropeptide S.

Fig 1. Anxiety-like behavior in rats with persistent nociception in EPM

(A, B) A typical locomotion pattern, recorded by a video tracing program, in an elevated plus maze (EPM) test of a sham-operated rat (A) and a CCI rat (B) 14 days after surgery. (C, D) In EPM test, CCI rats made fewer entries to open arms (C) and spent less time in open arms (D). (E) CCI rats exhibited a similar locomotor activity as sham rats in EPM. Mean $\pm$ SEM, $n = 8-10$ /test day, * $P < 0.05$.

Fig 2. Anxiety-like behavior in rats with persistent nociception in OFT

(A, B) A typical locomotion pattern, recorded by a video tracing program, in open field test (OFT) of a sham-operated rat (A) and a CCI rat (B) 14 days after surgery. (C, D) In open field test, CCI rats crossed fewer squares (C) and spent less time in the centre area compared with sham rats. Mean $\pm$ SEM, $n = 8-10$ /test day, * $P < 0.05$.

Fig 3. Reduction of brain NPS expression after CCI

(A) The NPS mRNA level was reduced in the rat's locus coeruleus 14 days after CCI. (B) The NPS content in microdialysates from the ipsilateral amygdala was also reduced 14 days after CCI. Mean $\pm$ SEM, n = 8, *P < 0.05.

Fig 4. NPSR expression in the amygdala after CCI

(A) NPSR mRNA level remained unchanged in the rat amygdala 14 days after CCI. (B) Immunostaining of NPSR of the rat amygdala. Bar: 100 μ m. (C, D) Western blot analysis of NPSR protein (65 kDa) in the amygdala of sham and CCI rats on day 14. Mean $\pm$ SEM, n = 8, *P < 0.05.

Fig 5. Reduced nociceptive and anxiety-like behaviors by exogenous NPS

(A, B) Intracerebroventricular injection of NPS (1nmol > 0.1 nmol = vehicle; once daily $\times$ 14 d), beginning on day 1 of CCI, attenuated thermal hyperalgesia and mechanical allodynia in rats. (C) NPS-treated rats made more entries into open arms in the EPM test on day 14. (D–E) NPS-treated rats showed increased explorative activity and spent more time in the centre area in the open field test. (F) In the marble burying test, NPS-treated rats buried fewer marbles, but the difference was not statistically significant. Mean $\pm$ SEM, $n=8$, * $P<0.05$, compared to the vehicle (Veh) group.

Fig 6. Nociceptive and anxiety-like behaviors in sham rats treated by NPS
 (A, B) Intracerebroventricular injection of NPS (1nmol; once daily $\times$ 14 d), beginning on day 1 of sham operation, did not alter thermal hyperalgesia and mechanical allodynia in these rats when examined on day 14. (C, D) NPS-treated rats made more entries into open arms in EPM and showed increased explorative activities in OFT. Mean $\pm$ SEM, $n=8$. * $P<0.05$, compared to the vehicle (Veh) group.

Fig 7. Increased sIPSCs by NPS of MeA interneurons (at -70 mV)

(A) A typical FS (fast spiking) pattern was evoked by depolarizing current pulses (-300 and 300 pA, 600 ms). (B) Representative tracings of sIPSCs of an MeA interneuron held at -70 mV during control, application of NPS (500 nM) and SHA68 (100 μ M). (C, D) Cumulative distribution plots of sIPSCs in control and NPS-treated neurons. (E, F) NPS increased the frequency and amplitude of sIPSCs ($n=15$, $*p<0.05$). SHA68 reversed the effects of NPS. (G) Exogenous GABA-induced current was not affected by NPS (500 nM; control: 883 ± 59.8 pA; NPS: 880 ± 64.6 pA; $P>0.05$; $n=6$). NBQX (20 μ M) and L-AP5 (50 μ M) were included in the bath solution.

Fig 8. Increased mIPSC frequency of MeA interneurons (at -70 mV)

(A) Representative tracings of mIPSCs in a MeA interneuron during control, application of 500 nM NPS and 100 μ M SHA68. (C, D) Cumulative probability plots of the distribution of the intervals and peak amplitude of interneurons during control and NPS application. (E, F) Summary data showing that the frequency but not the amplitude of mIPSCs was increased during application of NPS ($P < 0.05$; $n = 5$) and this increase was reversed during bath application of 100 μ M SHA68. NBQX (20 μ M) and L-AP5 (50 μ M) were included in the bath solution.

Fig 9. Increased sIPSCs of MeA interneurons (at 0 mV)

(A) Representative tracings of sIPSCs of an interneuron held at 0 mV during control, application of 500 nM NPS and 100 μ M SHA68. (B, C) Cumulative distribution plots of sIPSCs in control and NPS-treated neurons. (D, E) NPS increased the frequency and amplitude of sIPSCs ($n=15$, $*p<0.05$). SHA68 reversed the effects of NPS. (F) Bicuculline completely inhibited sIPSCs. TTX (1 μ M) was included in the bath solution.

Fig 10. Increased sIPSCs by NPS of MeA pyramidal neurons (at 0 mV)

(A) A typical FS (fast spiking) pattern was evoked by depolarizing current pulses (−300 and 300 pA, 600 ms) in MeA pyramidal neurons identified as large cells with an apical dendrite extending toward the pial surface. (B) Representative tracings show sIPSCs in MeA pyramidal neurons held at 0 mV during control, application of NPS (500 nM) and bath application of SHA68 (100 μM). (C, D) Cumulative distribution plots of sIPSCs in control and NPS-treated neurons. (D, E) NPS increased the frequency and amplitude of sIPSCs ($n=10$, $*p<0.05$). SHA68 reversed the effects of NPS.

Fig 11. Increased mIPSC frequency of MeA interneurons (at 0 mV)

(A) Representative tracings of mIPSCs in a MeA interneuron during control, application of 500 nM NPS and 100 μ M SHA68. (B, C) Cumulative probability plots of the distribution the intervals and peak amplitude of interneurons during control and NPS application. (D, E) Summary data showing that the frequency but not the amplitude of mIPSCs was increased during application of NPS ($P < 0.05$; $n = 5$) and this increase was reversed during bath application of 100 μ M SHA68. TTX (1 μ M) was included in the bath solution. (F) Immunostaining showing that NPSR and GAD67 were co-expressed on neurons in MeA. Bar: 100 μ m.

Fig 12. Role of brain NPS in pain and anxiety after nerve injury

A schematic presentation illustrating a role for NPS-mediated intra-amygdaloidal GABAergic activity in the interaction between pain and anxiety.