NEUROBIOLOGY

Spexin/NPQ Induces FBJ Osteosarcoma Oncogene (Fos) and Produces Antinociceptive Effect against Inflammatory Pain in the Mouse Model

Shuang-Yu Lv,^{*} Binbin Cui,^{*} Yanjie Yang,[†] Hua Du,[‡] Xiaomei Zhang,^{*} Yuchen Zhou,^{*} Wenling Ye,^{*} Xiaobo Nie,^{*} Yang Li,[§] Qun Wang,^{*} Wei-Dong Chen,^{*‡} and Yan-Dong Wang[¶]

From the Key Laboratory of Receptors-Mediated Gene Regulation and Drug Discovery^{*} and the Laboratory of Cell Signal Transduction,[†] School of Medicine, Henan University, Kaifeng; the Department of Pathology,[‡] College of Basic Medicine, Inner Mongolia Medical University, Hohhot; the Department of Orthopaedics,[§] The 969th Hospital of People's Liberation Army of China, Hohhot; and the State Key Laboratory of Chemical Resource Engineering,[¶] College of Life Science and Technology, Beijing University of Chemical Technology, Beijing, China

Accepted for publication
December 7, 2018.

Address correspondence to
Yan-Dong Wang, Ph.D., Col-
lege of Life Science and Tech-
nology, Beijing University of
Chemical Technology, No. 15
Beisanhuan Donglu, Beijing
100029, China; or Wei-Dong
Chen, Ph.D., Key Laboratory of
Receptors-Mediated Gene
Regulation and Drug Discov-
ery, School of Medicine, Henan
University, No. 1 Jinming
Road, Kaifeng, 475004,
China. E-mail:
ydwangbuct2009@163.com or
wdchen666@163.com.

Spexin/NPQ is a novel highly conserved neuropeptide. It has a widespread expression in the periphery and central nervous system. However, the effects of central spexin on acute inflammatory pain are still unknown. This study explored the mechanisms and effects of supraspinal spexin on inflammatory pain. The results from the mouse formalin test show that i.c.v. administration of spexin decreased licking/biting time during the late and early phases. The nonamidated spexin had no effect on pain response. The antinociception of spexin was blocked by galanin receptor 3 antagonist SNAP 37889. The *Galr3* and *Adcy4* mRNA levels in the brain were increased after injection with spexin. The antinociceptive effects of spexin were completely reversed by opioid receptor antagonist naloxone and κ -opioid receptor antagonist nor-binaltorphimine dihydrochloride. Spexin up-regulated the dynorphin and κ -opioid receptor gene and protein expression. PCR array assay and real-time PCR analysis show that spexin up-regulated the mRNA level of the FBJ osteosarcoma oncogene (*Fos*). T-5224, the inhibitor of c-Fos/activator protein 1 (AP-1), blocked the increased mRNA level of *Pdyn* and *Oprk1* induced by spexin. I.C.V. spexin (2.43 mg/kg) increased the number of c-Fos-positive neurons in most subsections of periaqueductal gray. In addition, in the acetic acid-induced writhing test, i.c.v. spexin produced an antinociceptive effect. Our results indicate that spexin might be a novel neuropeptide with an antinociceptive effect against acute inflammatory pain. (*Am J Pathol* 2019, 189: 886–899; <https://doi.org/10.1016/j.ajpath.2018.12.009>)

The novel peptide spexin was first identified as a pro-hormone containing an amidated 14-amino acid peptide using a hidden Markov model.¹ The precursor of spexin gene (*Spx*) has been found through a bioinformatics method by Sonmez et al.² The spexin prepropeptide contains mature peptides, cleavage sites, and a signal peptide sequence. And, for the record, a small amino acid region, NWTPQAM-LYLKGAQ-NH₂ (named NPQ peptide or spexin), is substantially conserved in different vertebrates during biological evolution,^{1–3} and it is considered most likely active neuropeptide.⁴ Spexin could activate human galanin receptor types 2 and 3 (GALR2/3) in ligand-receptor interaction assay *in vitro*, suggesting that spexin may be a natural ligand for GALR2/3.⁵

Recently, some physiological functions of spexin have been found, such as appetite, bowel movement, energy metabolism, cardiovascular function, and hyperoxia. Spexin

Supported by the National Natural Science Foundation of China grants 81472232 (W.-D.C.), 81270522 (W.-D.C.), 81672433 (Y.-D.W.), 81370537 (Y.-D.W.), and 81600974 (S.-Y.L.); Program for Science & Technology Innovation Talents in Universities of Henan Province (HASTIT) grant 13HASTIT024 (W.-D.C.) and Plan for Scientific Innovation Talent of Henan Province (W.-D.C.); the Key Scientific Research Program for Universities of Henan Province grant 17A310003 (S.-Y.L.) and the Scientific Research Foundation of Henan University grant 2015YBZR050 (S.-Y.L.); the Fundamental Research Funds for the Central Universities grants PYBZ1803 (Y.-D.W.), XK1802-8 (Y.-D.W.), and PYBZ1706 (Y.-D.W.); and Henan Provincial Natural Science Foundation grant 182300410323 (W.-D.C.).

Disclosures: None declared.

was involved in the regulation of rat adrenocortical cell proliferation.⁶ Under conditions of hyperoxia exposure, spexin expression is up-regulated in postnatal rats.⁷ Toll et al⁸ shows that spexin contributes to an increase of arterial blood pressure and a decrease of urine flow rate. Central administration of spexin can inhibit appetite in goldfish.³ Spexin suppressed the release of luteinizing hormone in pituitary cells *in vitro* and decreased the serum luteinizing hormone levels of goldfish.⁹ In Ya-fish, the gene expression of *Spx* was affected by different feeding conditions or metabolic status.¹⁰ In addition, spexin causes weight loss in diet-induced obesity mice¹¹ and could enhance bowel movement in mice.¹²

Spexin mRNA and protein were abundantly distributed in both the central nervous system (CNS) and the periphery in humans,¹³ rodents,⁴ and fish.^{3,10} In the periphery, spexin mRNA and protein have been detected in various organs, such as liver, kidney, thyroid, ovary, lung, stomach, and small intestine.^{4,13} In the CNS of rodents, immunohistochemical analysis shows that spexin-like immunoreactivity was detected in brainstem, trigeminal ganglia, and brain cortex,⁴ which is known to be closely related to nociceptive transmission. It indicates that spexin potentially plays a role in modulating nociceptive behavior.

The formalin test is used as a continuous tonic pain animal model, with the persistent rather than transient nociceptive stimulus and response,^{14,15} which differs from the tail immersion test. The formalin test presents a biphasic behavioral reaction. The early phase lasts 10 minutes, starting from injection with formalin instantly, and it is induced by a bursting activity from pain fibers, such as C fibers. The late phase is commonly between the 10th and 30th minute after treatment and is triggered by inflammation and central sensitization.^{16,17} The formalin test is a valid model for evaluating the antinociceptive effect of different potential therapeutic agents. The nociceptive response in the formalin test is quantifiable as well as reproducible.^{18,19} The acetic acid-induced writhing test is commonly used for evaluating and comparing the efficacy of new drugs in visceral pain treatment.²⁰ This test is used to model tonic pain because of its longer duration.²¹ Toll et al⁸ showed that spexin exerted antinociceptive activity in the tail-flick test, an acute pain model. However, as far as we know, there are no published articles that have reported the effect of spexin on tonic inflammatory pain or visceral pain.

Considering the distribution of spexin in the CNS, which is known to be closely related to nociceptive transmission, and the lack of research on spexin in tonic pain, it is necessary to determine the effect of spexin on inflammatory pain and visceral pain. The current investigation was designed to explore the antinociceptive effect of central spexin against tonic inflammatory pain using the mouse formalin test and against visceral pain using the writhing test. The expression levels of the related genes and proteins in brain were determined using real-time PCR, PCR array, Western blot analysis, enzyme-linked immunosorbent assay (ELISA), and immunohistochemistry.

Materials and Methods

Animals

Kunming mice (male, aged 6 to 8 weeks) were obtained from the Animal Center of Henan Province (Zhengzhou, China). The mice were acclimated to the controlled animal room with standard room temperature, humidity, and regular lighting. The animals with free access to dietary food and water were accommodated in five mice per cage for at least 1 week before the behavioral test. All animal experimental protocols were approved by the Committee of Medical Ethics and Welfare for Experimental Animals, Henan University School of Medicine.

Chemicals

The peptides used in this study [spexin, nonamidated spexin, and galanin (1–29)] were supplied by GL Biochem (Shanghai) Ltd (Shanghai, China). Bicuculline methiodide was supplied by Tokyo Chemical Industry Co Ltd (Tokyo, Japan). Naloxone, nor-binaltorphimine dihydrochloride, and β -funaltrexamine hydrochloride were supplied by Sigma-Aldrich (St. Louis, MO). M871 was supplied by Abcam Inc. (Burlingame, CA). SNAP 37889 (1-phenyl-3-[[3-(trifluoromethyl)phenyl]imino]-1*H*-indol-2-one) was obtained from MedChemExpress (Princeton, NJ). T-5224 was provided by APEX BIO (Houston, TX). The peptides were dissolved and diluted in sterilized saline at a working concentration before the experiment.

Drug Administration

I.C.V. injection was performed according to the protocols shown by Haley and McCormick²² and the previous work.²³ The injected point was 1.5 mm lateral, 1.0 mm posterior to bregma, and 3.0 mm below the skull surface. Chemicals or normal saline was treated in a volume of 4 μ L (10 μ L/minute) by a 10- μ L Hamilton microsyringe. The animals in the control group were treated with the same volume of sterilized normal saline. The correctness of the i.c.v. injection was confirmed by checking brain sections for the site of injection after treatment with methylene blue dye.

Formalin Test

The tonic inflammatory pain was assessed using the formalin test. This test was performed according to the previous reports.^{16,24} In brief, before testing, the animals were put singly into a transparent glass cylinder (height, 20 cm; diameter, 15 cm). A mirror was mounted at 45 degrees beneath the cylinder, allowing unobstructed observation of the hind paws. After acclimation for 30 minutes, the mice were taken out of the chamber and given an intraplantar injection of 20 μ L of 1.0% formalin solution into the dorsal surface of the right hind paw. Thereafter, the animals were immediately put back to the observation glass cylinder and

time (seconds) spent licking or biting the formalin-injected paw was counted by the timer for 30 minutes in a blinded manner (B.C. and X.Z.). The mouse hind paw exhibited a biphasic pain response: the first 10 minutes (0 to 10 minutes) was regarded as the acute phase (early phase), and the second 20 minutes (10 to 30 minutes) was known as the longer-lasting tonic phase (late phase).

Acetic Acid–Induced Writhing Test

The writhing response induced by acetic acid was used to evaluate visceral pain. This model of pain was based on the methods previously described.^{21,25} Each mouse was intraperitoneally treated with acetic acid (1.0%, 10 mL/kg of body weight), and then the animals were put in single transparent cages immediately. The number of writhes (contractions of the abdominal muscles) was counted for 30 minutes in a blinded manner (X.Z. and Y.Z.). The writhing behavior was defined as outstretching of the hind limbs and extension of the whole body. The antinociception was presented as the reduction of the number of writhes.

The Radiant Heat Paw Withdrawal Test

The nociceptive response was assessed by the radiant heat paw withdrawal test using PL-200 radiant heat apparatus (Chengdu Taimeng Technology & Market Corp., Chengdu, China), according to the previous report.²⁶ Each mouse was adapted to the testing environment for at least 30 minutes before experiments. The radiant heat source was positioned under the transparent floor directly beneath the hind paw. The light beam focused on the plantar of the hind draw, and the latency for paw withdrawal response against radiant heat stimulation was measured. The intensity of radiant heat was adjusted so that the control latency for paw withdrawal response was 5 to 10 seconds before drug administration. The mice were tested again after i.c.v. administration of drugs at different times. The paw withdrawal latency was then recorded, with a maximum cutoff score of 15 seconds to minimize the tissue damage.

Total RNA Isolation

Total RNA was extracted from brain tissue by TRIzol Reagent (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA), following the manufacturer's instruction. In brief, no more than 100 mg of tissue was mixed with 1 mL TRIzol Reagent by a homogenizer (PowerGen 125; Fisher Scientific, Pittsburgh, PA). The amount of the purified RNAs and their quality were assessed by NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE).

Reverse Transcription and RT-qPCR

Total RNA (0.5 µg) from each brain was reverse transcribed to synthesize cDNA using the High Capacity cDNA Reverse

Transcription kit (Applied Biosystems, Foster City, CA). The expression of *Galr2*, *Galr3*, adenylyl cyclase 1 (*Adcy1*) ~ *Adcy9*, proopiomelanocortin (*Pomc*), prodynorphin (*Pdyn*), proenkephalin (*Penk*), µ-opioid receptor (*Oprm1*), κ-opioid receptor (*Oprk1*), δ-opioid receptor (*Oprd1*), *Gabra1* ~ *Gabra5*, FBJ osteosarcoma oncogene (*Fos*), *Egr1*, and *Jun1* mRNAs was assessed using quantitative real-time RT-PCR (RT-qPCR). Real-time PCR was performed by the 7500HT thermal cycler and SYBR Green master mix (Applied Biosystems). Primer pairs used are shown in Table 1, which were designed following the previous reports.^{27–32} All samples were analyzed in duplicate, measuring both the gene of interest and *36B4* as an internal control. After each RT-qPCR, the dissociation curve analysis was performed. The normalized expression of the target genes was analyzed by the $2^{-\Delta\Delta Ct}$ method,³³ where $\Delta\Delta Ct = (Ct_{Target} - Ct_{36B4})_{drug} - (Ct_{Target} - Ct_{36B4})_{control}$.

PCR Array

Pathway-focused gene expression profiling was performed through a 96-well mouse PCR array, RT² Profiler PCR Array (PAMM-075Z, Mouse Transcription Factors; Qiagen, Hilden, Germany). This RT² Profiler PCR Array contains a total of 96 wells with 84 transcription factor genes and other internal and technical controls (positive, negative, and DNA contamination controls). All of the 84 genes in the array participate in various processes of transcription factor signaling. The RT² Profiler PCR Array and data analysis were done following the manufacturer's instruction (Qiagen).

ELISA

Mice in each group were sacrificed under ether anesthesia, and their brains were immediately taken out after 15 minutes of i.c.v. injection with spexin. Thereafter, each mouse brain was weighed, homogenized with 10% w/v phosphate buffer (0.1 mol/L, pH 7.4), and centrifuged for $6714 \times g$ at 4°C for 15 minutes. Then, the supernatants obtained were used for ELISA. The mouse dynorphin was quantified using the Mouse Dynorphin ELISA Kit (Shanghai Fusheng Shiye Co Ltd, Shanghai, China), following the manufacturer's instruction.

Western Blot Analysis

The mouse brain tissue samples were homogenized with radioimmunoprecipitation assay lysis buffer containing protease inhibitor. Then, the homogenate was centrifuged at $14,000 \times g$ for 10 minutes at 4°C to obtain the tissue protein. The quantitation of protein concentration was measured by bicinchoninic acid method. Equivalent amounts of protein were loaded into each lane of a gel, resolved by SDS-PAGE gel, and then blotted onto polyvinylidene difluoride membranes. After blocking with 5% milk, the blots were incubated with the first antibodies, κ-opioid receptor (KOR; 1:1000; Abcam, Cambridge, MA)

Table 1 Primer Sequence Used for RT-qPCR

Primer name	Primer sequence	Size, bp
<i>Galr2</i> -F	5'-TGGACGATTGGGTGTTTGCG-3'	130
<i>Galr2</i> -R	5'-AGCGGGTAGCGGATGGC-3'	
<i>Galr3</i> -F	5'-GGCCGTCTCAGTGGATAGGT-3'	137
<i>Galr3</i> -R	5'-AGCTTAGGTAGGGCGCGGA-3'	
<i>Adcy1</i> -F	5'-CCGGAACATGGACCTCTACTAC-3'	284
<i>Adcy1</i> -R	5'-ATAGGTGGGAGGAGATGGACTG-3'	
<i>Adcy2</i> -F	5'-CCTGGGACCAGGTGTCATTTC-3'	412
<i>Adcy2</i> -R	5'-CCTGCTTTGGGTCCCTGTAG-3'	
<i>Adcy3</i> -F	5'-TACTTCAAAAGGCAGCGCCA-3'	482
<i>Adcy3</i> -R	5'-TTGGCCAGGATCTCCCTCAG-3'	
<i>Adcy4</i> -F	5'-TTGACCCAAAGCGGGCAG-3'	248
<i>Adcy4</i> -R	5'-GCACACAGCACAGTTGTCAG-3'	
<i>Adcy5</i> -F	5'-ACTTGGCCATCTCTTCGCAC-3'	445
<i>Adcy5</i> -R	5'-TGATTCTCCGACGCCAAGTT-3'	
<i>Adcy6</i> -F	5'-GCGGTGAGGGAGAATCACTG-3'	163
<i>Adcy6</i> -R	5'-TCACACCTGTTACCTCACGC-3'	
<i>Adcy7</i> -F	5'-GCAGGTAACAGGGTCGGAG-3'	392
<i>Adcy7</i> -R	5'-AGGTCTCAGCTCTTTCGCAC-3'	
<i>Adcy8</i> -F	5'-TTGCGGAGTGGCGATAAGTT-3'	482
<i>Adcy8</i> -R	5'-ACAAAGTACTCTGGGTAGGAGC-3'	
<i>Adcy9</i> -F	5'-AAGACCAGCACCAAGCTTTC-3'	183
<i>Adcy9</i> -R	5'-GTTCTTGAACCTGAGCGGGA-3'	
<i>Pomc</i> -F	5'-AGATTCAAGAGGGAGCTGGA-3'	159
<i>Pomc</i> -R	5'-CTTCTCGGAGGTCATGAAGC-3'	
<i>Pdyn</i> -F	5'-CGGAACCTCTCTTGGGGTAT-3'	154
<i>Pdyn</i> -R	5'-TTTGGAACCGGAAAGAATC-3'	
<i>Penk</i> -F	5'-AACAGGATGAGAGCCACTTGC-3'	474
<i>Penk</i> -R	5'-CTTCATCGGAGGGCAGAGACT-3'	
<i>Oprm1</i> -F	5'-ATCCTCTCTTCTGCCATTGGT-3'	127
<i>Oprm1</i> -R	5'-TGAAGGCGAAGATGAAGACA-3'	
<i>Oprd1</i> -F	5'-AAGTACTTGGCGCTCTGGAA-3'	125
<i>Oprd1</i> -R	5'-GCTCGTCATGTTTGGCATC-3'	
<i>Oprk1</i> -F	5'-CCGATACACGAAGATGAAGAC-3'	341
<i>Oprk1</i> -R	5'-GTGCCCTCCAAGGACTATCGC-3'	
<i>Gabra1</i> -F	5'-ATGGGGATTAGGGCCAGAGT-3'	432
<i>Gabra1</i> -R	5'-TTGCACAGCTCGGGGC-3'	
<i>Gabra2</i> -F	5'-CCACGACTCCTCAAGCTCTC-3'	322
<i>Gabra2</i> -R	5'-TTAGCCAGCACCAACCTGAC-3'	
<i>Gabra3</i> -F	5'-GTGACACTCGATCTCACAGGT-3'	133
<i>Gabra3</i> -R	5'-CTTGGCTAGTGGTTCCAGGG-3'	
<i>Gabra4</i> -F	5'-AAGCTTGACAAGGTGCGGAG-3'	499
<i>Gabra4</i> -R	5'-TCTGTAAACAGGACCCCCAAAT-3'	
<i>Gabra5</i> -F	5'-CGCGTAGGCGTCAAGATCAA-3'	340
<i>Gabra5</i> -R	5'-TGGGCCAAAGCTGGTAACAT-3'	
<i>Fos</i> -F	5'-GGTGAAGACCGTGTGTCAGGAG- CAG-3'	117
<i>Fos</i> -R	5'-GCCATCTTATTCCGTTCCTT- CGG-3'	
<i>Jun</i> -F	5'-CTCATACCAGTTCGCACAGGCGGC-3'	298
<i>Jun</i> -R	5'-CCGCTAGCACTCACGTTGGTA- GCG-3'	
<i>Egr1</i> -F	5'-GAGCACCTGACCACAGAGTC-3'	172
<i>Egr1</i> -R	5'-AAAGGGGTTTCAGGCCACAAA-3'	
<i>36B4</i> -F	5'-CGAGCTGGAAGTCCAACTAC-3'	109
<i>36B4</i> -R	5'-ATCTGCTGCATCTGCTTG-3'	

F, forward; R, reverse; RT-qPCR, real-time quantitative RT-PCR.

or β -actin (1:1000; Beyotime, Shanghai, China), at 4°C overnight. Then, the blots were incubated with the proper horseradish peroxidase-labeled secondary antibodies for 1 hour. The membrane was washed, and the immunoreactive proteins were visualized by electrochemiluminescence.

Preparation of Brain Sections and c-Fos Immunohistochemistry

Fifteen minutes after i.c.v. injection with spexin or normal saline, mice were anesthetized with 100 mg/kg (intraperitoneally) of pentobarbital sodium, then perfused transcardially with 25 mL normal saline, followed by 25 mL of 4% paraformaldehyde in phosphate-buffered saline. Brains were taken out and fixed in fixation fluid overnight at 4°C and then embedded in paraffin. Coronal sections (5 μ m thick) of each example were stained by immunohistochemistry, according to the previous reports.^{34,35} After deparaffinization, the sections were incubated with 10% normal goat serum and incubated 12 hours with the polyclonal anti-c FBJ osteosarcoma oncogene (c-Fos) antibody (1:100; Abcam Inc., Burlingame, CA). The sections were incubated for 2 hours with the biotinylated secondary antibody and then incubated for 30 minutes at room temperature with the avidin-biotin-peroxidase complex (Corning Inc., Corning, NY). The sections of the examples were incubated with 3,3-diaminobenzidine containing hydrogen peroxide (ZSGB-bio, Beijing, China) to examine the antibody binding. The sections were then lightly counterstained with hematoxylin. To analyze the results, the periaqueductal gray (PAG) was divided into four areas, including dorsomedial, dorsolateral, lateral, and ventrolateral parts. Three sections (at -4.1, -4.5, and -4.9 mm from bregma) in rostral, intermediate, and caudal PAG, respectively, were determined in each brain. The number of c-Fos-like immunoreactive (FLI) neurons was calculated using Image-Pro Plus 6.0 software (Media Cybernetics, Silver Spring, MD) in a blinded manner (Y.Z.).

Experimental Design

To study the role of spexin in tonic inflammatory pain, spexin (0.081, 0.81, and 2.43 mg/kg) or galanin (0.32 mg/kg) was intracerebroventricularly administrated at 5 minutes before the formalin injection. After that, the formalin-induced pain behavior was detected. In addition, to compare the analgesic effect of spexin with nonamidated spexin, the nonamidated spexin (2.43 mg/kg) was intracerebroventricularly administrated with the identical protocol. To evaluate the analgesic effect of spexin after the induction of the pain state, spexin (0.81 and 2.43 mg/kg) was intracerebroventricularly injected at 5 minutes after formalin treatment.

To further explore the mechanism of the antinociception caused by spexin, the GALR2 antagonist M871 (0.11 mg/kg) and GALR3 antagonist SNAP 37889 (0.018 mg/kg) were co-administrated with 2.43 mg/kg spexin (ie, each

Figure 1 The antinociceptive effect of spexin in the formalin test in mice. **A** and **B**: Normal saline (control), spexin (0.081, 0.81, and 2.43 mg/kg), or galanin (GAL; 0.32 mg/kg) was intracerebroventricularly administered to mice at 5 minutes before the intraplantar (i.pl.) injection of formalin. **C** and **D**: Normal saline or nonamidated spexin (2.43 mg/kg) was intracerebroventricularly administered to mice at 5 minutes before the i.pl. injection of formalin. Antinociception was recorded at 0 to 10 minutes (early phase) and 10 to 30 minutes (late phase) after formalin injection. **E** and **F**: Normal saline or spexin (0.81 and 2.43 mg/kg) was intracerebroventricularly administered at 5 minutes after formalin treatment. Each point is the mean licking/biting time. The unpaired *t*-test was performed to test the difference between nonamidated spexin and the control group. Data are expressed as means $\pm$ SEM (**A–F**). *n* = 7 to 10 per group (**A–F**). **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 versus control.

antagonist was mixed with spexin and given in one i.c.v. administration of 4 μ L). And the *Galr2*, *Galr3*, and *Adcy1* ~ *Adcy9* mRNAs were determined by RT-qPCR. The opioid receptor antagonist naloxone (0.2 mg/kg) and γ -aminobutyric acid A (GABA_A) receptor antagonist bicuculline (20 μ g/kg) were co-administered with spexin (2.43 mg/kg). The doses of these antagonists were used following the previous reports.^{21,36–38}

To investigate which subtypes of opioid peptide(s), opioid receptor(s), or transcription factor(s) were involved in the antinociceptive effect of spexin in the formalin test, after 15 minutes of administration with spexin or normal saline (control), brains were quickly removed, frozen in liquid nitrogen, and preserved at -80°C for further studies (RT-qPCR, PCR array, ELISA, or Western blot analysis). And the related mRNA levels were determined. The κ -opioid receptor antagonist nor-binaltorphimine dihydrochloride (73.47 μ g/kg) was co-administered with spexin (2.43 mg/kg), and the nociceptive response was evaluated. T-5224 (100 mg/kg), the inhibitor of c-Fos/activator protein 1 (AP-1), was administered orally to block the Fos activity, and then the *Pdyn* and *Oprk1* mRNA levels were detected. The doses of the antagonists were chosen following the previous reports.^{23,39}

To further determine the sites of the up-regulated Fos induced by spexin in the mouse brain, the c-Fos neuronal activity in the PAG, the key component of descending

inhibitory pain circuitry, was detected by immunohistochemistry method.

To further study whether spexin could produce an antinociceptive effect against visceral pain, spexin (0.081, 0.81, and 2.43 mg/kg) or galanin (0.32 mg/kg) was intracerebroventricularly administrated at 5 minutes before acetic acid injection. The brain tissue was quickly collected at 15 minutes after spexin treatment, then frozen in liquid nitrogen, and stored at -80°C for further gene expression analysis.

Statistical Analysis

Statistical results were presented as means $\pm$ SEM. Analysis was performed by one-way analysis of variance, followed by Dunnett's test for multiple comparisons. The unpaired *t*-test was used to test the difference between the two groups. *P* < 0.05 was considered statistically significant.

Results

The Effect of I.C.V. Spexin on Nociceptive Response in the Mouse Formalin Test

I.C.V. spexin induced a dose-related decrease in licking/biting time in the early phase [$F(3, 31) = 8.090$, *P* < 0.001] (Figure 1A). Spexin markedly decreased the licking/biting

Figure 2 The effects of GALR2/3 antagonists on the antinociception of spexin and the influence of spexin on *Galr2*, *Galr3*, and *Adcy1* ~ *Adcy9* gene expression. The effects of GALR2 antagonist M871 (0.11 mg/kg; **A** and **B**) and GALR3 antagonist SNAP 37889 (0.018 mg/kg; **C** and **D**) on the antinociception induced by spexin (2.43 mg/kg) in the formalin test. Formalin was injected at 5 minutes after spexin or normal saline treatment. Data are presented as mean licking/biting time. Histogram showing the relative mRNA levels of the *Galr2* and *Galr3* (**E**) and *Adcy1*, *Adcy2*, *Adcy3*, *Adcy4*, *Adcy5*, *Adcy6*, *Adcy7*, *Adcy8*, and *Adcy9* (**F**) normalized with the housekeeping gene *36B4* mRNA expression. The gene expression was detected at 15 minutes after spexin (2.43 mg/kg) or normal saline (control) treatment. Formalin was injected at 5 minutes after spexin or normal saline administration. Data are expressed as means $\pm$ SEM (**A**–**F**). $n = 9$ to 10 per group (**A**–**D**); $n = 6$ to 8 per group (**E** and **F**). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

time at the doses of 0.81 and 2.43 mg/kg ($P < 0.01$ and $P < 0.001$, respectively), whereas no obvious difference was found at the lower dose of 0.081 mg/kg ($P = 0.80$) compared with the control group. In the late phase (Figure 1B), spexin only at the dose of 2.43 mg/kg significantly decreased the time spent on licking/biting, but not the doses of 0.081 or 0.81 mg/kg (0.081 mg/kg, $P = 0.97$; 0.81 mg/kg, $P = 0.36$; 2.43 mg/kg, $P < 0.05$), compared with the control group. The nonselective galanin receptor agonist galanin (0.32 mg/kg), as a positive control, exhibited a significant reduction in licking/biting time in both early ($P < 0.05$) and late ($P < 0.01$) phases. The antinociceptive effect of 0.32 mg/kg of galanin is equivalent to 2.43 mg/kg of spexin (Figure 1, A and B).

To further determine whether the C-terminal amidation is necessary for spexin to exert its activity, the nonamidated spexin was used. I.C.V. nonamidated spexin (2.43 mg/kg) had no influence on paw licking/biting time in either the early phase ($P = 0.30$) or the late phase ($P = 0.12$) (Figure 1, C and D). Spexin at 2.43 mg/kg produced an analgesic effect in the late phase ($P < 0.01$) (Figure 1F), not

the early phase ($P > 0.05$) (Figure 1E), after the induction of the pain state in the formalin test.

The nociceptive response was also assessed by the radiant heat paw withdrawal test. Compared with the control group, i.c.v. administration of spexin (0.81 mg/kg) significantly increased the paw withdrawal latency at 15 minutes after treatment in the radiant heat paw withdrawal test ($P < 0.05$), although it did not reach a statistically significant level at 30 or 45 minutes after injection. The result indicates that i.c.v. spexin (0.81 mg/kg) produced an antinociceptive effect in the radiant heat paw withdrawal test in mice (Supplemental Figure S1).

The Effect of I.C.V. Spexin on GALR2/3

A previous report shows that spexin activates GALR2/3.⁵ To identify which type of galanin receptor mediated the antinociception of spexin in the formalin test, the GALR2 antagonist M871 and the GALR3 antagonist SNAP 37889 were used, and the *Galr2* and *Galr3* gene expression in the whole brain was detected at 15 minutes after spexin

Figure 3 The effect of i.c.v. administration of naloxone (NLX) and bicuculline (Bic) on spexin-induced antinociception in the mouse formalin test. The opioid receptor antagonist naloxone (0.2 mg/kg; **A** and **B**) or GABA_A receptor antagonist bicuculline (20 µg/kg; **C** and **D**) was co-administrated (intracerebroventricularly) with spexin (2.43 mg/kg). Antinociception was recorded at 0 to 10 minutes (early phase) and 10 to 30 minutes (late phase) after formalin injection. Data are presented as means ± SEM (**A–D**). $n = 7$ to 10 per group (**A–D**). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

injection. M871 (0.11 mg/kg) could not block the decreasing licking/biting time caused by spexin in either the early phase (Figure 2A) or the late phase (Figure 2B) of the formalin test. However, SNAP 37889 (0.018 mg/kg) significantly antagonized the antinociceptive effect of spexin in the early phase ($P < 0.01$) (Figure 2C) and late phase ($P < 0.05$) (Figure 2D) of the formalin test. I.C.V. spexin significantly increased the mRNA level of *Galr3*, but not *Galr2* (Figure 2E). These results indicate that *Galr3* may be involved in the antinociception of spexin. The gene expression of *Adcy* (from type 1 to type 9), as GALR2/3's downstream signal, was also analyzed. Only *Adcy4* mRNA levels were markedly increased (Figure 2F).

The Effects of Naloxone and Bicuculline on the Antinociception of Spexin in the Formalin Test

To explore whether the opioid receptor was involved in the antinociceptive effect of spexin, the opioid receptor antagonist naloxone was selected. Compared with the control group, i.c.v. naloxone (0.2 mg/kg) alone did not influence the nociceptive behavior induced by formalin in either the early phase ($P = 0.94$) or the late phase ($P = 0.79$) (Figure 3, A and B). However, i.c.v. co-administration of naloxone significantly reversed the antinociception of spexin in the early phase (Figure 3A) and late phase (Figure 3B).

To ascertain whether the antinociceptive effect of spexin was related to GABA_A receptor, bicuculline was used. The licking/biting responses in the two phases were not affected by i.c.v. administration of bicuculline compared with the control group (Figure 3, C and D). I.C.V. bicuculline (20 µg/kg) failed to block the antinociceptive response induced by 2.43 mg/kg of spexin in the early phase (Figure 3C) and late phase (Figure 3D) in the mouse formalin test.

The mRNA and Protein Levels of the Endogenous Opioid Peptides and Opioid Receptors after Spexin Administration in Mice

To understand which kind of endogenous opioid peptides were involved in the analgesia of spexin, the mRNA levels of the endogenous ligand for *Oprm1* *Pomc*, the endogenous ligand for *Oprd1* *Penk*, and the endogenous ligand for *Oprk1* *Pdyn* were determined. Spexin did not affect the *Pomc* or *Penk* mRNA levels compared with the control group (Figure 4A). However, a significant increase in *Pdyn* mRNA level was demonstrated in the spexin-treated group (Figure 4A).

Furthermore, it was examined which subtype of opioid receptors was involved in the analgesic effect of spexin. The mRNA levels of three classic subtypes of opioid receptors, including *Oprm1*, *Oprd1*, and *Oprk1*, were detected. The expression levels of *Oprm1* or *Oprd1* mRNA did not change markedly compared with the control group (Figure 4B). However, the *Oprk1* mRNA levels significantly increased (Figure 4B), which is consistent with the previous result (Figure 4A) that the mRNA levels of *Pdyn*, the endogenous ligand for KOR, were up-regulated by central spexin.

The ELISA test result shows that the level of dynorphin was increased compared with the control group (Figure 4C). Western blot analysis demonstrates that the protein level of KOR was up-regulated compared with the control group (Figure 4, D and E). The KOR antagonist nor-binaltorphimine dihydrochloride (73.47 µg/kg) blocked the analgesic response of spexin (2.43 mg/kg) in the early phase ($P < 0.01$) (Figure 4F) and late phase ($P < 0.01$) (Figure 4G) of the formalin test. The µ-opioid receptor (MOR) antagonist β-funaltrexamine hydrochloride (49 µg/kg) could not reverse the antinociception of spexin (2.43 mg/kg) in either the early or the late phase of the formalin test (Supplemental Figure S2).

Figure 4 The gene and protein expression levels of endogenous opioid peptides and opioid receptors in the mouse brain after administration of spexin and the effect of KOR antagonist on the antinociception of spexin. **A** and **B**: Histogram showing the relative mRNA expression levels of *Pomc*, *Penk*, *Pdyn*, *Oprm1*, *Oprd1*, and *Oprk1* normalized with the housekeeping gene *36B4* mRNA expression using real-time PCR. **C**: The concentration of dynorphin in brain was determined using enzyme-linked immunosorbent assay. **D** and **E**: Western blot analysis and densitometric determination of the protein levels of KOR. The brain tissues were removed at 15 minutes after spexin (2.43 mg/kg) or normal saline (control) treatment. Formalin was injected at 5 minutes after spexin or normal saline administration. **F** and **G**: The effect of KOR antagonist nor-binaltorphimine dihydrochloride (nor-BNI; 73.47 μ g/kg) on the antinociception caused by spexin (2.43 mg/kg) in the formalin test. Formalin was injected at 5 minutes after spexin or normal saline treatment. Data are presented as mean of licking/biting time. Data are expressed as means $\pm$ SEM (**A–C** and **E–G**). $n = 4$ to 6 per group (**A–C** and **E**); $n = 9$ to 10 per group (**F** and **G**). * $P < 0.05$, *** $P < 0.001$ versus control; † $P < 0.05$, †† $P < 0.01$.

Transcription Factor–Linked Gene Assay and Real-Time PCR Analysis

To further explore the mechanism by which spexin up-regulates PDYN and KOR expression, a Mouse Transcription Factors RT² Profiler PCR Array was performed to determine which genes in transcription factors were regulated by spexin. The results show that Fos gene expression was increased, with approximately 2.3-fold in brain after spexin treatment (Figure 5). Moreover, the result from real-time PCR showed that spexin significantly up-regulated the Fos gene expression (Figure 6A), which was consistent with the result of PCR array. Spexin did not change the expression of Jun or Egr1 (Figure 6A). T-5224 (100 mg/kg), an inhibitor of c-Fos/AP-1, obviously blocked the up-regulated *Pdyn* and *Oprk1* mRNA levels caused by 2.43 mg/kg of spexin (Figure 6, B and C). This suggests that spexin may induce Fos expression, resulting in stimulation of opioid signaling.

Effect of Spexin on c-Fos Protein Expression in the PAG

The number of FLI neurons was calculated in up to three sections, which include rostral, intermediate, and caudal parts. Each part contains dorsomedial, dorsolateral, lateral, and ventrolateral columns of PAG. The numbers of c-Fos–positive cells were compared within each subdivision at each bregma level. Spexin at 2.43 mg/kg markedly increased the number of

FLI neurons in the caudal dorsomedial PAG ($P < 0.05$), the caudal lateral PAG ($P < 0.05$), and caudal ventrolateral PAG ($P < 0.05$), compared with saline control (Figure 7, A–D). Figure 7, E and F, illustrate the examples of FLI neurons in the caudal PAG (at -4.9 mm from bregma).

The Role of Central Spexin on Nociceptive Response in the Acetic Acid–Induced Writhing Test

I.C.V. spexin dose relatedly decreased the number of writhes [$F(3, 34) = 4.578$, $P < 0.01$] (Figure 8A). The decrease on the number of writhes caused by i.c.v. spexin reached statistical significance at the doses of 0.81 and 2.43 mg/kg ($P < 0.05$ and $P < 0.01$, respectively). Galanin (0.32 mg/kg, the positive control) markedly reduced the number of writhes.

In the writhing test, i.c.v. spexin up-regulated the *Galr2* mRNA levels, but not *Galr3* (Figure 8B). All of the nine types of *Adcy* mRNA were detected, and *Adcy1* gene levels were obviously increased (Figure 8C). To understand whether opioid receptor or GABA_A was involved in the antinociception of spexin in the acetic acid–induced visceral pain model, the mRNA levels of endogenous opioid peptides (*Pomc*, *Penk*, and *Pdyn*), opioid receptors (*Oprm1*, *Oprk1*, and *Oprd1*), and GABA_A receptors (*Gabra1*, *Gabra2*, *Gabra3*, *Gabra4*, and *Gabra5*) were determined. The mRNA levels of *Pomc* and *Oprm1* were markedly elevated (Figure 8, D and E), whereas the mRNA levels of each GABA_A receptor were not changed

Figure 5 Gene analysis using transcription factor PCR array. **A:** Spexin treatment increases *Fos* expression in mouse brain. Mice were treated with spexin (2.43 mg/kg). After 5 minutes, mice were intraplantarly injected with formalin. After 10 minutes of injection, total RNAs were prepared for PCR array analysis. **B:** Scatter plot between spexin-treated group and nontreated control group. $n = 3$ per group (**A**).

(Figure 8F). To determine which signal molecule was involved in the effect of spexin on POMC/MOR, the *Fos*, *Jun*, and *Egr1* gene expression levels were measured. The *Fos* mRNA levels were markedly increased (Figure 8G).

Discussion

This study indicates that spexin produced an analgesic effect in the tonic inflammatory pain mouse model using the formalin test and in the visceral pain model using the acetic acid-induced writhing test at the supraspinal level. These results demonstrate that i.c.v. spexin enhanced *Galr3* and *Adcy4* gene expression and up-regulated the mRNA and

protein levels of dynorphin and KOR, which were mediated by Fos (especially in PAG), thereby producing an inhibitory effect against inflammatory pain in the tonic inflammatory pain mouse model. However, in the visceral pain model, the antinociception of spexin was mediated by stimulating *Galr2* and *Adcy1* gene expression and then up-regulating the mRNA levels of *Pomc* and *Oprm1*.

The formalin test is considered as a valid model of persistent pain and is commonly used to evaluate potential analgesic compounds.¹⁵ In this study, a mouse injected with 1.0% formalin in the paw displays a nociceptive behavior with a biphasic characteristic, as shown in the previous reports.^{14,40} This result indicates that i.c.v. administration of 0.81 and 2.43 mg/kg spexin significantly decreased the

Figure 6 Quantitative determination of the effects of spexin on the mRNA levels of *Fos*, *Jun*, and *Egr1* and the effects of the T-5224 on the increased *Pdyn* and *Oprk1* mRNA levels induced by spexin. Histogram showing the relative mRNA expression levels of *Fos*, *Jun*, and *Egr1* (A), *Pdyn* (B), and *Oprk1* (C) normalized with the housekeeping gene *36B4* mRNA expression. The gene expression levels were detected at 15 minutes after spexin (2.43 mg/kg) or normal saline (control) treatment. T-5224 (100 mg/kg), the inhibitor of c-Fos/AP-1, was administered orally at 2 hours before spexin treatment. Formalin was injected at 5 minutes after spexin or normal saline administration. Data are expressed as means $\pm$ SEM (A–C). $n = 4$ to 6 per group (A–C). * $P < 0.05$.

licking/biting time in the early phase, whereas i.c.v. spexin at the dose of 2.43 mg/kg significantly reduced the licking/biting time in the late phase. These results were consistent with the previous report that central spexin produced an analgesic effect in the tail-flick test.⁸ The tail-flick test is an acute pain model using a short-lasting thermal stimuli,

whereas the formalin test is a tonic pain model with a continuous chemical stimuli.¹⁴ The consistent analgesic effects of spexin in both models indicate that spexin is a potent central modulator of pain at the supraspinal level.^{4,41}

The C-terminal residue of spexin is aminated. Our results show that i.c.v. nonamidated spexin could not markedly

Figure 7 The effect of spexin on c-Fos protein expression in the periaqueductal gray (PAG) in mice. A–D: Comparison of the number of c-Fos-like immunoreactive (FLI) cells between the two groups (normal saline and 2.43 mg/kg spexin) within the four columns of the PAG dorsomedial (A), dorsolateral (B), lateral (C), and ventrolateral (D) areas. The three sections are in the rostral, the intermediate, and the caudal PAG, which are correspondingly located at bregma -4.1, -4.5, and -4.9 mm. Formalin was treated at 5 minutes after spexin injection. The values are expressed as the mean number of FLI neurons. E and F: Photomicrographs showing c-Fos-labeled neurons in the PAG (at bregma -4.9 mm) in representative sections from the two groups separately intracerebroventricularly injected with normal saline (E) and 2.43 mg/kg spexin (F). Data are expressed as means $\pm$ SEM (A–D). $n = 7$ to 8 per group (A–D). * $P < 0.05$. Scale bars = 200 μ m (E and F).

Figure 8 The antinociceptive effects of spexin on the acetic acid-induced mouse visceral pain model and the related gene expression analysis. **A:** The effects of i.c.v. administration of spexin (0.081, 0.81, and 2.43 mg/kg) and galanin (GAL; 0.32 mg/kg) on the number of writhes in the acetic acid-induced writhing test. Normal saline (control), spexin, or GAL was administered at 5 minutes before i.p. injection of acetic acid. **B** and **C:** Quantitative analysis of *Galr2*, *Galr3*, *Adcy1*, *Adcy2*, *Adcy3*, *Adcy4*, *Adcy5*, *Adcy6*, *Adcy7*, *Adcy8*, and *Adcy9* mRNA expression in the brain of mice at 15 minutes after spexin or normal saline treatment. Acetic acid was injected at 5 minutes after spexin administration. **D** and **E:** Quantitative analysis of *Pomc*, *Penk*, *Pdyn*, *Oprm1*, *Oprd1*, and *Oprk1* mRNA expression in brain of mice at 15 minutes after spexin or normal saline treatment. Acetic acid was injected at 5 minutes after spexin administration. **F:** Quantitative analysis of *Gabra1*, *Gabra2*, *Gabra3*, *Gabra4*, and *Gabra5* mRNA expression in brain of mice at 15 minutes after spexin or normal saline treatment. Acetic acid was injected at 5 minutes after spexin administration. **G:** Quantitative analysis of *Fos*, *Jun*, and *Egr1* mRNA expression in brain of mice at 15 minutes after spexin or normal saline treatment. Acetic acid was injected at 5 minutes after spexin administration. Data are expressed as means $\pm$ SEM (A–G). $n = 8$ to 10 animals per group (A); $n = 4$ to 6 in each group (B–E); $n = 6$ in each group (F); $n = 5$ to 6 in each group (G). * $P < 0.05$, ** $P < 0.01$ versus control.

influence the licking/biting response in the formalin test. In addition, the molecular form of spexin that exerts its effect on obesity,¹³ bowel movement in mice,¹² and cardiovascular and renal function⁸ is amidated spexin. The results were consistent with the previous study in which most of the prepropeptides were processed through modification after translation, and >50% of the peptides underwent C-terminal amidation.⁴²

Spexin could activate GALR2/3 in the ligand-receptor interaction study, indicating that spexin is a natural ligand for GALR2/3.⁵ Our result shows that the antinociceptive response induced by spexin was blocked by GALR3 antagonist SNAP 37889, but not M871, in the mouse formalin test. The real-time PCR result shows that i.c.v. spexin increased *Galr3* mRNA expression, but not *Galr2*. ADCY was GALR2/3's downstream signaling molecule.⁴³ The *Adcy4* mRNA level was increased. These results indicate that central spexin produces an antinociceptive effect by activating GALR3/ADCY4 in the formalin test.

Opioid receptors are involved in controlling many regions of the nervous system.⁴⁴ Our result shows that naloxone, a classic opioid receptor antagonist, could significantly block the analgesia induced by i.c.v. spexin in both early and late phases in the formalin test. It suggests the involvement of opioid receptor in the analgesia of spexin. However, Toll et al⁸ show that naloxone could not antagonize the antinociception of spexin in the tail-flick assay. The tail-flick test is an acute pain test, whereas the formalin test is a persistent pain model.¹⁵ The neurotransmitters and neuromodulators mediating pain responses in each of these models may differ.¹⁵ The inconsistent effects of naloxone may be due to the different mechanisms of the two classes of pain models.¹⁴ In addition, it is well known that GABA_A receptor participates in pain control in the CNS, essentially through the descending inhibitory system.^{36,45} The GABA_A receptor antagonist bicuculline failed to antagonize the

antinociceptive effect of spexin, indicating that GABA_A receptor is not related to the antinociception of spexin.

The classic opioid receptor comprises three receptor subtypes: MOR, δ -opioid receptor (DOR), and KOR. Our result shows that spexin did not affect the *Pomc*, *Penk*, *Oprm1*, or *Oprdl* mRNA levels, whereas spexin significantly increased the mRNA levels of *Pdyn* and *Oprk1*. The ELISA and Western blot analysis show that the protein levels of dynorphin and KOR were increased. The synchronous changes of mRNA and protein levels of dynorphin and KOR suggest that the antinociceptive effect induced by i.c.v. spexin was mediated by the dynorphin/KOR system. Moreover, the KOR antagonist nor-binaltorphimine dihydrochloride antagonized the antinociception of spexin in the formalin test, which further supports the involvement of KOR. KOR is involved in antinociceptive activity in a variety of animal pain models.⁴⁶ KOR agonists dynorphin A and ICI 199,441 induce an antinociceptive response in the formalin test,⁴⁷ which is in accordance with the result in this study. However, the activation of the dynorphin/KOR system could induce aversive effects, such as depression,⁴⁸ which would limit the effectiveness of spexin-derived therapies in the inflammatory pain state.

Fos and pain are closely related, and the Fos expression could be considered as a special factor to map out the effect of antinociceptive drugs in the study of pain.^{49,50} Fos influences the transcription of opioid family genes by acting on activation protein-1.⁵¹ These results show that i.c.v. spexin induced a significant increase of Fos mRNA level. T-5224, an inhibitor of c-Fos/AP-1, notably reversed the increase of *Pdyn* and *Oprk1* mRNA levels caused by spexin. Considering the fact that spexin significantly increased the mRNA and protein levels of dynorphin and KOR, we proposed that central spexin may activate the dynorphin/KOR by the Fos pathway, inducing release of endogenous opioid peptide dynorphin and increase of KOR and producing antinociception in the mouse model of acute inflammatory pain. The result was also supported by the previous report that Fos directly led to dynorphin gene expression.^{52,53}

c-Fos protein expression, commonly as a marker of neuronal activity, has generally been used to determine the nervous pathway.⁵⁴ The PAG, as a key position of the pain regulatory system, participated in control of pain signaling in the CNS.^{55,56} Spexin up-regulated the quantity of FLI cells in almost all subdivisions of the PAG detected, particularly the caudal dorsomedial PAG, the caudal lateral PAG, and the caudal ventrolateral PAG. The result indicated that spexin might activate neurons in the PAG, which integrated the ascending pain signals and information from supraspinal pain circuitry,⁵⁷ subsequently exerting antinociceptive activity.

In the acetic acid-induced writhing test, i.c.v. spexin produced an antinociceptive effect against visceral pain. This result is consistent with that in the formalin test. Spexin increased *Galr2* and *Adcy4* gene expression. GABA_A receptor (*Gabra1* ~ *Gabra5*) mRNA levels were not affected by spexin, whereas *Pomc* and *Oprm1* mRNA levels were

elevated, suggesting the involvement of POMC/MOR in the antinociception of spexin in the mouse visceral pain model. The molecules induced by spexin in the acetic acid-induced writhing test differ from those in the formalin test, which may be due to different molecular mechanisms between visceral pain and inflammatory pain.¹⁴ Furthermore, i.c.v. spexin up-regulated the Fos gene expression in the visceral pain model, which is consistent with that in the formalin test. This result conformed with the involvement of Fos in the antinociceptive response induced by spexin, and it was supported by the evidence that Fos was involved in transactivation of the opioid gene.^{58,59}

In summary, the present study shows that central spexin produced an antinociceptive effect in the formalin test and acetic acid-induced writhing test. In the formalin test, i.c.v. spexin stimulates *Galr3* and *Adcy4* gene expression, and then activates the endogenous dynorphin/KOR system through Fos, which were particular in PAG, accordingly producing an antinociceptive effect against inflammatory pain. In the writhing test, central spexin up-regulates *Galr2* and *Adcy1* mRNAs, and then activates POMC/MOR via Fos, accordingly inducing an antinociceptive effect against visceral pain. This study supports a key role for spexin in central nociceptive behavior, and spexin could be considered as a novel target for treatment of inflammatory pain as well as visceral pain.

Supplemental Data

Supplemental material for this article can be found at <https://doi.org/10.1016/j.ajpath.2018.12.009>.

References

1. Mirabeau O, Severini C, Audero E, Gascuel O, Possenti R, Birney E, Rosenthal N, Gross C: Identification of novel peptide hormones in the human proteome by hidden Markov model screening. *Genome Res* 2007, 17:320–327
2. Sonmez K, Zaveri NT, Kerman IA, Burke S, Neal CR, Xie X, Watson SJ, Toll L: Evolutionary sequence modeling for discovery of peptide hormones. *PLoS Comput Biol* 2009, 5:e1000258
3. Wong MK, Sze KH, Chen T, Cho CK, Law HC, Chu IK, Wong AO: Goldfish spexin: solution structure and novel function as a satiety factor in feeding control. *Am J Physiol Endocrinol Metab* 2013, 305: E348–E366
4. Porzionato A, Rucinski M, Macchi V, Stecco C, Malendowicz LK, De Caro R: Spexin expression in normal rat tissues. *J Histochem Cytochem* 2010, 58:825–837
5. Kim D-K, Yun S, Son GH, Hwang J-I, Park CR, Kim JI, Kim K, Vaudry H, Seong JY: Coevolution of the spexin/galanin/kisspeptin family: spexin activates galanin receptor type II and III. *Endocrinology* 2014, 155:1864–1873
6. Rucinski M, Porzionato A, Ziolkowska A, Szyszka M, Macchi V, De Caro R, Malendowicz LK: Expression of the spexin gene in the rat adrenal gland and evidences suggesting that spexin inhibits adrenocortical cell proliferation. *Peptides* 2010, 31:676–682
7. Porzionato A, Rucinski M, Macchi V, Stecco C, Sarasin G, Sfriso MM, Di Giulio C, Malendowicz LK, De Caro R: Spexin is

- expressed in the carotid body and is upregulated by postnatal hyperoxia exposure. *Adv Exp Med Biol* 2012, 758:207–213
8. Toll L, Khroyan TV, Sonmez K, Ozawa A, Lindberg I, McLaughlin JP, Eans SO, Shahien AA, Kapusta DR: Peptides derived from the prohormone proNPQ/spexin are potent central modulators of cardiovascular and renal function and nociception. *FASEB J* 2012, 26:947–954
 9. Liu Y, Li S, Qi X, Zhou W, Liu X, Lin H, Zhang Y, Cheng CH: A novel neuropeptide in suppressing luteinizing hormone release in goldfish, *Carassius auratus*. *Mol Cell Endocrinol* 2013, 374: 65–72
 10. Wu H, Lin F, Chen H, Liu J, Gao Y, Zhang X, Hao J, Chen D, Yuan D, Wang T, Li Z: Ya-fish (*Schizothorax prenanti*) spexin: identification, tissue distribution and mRNA expression responses to periprandial and fasting. *Fish Physiol Biochem* 2016, 42:39–49
 11. Walewski JL, Ge F, Lobdell H, Levin N, Schwartz GJ, Vasselli JR, Pomp A, Dakin G, Berk PD: Spexin is a novel human peptide that reduces adipocyte uptake of long chain fatty acids and causes weight loss in rodents with diet-induced obesity. *Obesity* 2014, 22: 1643–1652
 12. Lin C-Y, Zhang M, Huang T, Yang L-L, Fu H-B, Zhao L, Zhong LL, Mu H-X, Shi X-K, Leung CF: Spexin enhances bowel movement through activating L-type voltage-dependent calcium channel via galanin receptor 2 in mice. *Sci Rep* 2015, 5:12095
 13. Gu L, Ma Y, Gu M, Zhang Y, Yan S, Li N, Wang Y, Ding X, Yin J, Fan N: Spexin peptide is expressed in human endocrine and epithelial tissues and reduced after glucose load in type 2 diabetes. *Peptides* 2015, 71:232–239
 14. Le Bars D, Gozariu M, Cadden SW: Animal models of nociception. *Pharmacol Rev* 2001, 53:597–652
 15. Bannon AW, Malmberg AB: Models of nociception: hot-plate, tail-flick, and formalin tests in rodents. *Curr Protoc Neurosci* 2007, Chapter 8:Unit 8.9
 16. Tjølsen A, Berge O-G, Hunskaar S, Rosland JH, Hole K: The formalin test: an evaluation of the method. *Pain* 1992, 51:5–17
 17. McNamara CR, Mandel-Brehm J, Bautista DM, Siemens J, Deranian KL, Zhao M, Hayward NJ, Chong JA, Julius D, Moran MM: TRPA1 mediates formalin-induced pain. *Proc Natl Acad Sci U S A* 2007, 104:13525–13530
 18. Reeta K, Mediratta P, Rath N, Jain H, Chugh C, Sharma K: Role of κ - and δ -opioid receptors in the antinociceptive effect of oxytocin in formalin-induced pain response in mice. *Regul Pept* 2006, 135:85–90
 19. Padi S, Kulkarni SK: Role of cyclooxygenase-2 in lipopolysaccharide-induced hyperalgesia in formalin test. *Indian J Exp Biol* 2005, 43:53–60
 20. Shamsi Meymandi M, Keyhanfar F: Assessment of the antinociceptive effects of pregabalin alone or in combination with morphine during acetic acid-induced writhing in mice. *Pharmacol Biochem Behav* 2013, 110:249–254
 21. Lv S-Y, Qin Y-J, Wang N-B, Yang Y-J, Chen Q: Supraspinal antinociceptive effect of apelin-13 in a mouse visceral pain model. *Peptides* 2012, 37:165–170
 22. Haley T, McCormick W: Pharmacological effects produced by intracerebral injection of drugs in the conscious mouse. *Br J Pharmacol Chemother* 1957, 12:12–15
 23. Lv S-Y, Qin Y-J, Wang H-T, Xu N, Yang Y-J, Chen Q: Centrally administered apelin-13 induces depression-like behavior in mice. *Brain Res Bull* 2012, 88:574–580
 24. Lv S-Y, Yang YJ, Hong S, Wang N-B, Qin Y-J, Li W-X, Chen Q: Intrathecal apelin-13 produced different actions in formalin test and tail-flick test in mice. *Protein Pept Lett* 2013, 20:926–931
 25. Collier HO, Dinneen LC, Johnson CA, Schneider C: The abdominal constriction response and its suppression by analgesic drugs in the mouse. *Br J Pharmacol Chemother* 1968, 32:295–310
 26. Wang CL, Yang DJ, Yuan BY, Wang Y: C-terminal hydrazide modification changes the spinal antinociceptive profiles of endomorphins in mice. *Peptides* 2018, 99:128–133
 27. Chadzinska M, Starowicz K, Scislowska-Czarnecka A, Bilecki W, Pierzchala-Koziec K, Przewlocki R, Przewlocka B, Plytycz B: Morphine-induced changes in the activity of proopiomelanocortin and prodynorphin systems in zymosan-induced peritonitis in mice. *Immunol Lett* 2005, 101:185–192
 28. Wang HB, Laverghetta AV, Foehring R, Deng YP, Sun Z, Yamamoto K, Lei WL, Jiao Y, Reiner A: Single-cell RT-PCR, in situ hybridization histochemical, and immunohistochemical studies of substance P and enkephalin co-occurrence in striatal projection neurons in rats. *J Chem Neuroanat* 2006, 31:178–199
 29. Gaveriaux-Ruff C, Nozaki C, Nadal X, Hever XC, Weibel R, Matifas A, Reiss D, Filliol D, Nassar MA, Wood JN: Genetic ablation of delta opioid receptors in nociceptive sensory neurons increases chronic pain and abolishes opioid analgesia. *Pain* 2011, 152:1238–1248
 30. Gabrilovac J, Čupić B, Zapletal E, Brozovic A: IFN- γ up-regulates kappa opioid receptors (KOR) on murine macrophage cell line J774. *J Neuroimmunol* 2012, 245:56–65
 31. Hashikawa-Hobara N, Ogawa T, Sakamoto Y, Matsuo Y, Ogawa M, Zamami Y, Hashikawa N: Calcitonin gene-related peptide pre-administration acts as a novel antidepressant in stressed mice. *Sci Rep* 2015, 5:12559
 32. Simpson DA, Feeney S, Boyle C, Stitt AW: Technical brief: retinal VEGF mRNA measured by SYBR green I fluorescence: a versatile approach to quantitative PCR. *Mol Vis* 2000, 6:178–183
 33. Livak KJ, Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2- $\Delta\Delta CT$ method. *Methods* 2001, 25:402–408
 34. Singewald N, Salchner P, Sharp T: Induction of c-Fos expression in specific areas of the fear circuitry in rat forebrain by anxiogenic drugs. *Biol Psychiatry* 2003, 53:275–283
 35. Saito Y, Miyasaka T, Hatsuta H, Takahashi-Niki K, Hayashi K, Mita Y, Kusano-Arai O, Iwanari H, Ariga H, Hamakubo T, Yoshida Y, Niki E, Murayama S, Ihara Y, Noguchi N: Immunostaining of oxidized DJ-1 in human and mouse brains. *J Neuropathol Exp Neurol* 2014, 73:714–728
 36. Mahmoudi M, Zarrindast M-R: Effect of intracerebroventricular injection of GABA receptor agents on morphine-induced antinociception in the formalin test. *J Psychopharmacol* 2002, 16: 85–91
 37. de Souza MM, Silote GP, Herbst LS, Funck VR, Joca SRL, Bejjani V: The antidepressant-like effect of galanin in the dorsal raphe nucleus of rats involves GAL2 receptors. *Neurosci Lett* 2018, 681:26–30
 38. Swanson CJ, Blackburn TP, Zhang X, Zheng K, Xu Z-QD, Hokfelt T, Wolinsky TD, Konkel MJ, Chen H, Zhong H, Walker MW, Craig DA, Gerald CPG, Branchek TA: Anxiolytic- and antidepressant-like profiles of the galanin-3 receptor (Gal(3)) antagonists SNAP 37889 and SNAP 398299. *Proc Natl Acad Sci U S A* 2005, 102:17489–17494
 39. Miyazaki H, Morishita J, Ueki M, Nishina K, Shiozawa S, Maekawa N: The effects of a selective inhibitor of c-Fos/activator protein-1 on endotoxin-induced acute kidney injury in mice. *BMC Nephrol* 2012, 13:153
 40. Spooner M-F, Robichaud P, Carrier J, Marchand S: Endogenous pain modulation during the formalin test in estrogen receptor beta knockout mice. *Neuroscience* 2007, 150:675–680
 41. Basbaum AI, Bráz JM, Kruger L, Light A: Transgenic mouse models for the tracing of “pain” pathways. Edited by Kruger L, Light AR. In *Translational Pain Research: From Mouse to Man*. Boca Raton, FL: CRC Press/Taylor & Francis, 2010. pp. 1–17
 42. Eipper BA, Stoffers DA, Mains RE: The biosynthesis of neuropeptides: peptide alpha-amidation. *Annu Rev Neurosci* 1992, 15:57–85
 43. Lang R, Gundlach AL, Holmes FE, Hobson SA, Wynick D, Hokfelt T, Kofler B: Physiology, signaling, and pharmacology of galanin peptides and receptors: three decades of emerging diversity. *Pharmacol Rev* 2015, 67:118–175
 44. Dirksen R: Opioid receptors and pain. *Pharm Weekbl* 1990, 12: 41–45

45. Millan MJ: The induction of pain: an integrative review. *Prog Neurobiol* 1999, 57:1–164
46. Kivell B, Prisinzano TE: Kappa opioids and the modulation of pain. *Psychopharmacology* 2010, 210:109–119
47. Obara I, Parkitna JR, Korostynski M, Makuch W, Kaminska D, Przewlocka B, Przewlocki R: Local peripheral opioid effects and expression of opioid genes in the spinal cord and dorsal root ganglia in neuropathic and inflammatory pain. *Pain* 2009, 141:283–291
48. Muschamp JW, Carlezon WA Jr: Roles of nucleus accumbens CREB and dynorphin in dysregulation of motivation. *Cold Spring Harb Perspect Med* 2013, 3:a012005
49. Munglani R, Hunt SP: Molecular biology of pain. *Br J Anaesth* 1995, 75:186–192
50. Sun WZ, Shyu BC, Shieh JY: Nitrous oxide or halothane, or both, fail to suppress c-fos expression in rat spinal cord dorsal horn neurones after subcutaneous formalin. *Br J Anaesth* 1996, 76:99–105
51. Ahmad AH, Ismail Z: c-fos and its consequences in pain. *Malays J Med Sci* 2002, 9:3–8
52. Corvello CM, Metz R, Bravo R, Armelin MC: Expression and characterization of mouse cFos protein using the baculovirus expression system: ability to form functional AP-1 complex with coexpressed cJun protein. *Cell Mol Biol Res* 1995, 41:527–535
53. Dubner R, Ruda MA: Activity-dependent neuronal plasticity following tissue injury and inflammation. *Trends Neurosci* 1992, 15:96–103
54. Dragunow M, Faull R: The use of c-fos as a metabolic marker in neuronal pathway tracing. *J Neurosci Methods* 1989, 29:261–265
55. Fields HL, Heinricher MM, Mason P: Neurotransmitters in nociceptive modulatory circuits. *Annu Rev Neurosci* 1991, 14:219–245
56. Bandler R, Shipley MT: Columnar organization in the midbrain periaqueductal gray: modules for emotional expression? *Trends Neurosci* 1994, 17:379–389
57. Maletic V, Raison CL: Neurobiology of depression, fibromyalgia and neuropathic pain. *Front Biosci (Landmark Ed)* 2009, 14:5291–5338
58. Sonnenberg JL, Rauscher FJ 3rd, Morgan JI, Curran T: Regulation of proenkephalin by Fos and Jun. *Science* 1989, 246:1622–1625
59. Ziolkowska B, Przewlocka B, Mika J, Labuz D, Przewlocki R: Evidence for Fos involvement in the regulation of proenkephalin and prodynorphin gene expression in the rat hippocampus. *Brain Res Mol Brain Res* 1998, 54:243–251