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Research article

Collateral projections from the lateral parabrachial nucleus to the paraventricular thalamic nucleus and the central amygdaloid nucleus in the rat

Shao-Hua Liang[a], Jun-Bin Yin[b], Yi Sun[a], Yang Bai[b], Kai-Xiang Zhou[b,c], Wen-Jun Zhao[b,c], Wei Wang[a,**], Yu-Lin Dong[b,**], Yun-Qing Li[a,b,d,*]

[a] Department of Human Anatomy, Histology and Embryology, School of Basic Medical Sciences, Fujian Medical University, Fuzhou, Fujian 350108, China
[b] Department of Anatomy, Histology and Embryology and K.K. Leung Brain Research Centre, The Fourth Military Medical University, Xi'an 710032, China
[c] Student Brigade, The Fourth Military Medical University, Xi'an 710032, China
[d] Collaborative Innovation Center for Brain Science, Fudan University, Shanghai 200032, China

HIGHLIGHTS

- Some neurons in the LPB can collateralize to PVT and CeA.
- Almost all the LPB neurons (95%) sending fibers to both PVT and CeA are CGRPergic.
- About 94% of the LPB neurons with axon collaterals to PVT and CeA are activated (FOS immunoreactivity) in the chronic pain.

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ABSTRACT

Combined the retrograde double tracing with immunofluorescence histochemical staining, we examined the neurons in the lateral parabrachial nucleus (LPB) sent collateral projections to the paraventricular thalamic nucleus (PVT) and central amygdaloid nucleus (CeA) and their roles in the nociceptive transmission in the rat. After the injection of Fluoro-gold (FG) into the PVT and tetramethylrhodamine-dextran (TMR) into the CeA, respectively, FG/TMR double-labeled neurons were observed in the LPB. The percentages of FG/TMR double-labeled neurons to the total number of FG- or TMR-labeled neurons were 6.18% and 9.09%, respectively. Almost all of the FG/TMR double-labeled neurons (95%) exhibited calcitonin gene-related peptide (CGRP) immunoreactivity. In the condition of neuropathic pain, 94% of these neurons showed FOS immunoreactivity. The present data indicates that some of CGRP-expressing neurons in the LPB may transmit nociceptive information toward the PVT and CeA by way of axon collaterals.
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1. Introduction

As the relay station of sensory information, both the lateral parabrachial nucleus (LPB) and the thalamic nuclei are thought to be the critical sites for central perception, integration and transmission of sensory information especially nociceptive information [1,19,20,26]. However, in the rodents, the LPB receives about 90% noxious stimuli from the projection neurons in spinal dorsal horn, and then sends strong efferent projections to the central amygdaloid nucleus (CeA), which is the classical spino-parabrachio-amygdaloid pain-processing pathway in the central nervous system [13]. Recent studies have shown that this LPB-CeA pathway is not only involved in nociceptive transmission but also in regulation of pain-related negative emotions [11,25,28].

As one of the thalamic midline nuclei, paraventricular thalamic nucleus (PVT) is considered to be an important site for the integration of many descending and ascending signals to modulate both visceral information and negative emotions [5,12,18,22]. It has been reported that the fibrous projections between PVT and the subnuclei of parabrachial nucleus (PBN) display accurate topographical organization [15]. However, the role of LPB-PVT pathway played in pain processing is still unknown. Thus, we aimed to explore whether the LPB neurons sending fibers to the CeA might also

* Corresponding author at: Department of Human Anatomy, Histology and Embryology, School of Basic Medical Sciences, Fujian Medical University, Fuzhou, Fujian 350108, China.
** Corresponding authors.
E-mail addresses: wangwei0976@163.com (W. Wang), donganat@fmmu.edu.cn (Y.-L. Dong), deptanat@fmmu.edu.cn (Y.-Q. Li).

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project to the PVT and their roles in the nociceptive transmission by retrograde fluorescent double labeling and immunofluorescence histochemical staining.

2. Materials and methods

2.1. Animals

A total of sixteen Sprague-Dawley male rats weighting from 280 to 320 g were used for the experiment and were housed on a standard 12 h light/dark cycle and given food and water ad libitum. Before surgery, the rats were handled for 3–5 days to avoid stress. All protocols had been approved by the Ethics Committee of Animal Use for Research and Education of the Fourth Military Medical University (Xi'an, P.R. China). The number of animals was used as little as possible and their suffering was minimized to the lowest degree according to IASP guidelines [29].

2.2. Brain stereotaxic injection

All the rats were performed the brain stereotaxic injection. The procedures were shown below: the rats were fixed on a stereotaxic apparatus (NARISHIGE Scientific Instrument Lab, Tokyo, Japan). Fluoro-gold (FG, 80014, Biotium, Hayward, CA, USA) and tetramethylrhodamine-dextran (TMR, D3308, 3000MW, Molecular Probe, Eugene, OR, USA) were used as retrograde tracers to delineate the PVT and CeA, respectively. 0.04 μl of 4% FG dissolved in distilled water was stereotaxically pressure-injected into the right PVT through a glass micropipette (internal tip diameter 50–80 μm) attached to a 1 μl Hamilton microsyringe. And then the same injection was made into the ipsilateral CeA with 0.05 μl of 10% TMR dissolved in trisodium citrate solution (pH 3.0). Injection coordinates were shown as follows: anteroposterior, −3.5 mm; lateral to midline, 0.3 mm; dorsoventral, 5.6 mm for the PVT and anteroposterior, −2.3 mm; lateral to midline, 4.3 mm; dorsoventral, 8.3 mm for the CeA. After surgery, the rats were allowed to survive for 7 days.

2.3. Spared nerve injury (SNI) surgery

The SNI surgery, a neuropathic pain model, was performed on seven rats as reported previously according to Wolf [4]. Briefly, rats were anesthetized with pentobarbital sodium (40 mg/kg, i.p.). A small incision was made on the skin and then on the biceps femoris muscle in the left thigh to expose the sciatic nerve and its three terminal branches. The common peroneal and tibial branches were carefully separated and two ligations were made on each nerve with 5-0 silk sutures. The nerves were then cut off between the ligations with 2–4 mm of the distal nerve stump removed at the same time. Muscular fascia and skin were closed, respectively. After the SNI surgery, brain stereotaxic surgery was immediately carried out as described above. The rats were allowed to survive for 10 days. The SNI rats with obviously decreased mechanical pain threshold by von Frey text (Stoelting, Kiel, WI, USA) were chosen for further study.

2.4. Brain slice preparation

All animals were anesthetized with pentobarbital sodium (60 mg/kg, i.p.) and then transcardially perfused with 150 ml of 0.01 M phosphate-buffered saline (PBS, pH 7.4), followed by 500 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). After perfusion, the brains were removed immediately and immersed in a solution of 30% sucrose in 0.1 M PB (4 °C) until the brains completely sunk to the bottom of the container. Subsequently, the injections of PVT and CeA were serially cut into coronal sections at 40 μm and the brain tissue of LPB were serially cut into 35-μm-thick coronal slices on a freezing microtome (CM1950, Leica, Heidelberg, Germany). All sections were divided into six series and collected into 0.01 M PBS.

2.5. Immunofluorescent histochemical staining

The brain series of PVT and CeA were used for observation of the injection sites under an epifluorescence microscope (BX-60; Olympus, Tokyo, Japan). If the injection sites were successful, their brain slices of the LPB were used for further study.

Double-(FG and TMR) and triple- (FG, TMR and CGRP; FG, TMR and FOS) immunofluorescence staining were performed. All the antisera used here were shown in Table 1. The brain slices of the LPB were blocked in the 10% normal donkey serum for 30 min at room temperature and then incubated at 4 °C for 18–24 h with primary antisera in 0.01 M PBS contained 1% normal donkey serum, 0.3% Triton X-100, 0.02% sodium azide, and 0.12% carrageenan (pH 7.4). However, the sections needed to be incubated with Goat anti-CGRP at 4 °C for 3 d. Then, they were incubated with secondary antisera (fluorescein-labeled IgG) for 6–8 h at 4 °C. In the negative control experiments, the primary antisera were omitted, and the other steps were the same as the experimental groups.

2.6. Images acquisition and stereological analysis

After the immunofluorescence histochemical staining, microscopic images of mounted sections were taken using a confocal

Table 1
Antisera used for double- and triple-labeling immunofluorescence staining.

Group	Primary antisera	Secondary antisera
FG/TMR	Guinea pig anti-FG (1:200,NM-101,ProtosBiotech, NewYork, NY, USA) Rabbit anti-TMR (1:200,A6397,Invitrogen, Carlsbad, CA, USA)	Alexa 488 donkey anti-guinea pig (1:500,706-545-148,Jackson Immunoresearch,West Grove, PA, USA) Alexa 594 donkey anti-rabbit (1:500, A21207, Invitrogen)
FG/TMR/CGRP	Goat anti-CGRP (1:200,Ab36001,abcam, Cambridge, MA, USA) Rabbit anti-TMR (1:200, A6397, Invitrogen) Guinea pig anti-FG (1:200, NM-101, ProtosBiotech)	Alexa 488 donkey anti-goat (1:500, A11055, Invitrogen) Alexa 594 donkey anti-rabbit (1:500, A21207, Invitrogen) Alexa 647 goat anti-guinea pig (1:500, A21450, Invitrogen)
FG/TMR/FOS	Guinea pig anti-FG (1:200,NM-101,ProtosBiotech) Rabbit anti-TMR (1:200, A6397, Invitrogen) Mouse anti-FOS (1:500, ab11959, Abcam)	Alexa 488 donkey anti-guinea pig (1:500, 706-545-148,JacksonImmunoresearch) Alexa 594 donkey anti-rabbit (1:500, A21207, Invitrogen) Alexa 647 donkey anti-mouse (1:500, A31571, Invitrogen)

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