Collateral Projections from the Lateral Parabrachial Nucleus to the Central Amygdaloid Nucleus and the Ventral Tegmental Area in the Rat

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ABSTRACT

Lateral parabrachial nucleus (LPB) is a critical region in the integration and transmission of peripheral nociceptive information. The parabrachio-amygdaloid (P-Amy) pathway and parabrachio-ventral tegmental area (P-VTA) pathway is thought to be significant in regulation of pain-related negative emotions. In present study, retrograde tract tracers Fluoro-gold (FG) and tetramethylrhodramine-dextran (TMR) were stereotaxically injected into the right central amygdaloid nucleus (CeA) and right VTA, respectively. Then, part of these rats were performed with the spare nerve injury (SNI) in the controlateral side of FG and TMR injection. Afterwards, double- or triple-immunofluorescent histochemistry was used to examine FG/TMR double- and FG/TMR/FOS or FG/TMR/CGRP triple-labeled neurons in the LPB. The results showed that all of FG, TMR single- and FG/TMR double-labeled neurons were distributed in the LPB bilaterally with an ipsilateral predominance. The proportion of FG/TMR double-labeled neurons to the total number of FG- and TMR-labeled neurons was 10.78% and 13.07%, respectively. Nearly all of the FG/TMR double-labeled neurons (92.67%) showed calcitonin gene-related peptide (CGRP) immunopositive. On the other hand, in the SNI rats, about 89.49% and 77.87% of FG- and TMR-labeled neurons were FG/FOS- and TMR/FOS-positive neurons; about 93.33% of the FG/TMR double-labeled neurons were FOS-LI. Our results suggest that the part of CGRP immunopositive neurons in the LPB send projection fibers to both the CeA and VTA by the way of axon collaterals, which are activated by the nociceptive stimulation in the SNI condition, and may play an important role in the transmission of peripheral nociceptive information. Anat Rec, 302:1178–1186, 2019. © 2018 The Authors. *The Anatomical Record* published by Wiley Periodicals, Inc. on behalf of American Association of Anatomists.

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INTRODUCTION

The lateral parabrachial nucleus (LPB) is considered to be a critical region in the integration and transmission of peripheral nociceptive information (Menani and Johnson, 1995; Aicher et al., 2013; Missig et al., 2014; Strobel et al., 2014; Lu et al., 2015). Previous studies suggested that the LPB receives about 90% noxious stimuli from the projection neurons in the spinal dorsal horn (Spike et al., 2003), and then sends projection fibers to the central amygdaloid nucleus (CeA), which constitute the spino-parabrachio-amygdaloid pain-processing pathway in the central nervous system and play an important role in regulation of pain-related negative emotions (Hunt and Mantyh, 2001; Watabe et al., 2013; Han et al., 2015; Sato et al., 2015). Further studies revealed that a large numbers of neurons relaying pain signals to the CeA in the LPB of rats express calcitonin gene-related peptide (CGRP)-like immunoreactivity (Hunt and Mantyh, 2001; Watabe et al., 2013; Han et al., 2015; Sato et al., 2015).

As one of the relay station between the anterior brain and the brainstem, the ventral tegmental area (VTA) is considered that not only related to reward, addiction, internal secretion, and fear-conditioning, but also an important gathering station of pain regulation and the generation of depression (Bair et al., 2003; Ikemoto, 2007; Coizet et al., 2010; Hausknecht et al., 2017). A number of studies suggested that the VTA dopaminergic neurons are activated in the case of acute injury to animals, including the increase of the dopamine (DA) release and the enhancement of neuronal electrical activity (Holly and Miczek, 2016; Hausknecht et al., 2017; Lohani et al., 2017). And the behavioral studies reported that suppressing the projections of the VTA dopaminergic neurons to the prefrontal cortex are able to rapidly regulate depression-like behavior in mice (Chaudhury et al., 2013). Therefore, the VTA is thought to be a key region associated with chronic pain and depression (Chaudhury et al., 2013; Tye et al., 2013; Lohani et al., 2017). Recently, it has been reported that in the VTA, some projections fibrous originating from the LPB neurons may modulate salt appetite related functions (Miller et al., 2011). However, whether LPB–VTA projection pathway is involved in pain processing is still unknown. On the other hand, whether some LPB–CeA projection neurons also send projection fibers to the VTA is still unclear.

The aim of this study was to examine whether the LPB neurons of rats project to the CeA and VTA by way of axon collaterals and express CGRP-like immunoreactivity, by using double-retrograde-tract tracer FG and TMR injected into the CeA and VTA, respectively, and combining triple-immunofluorescent histochemical staining. Additionally, we explored whether the collateral projection neurons in the LPB of rats might be related with nociceptive signal transmission by detecting the immunoreactivity for FOS, protein product of *c-fos* proto oncogene after giving the nociceptive stimulation in the spared nerve injury (SNI) neuropathic model of rats.

MATERIALS AND METHODS

Animals

A total of 23 Sprague–Dawley male rats (280–320 g) were used in this experiment. All animal procedures in the present experiments were approved by the Animal Care and Use Committee at the Fourth Military Medical University (Xi'an, China). All efforts were made to minimize animal suffering and the number of animals used.

Brain Stereotaxic Injection of FG and TMR into the CeA and VTA for Retrograde Tract-Tracing

All surgical procedures for 23 rats were performed under general anesthesia by intraperitoneal injection of sodium pentobarbital (40 mg/kg body weight). The anesthetized rats were placed onto a stereotaxic frame (NARISHIGE, Tokyo, Japan). According to the stereotaxic coordinates in the stereotaxic atlas of Paxinos and Watson (2007), 0.04 μl of 4% Fluoro-gold (FG, 80014, Biotium, Hayward, CA) dissolved in distilled water was stereotaxically injected into the right CeA (2.30 mm posterior to the Bregma, 4.10 mm right to the midline, and 8.30 mm deep from the brain surface) of the rats, for which a glass micropipette (internal tip diameter 15–25 μm) attached to a 1 μl Hamilton microsyringe was used. Each injection was made by pressure over a period of 10 min and the micropipette was left in the place for additional 20 min after the injection. By the same procedure, a single injection of 0.05 μl of 10% tetramethylrhodamine-dextran (TMR, D3308, 3,000 MW, Molecular Probe, Eugene, OR) dissolved in trisodium citrate solution (pH 3.0) was made stereotaxically into the right VTA of the rats (5.76 mm posterior to the Bregma, 0.7 mm right to the midline, and 8.30 mm deep from the brain surface).

Spared Nerve Injury (SNI) Surgery

After the brain stereotaxic injection, the SNI surgery was performed (Li et al., 2006). Briefly, a small incision was made on the skin of the left hind limb of rats. Then subcutaneous tissue and muscle were separated to expose the sciatic nerve and its three terminal branches. The common peroneal and tibial branches were carefully separated and tightly ligated with 5.0 silk. 2–3 mm of the nerves were then cut off distal to the ligation. The sural nerve was left intact, and the muscular fascia and skin were closed. The rats were allowed to survive for 7 days after the SNI surgery. Those rats with significantly decreased mechanical pain threshold by von Frey filements text (Stoelting, Kiel, WI) were chosen for further study.

Tissue Preparation

All rats were reanesthetized deeply by intraperitoneal injection of an overdose of sodium pentobarbital (100 mg/kg body weight) and then perfused transcardially with 150 ml of 0.01 M phosphate-buffered saline (PBS, pH 7.4), followed by 500 ml of 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). After perfusion, the brains of rats were removed and placed in 0.1 M PB containing 30% sucrose (w/v) overnight at 4°C. Subsequently, the brains were serially cut into coronal sections at 30 μm thickness on a freezing microtome (CM1950, Leica, Heidelberg, Germany). All sections were divided into five series of alternate serial sections.

One series of the sections was directly mounted onto clean glass slides and air dried. In these sections, the location and extent of the FG and TMR injection sites, as well as the distribution of FG- and TMR-labeled neurons, were observed with an epifluorescence microscope (BX60; Olympus, Tokyo, Japan) under an appropriate filter for FG (excitation 360–370 nm; emission ≥395 nm) and TMR (excitation 540–552 nm; emission 575–625 nm). Only the rats of which both FG injection site in CeA and TMR injection site in VTA were accurate, the brain slices of LPB could be used for further study.

Examination of FG and TMR Double-Labeled Neurons in the LPB

The second series of sections through the LPB was processed for FG and TMR double-labeled staining. The sections were incubated at room temperature sequentially with (1) guinea pig anti-FG (1:200, NM-101, Protos Biotech, New York, NY) and rabbit anti-TMR (1:200, A6397, Invitrogen, Carlsbad, CA) overnight, and (2) Alexa488-conjugated donkey antibody to guinea pig IgG (1:500, 706-545-148, Jackson Immunoresearch, West Grove, PA) and Alexa594-conjugated donkey antibody to rabbit (1:500, A21207, Invitrogen, Eugene, OR) for 6 hr. The (1)incubation was carried out at room temperature in PBS containing 0.3% (v/v) Triton X-100, 0.25% (w/v) λ-carrageenan, 0.05% NaN_3, and 1% (v/v) donkey serum (PBS-XCD). The (2) incubation was carried out at room temperature in PBS containing 0.3% (v/v) Triton X-100.

Triple-Immunofluorescence Histochemistry for FG/TMR/CGRP or FG/TMR/FOS

The third and fourth series of sections through the LPB were processed as the following procedure for FG/TMR/CGRP or FG/TMR/FOS. The sections of rats' LPB were incubated with a mixture of guinea pig anti-FG (1:200, Protos Biotech), rabbit anti-TMR (1:200, Invitrogen), and goat anti-CGRP (1:200, b36001, Abcam, Cambridge, MA), or mouse anti-FOS (1:500, ab11959, Abcam) overnight at room temperature. Then the sections were further incubated for 6 hr with Alexa 647-conjugated goat anti-guinea pig (1:500, A21450, Invitrogen), Alexa 594-conjugated donkey anti-rabbit (1:500, A21207, Invitrogen) and Alexa 488-conjugated donkey anti-goat (1:500, A11055, Invitrogen) or Alexa 488-labeled donkey anti-mouse (1:500; Invitrogen). The incubations for different steps were done in the same medium as abovementioned. Another set of sections was treated with the same procedures except that the primary antibodies were removed or replaced by normal serum, and the labeling results were negative.

Stereological Analysis

After the immunofluorescence histochemical staining, all sections were mounted onto glass slides and cover slipped with 50% (v/v) glycerol and 2.5% (w/v) triethylenediamine in PBS followed by observation with a confocal laser scanning microscope (FV-1000; Olympus, Tokyo, Japan). Digital images were captured and analyzed with Fluoview software (FV10-ASW 1.7, Olympus). The immunoreactive neurons in the LPB were estimated by stereological methods (Coggeshall, 1992). The analysis was performed by using the modification of the optical dissector method with the aid of the Stereo Investigator software (Micro Bright-Field, Williston, VT) (Polgar et al., 2004). One out of 3 series of alternate serial sections was observed, and all sequential sections, were evenly divided into 3 groups that represented the rostral, middle, and caudal part of the LPB. Then, by a completely randomized digital table, 1, 3, and 1 sections were randomly selected from the rostral, middle, and caudal groups of the LPB sections, respectively. Thus, 5 sections of 35 μm thickness covering the whole rostral–caudal axis of the LPB were selected from each rat (n = 5 rats, CGRP and FOS, respectively; total 25 sections) that exhibited accurate injection sites into both the CeA and VTA. Subsequently, we counted the number of double-labeled neurons and triple-labeled neurons. All the data were shown in mean ± standard deviation (SD).

RESULTS

The Injection Sites of FG in the CeA and TMR in the VTAs

In 23 rats, 10 rats showed the successful injection sites, including five with SNI models. In the 10 rats, both the FG injection into the CeA region and the TMR injection into the VTA were made and tracer injection sites were displayed in Figure 1. In the site of the FG injection into the CeA (Fig. 1A1,A2) and TMR injection into the VTA (Fig. 1A1,A3), a dense core of the tracer was surrounded by a diffuse halo of the tracer, and the brightest injection area was considered to represent the injection site (Fig. 1A2,A3). The diffusion extent of the FG (Fig. 1B1–B3,D1–D3) and TMR (Fig. 1C1–C3,E1–E3) injection sites at different coronal sections were exhibited according to the stereotaxic coordinates by Paxinos and Watson (2007). Although the location and extent of the injection sites in these 10 rats (Rat 1, Rat 3, Rat 6, Rat 7, and Rat 9 in TMR/FG/CGRP; Rat 11, Rat 12, Rat 15, Rat 19, and Rat 21 in TMR/FG/FOS) were somewhat variable, the distribution patterns of retrograde labeling in the LPB were all quite similar (seen in Tables 1–3).

Neurons in the LPB Sent Collateral Projections to the CeA and VTA

The brain slices of the five rats (Rat 1, Rat 3, Rat 6, Rat 7, and Rat 9) with successful injections were carried out by FG/TMR double-labeled immunofluorescence histochemistry. By confocal laser microscopic detection, almost all retrogradely labeled neurons showed the

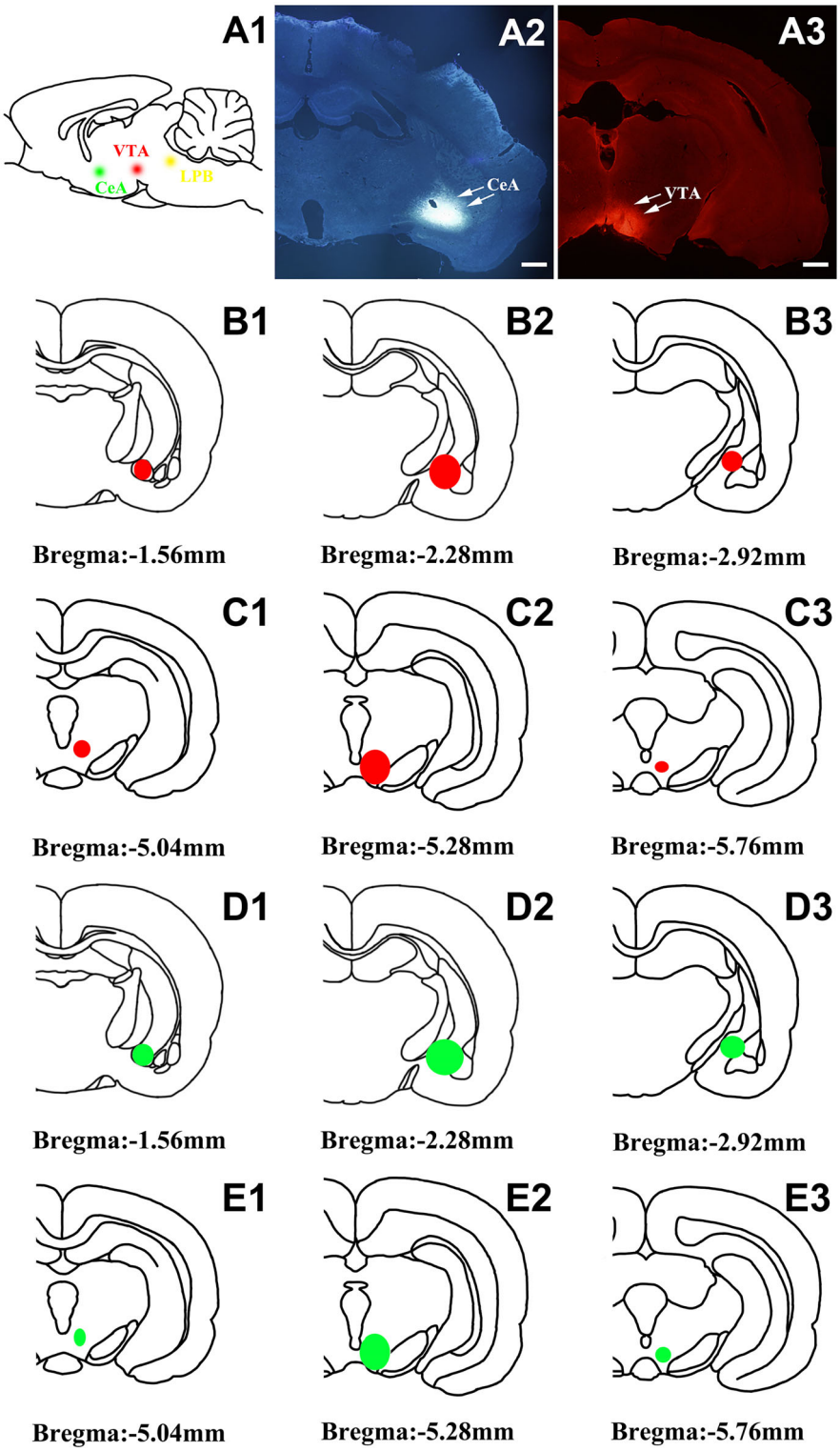

Fig. 1. Injection sites of FG into CeA and TMR into VTA. Brief drawing (A1) displayed the site of injection and projection in the whole brain. The photomicrographs showed the injection sites of FG into the CeA (A2) and TMR into the VTA (A3). Brief drawings showed the rostrocaudal extent of the FG (B1, B2, and B3) and TMR (C1, C2, and C3) injection sites at different levels by the red areas with the CGRP rats, the FG (D1, D2, and D3) and TMR (E1, E2, and E3) injection sites at different levels by the green areas with the FOS rats. The numbers in B1–B3, C1–C3, D1–D3, and E1–E3 indicated the distance in millimeters (mm) posterior to the Bregma in rats. Scale bar = 200 μm in A2 and A3.

TABLE 1. The number and percentages of FG, TMR, and FG/TMR double-labeled neurons in the LPB

	FG	TMR	FG + TMR	(FG + TMR)/FG(%)	(FG + TMR)/TMR(%)
Rat 1	53	46	6	11.32	13.04
Rat 3	57	43	6	10.53	13.95
Rat 6	53	40	5	9.43	12.50
Rat 7	46	41	5	10.87	12.20
Rat 9	51	44	6	11.76	13.64
Total	260 ± 4	214 ± 2	28 ± 1	10.78 ± 0.89	13.07 ± 0.74

FG was injected into the right side CeA, whereas TMR was injected into the right side VTA. Counts were made on five sections from a series of every fifth 30-μm-thick section in the right LPB. FG + TMR/FG(%) represents the percentage of FG/TMR double-labeled neurons to total number of FG-labeled neurons. FG + TMR/TMR(%) represents the percentage of FG/TMR double-labeled neurons to total number of TMR-labeled neurons. All the data of total were shown in mean ± standard deviation (SD).

TABLE 2. The number and percentages of FG/TMR/CGRP triple-labeled neurons in the LPB

	FG	TMR	F + C	(F + C)/F (%)	T + C	(T + C)/T (%)	F + T	F + T + C	(F + T + C)/(F + T) (%)
Rat 1	55	47	51	92.73	9	19.15	6	5	83.33
Rat 3	52	41	45	86.54	13	31.71	5	5	100
Rat 6	51	42	45	88.24	15	35.71	5	5	100
Rat 7	47	39	37	78.72	10	25.64	5	4	80.00
Rat 9	49	42	40	81.63	9	21.43	5	5	100
Total	254 ± 3	211 ± 3	218 ± 5	85.57 ± 5.52	56 ± 3	26.73 ± 6.93	26 ± 1	24 ± 1	92.67 ± 10.11

FG was injected into the right side CeA, whereas TMR was injected into the right side VTA. Counts were made on five sections from a series of every fifth 30-μm-thick section in the right LPB. F + C represents the number of FG/CGRP double-labeled neurons. (F + C)/F(%) represents the percentage of FG/CGRP double-labeled neurons to total number of FG-labeled neurons. T + C represents the number of TMR/CGRP double-labeled neurons. (T + C)/T (%) represents the percentage of TMR/CGRP double-labeled neurons to total number of TMR-labeled neurons. F + T indicates the number of FG/TMR double-labeled neurons. F + T + C represents the number of FG/TMR/CGRP triple-labeled neurons. (F + T + C)/(F + T) (%) represents the percentage of FG/TMR/CGRP triple-labeled neurons to total number of FG/TMR double-labeled neurons. All the data of total were shown in mean ± standard deviation (SD).

TABLE 3. The number and percentages of FG/TMR/FOS triple-labeled neurons in the LPB

	FG	TMR	F + S	(F + S)/F (%)	T + S	(T + S)/T (%)	F + T	F + T + S	(F + T + S)/(F + T) (%)
Rat 11	55	48	51	92.73	38	79.17	6	5	83.33
Rat12	47	46	40	85.11	37	80.43	4	4	100
Rat 15	49	43	45	91.84	32	74.42	5	5	100
Rat 19	56	50	50	89.29	41	82.00	6	5	83.33
Rat 21	52	45	46	88.46	33	73.33	5	5	100
Total	259 ± 4	232 ± 3	232 ± 4	89.49 ± 3.01	181 ± 4	77.87 ± 3.80	26 ± 1	24 ± 1	93.33 ± 9.13

FG was injected into the right side CeA, whereas TMR was injected into the right side VTA. Counts were made on five sections from a series of every fifth 30-μm-thick section in the right LPB. F + S represents the number of FG/FOS double-labeled neurons. (F + S)/F (%) represents the percentage of FG/FOS double-labeled neurons to total number of FG-labeled neurons. T + S represents the number of TMR/FOS double-labeled neurons. (T + S)/T(%) represents the percentage of TMR/FOS double-labeled neurons to total number of TMR-labeled neurons. F + T indicates the number of FG/TMR double-labeled neurons. F + T + S represents the number of FG/TMR/FOS triple-labeled neurons. (F + T + S)/(F + T) (%) represents the percentage of FG/TMR/FOS triple-labeled neurons to total number of FG/TMR double-labeled neurons. All the data of total were shown in mean ± standard deviation (SD).

neuronal soma and process, which were located in the LPB with an ipsilateral predominance of FG and TMR injection. Thus, all the following description and statistical results in Table 1 were carried out in the ipsilateral side. FG- and TMR-labeled neurons densely distributed throughout the rostral–caudal extent of the LPB, and only a few FG/TMR double-labeled neurons were sparsely distributed in the LPB (Fig. 2). By cell counting, the proportion of FG/TMR double-labeled neurons to the total number of FG- and TMR-labeled neurons in LPB were 10.78% and 13.07%, respectively (Table 1).

The LPB Neurons Collateralized Projecting to the CeA and VTA Express CGRP-Like Immunoreactivity

To examine whether the LPB collateral projection neurons identified above expressed CGRP-like immunoreactivity, FG/TMR/CGRP triple-immunofluorescence labeling was used. By confocal laser scanning microscopic detection, CGRP-labeled neurons were mainly observed in the neuronal soma and labeled in a high density in the LPB (Fig. 3). By cell counting, the FG/CGRP double-labeled neurons accounted for 85.57% of the total FG-labeled

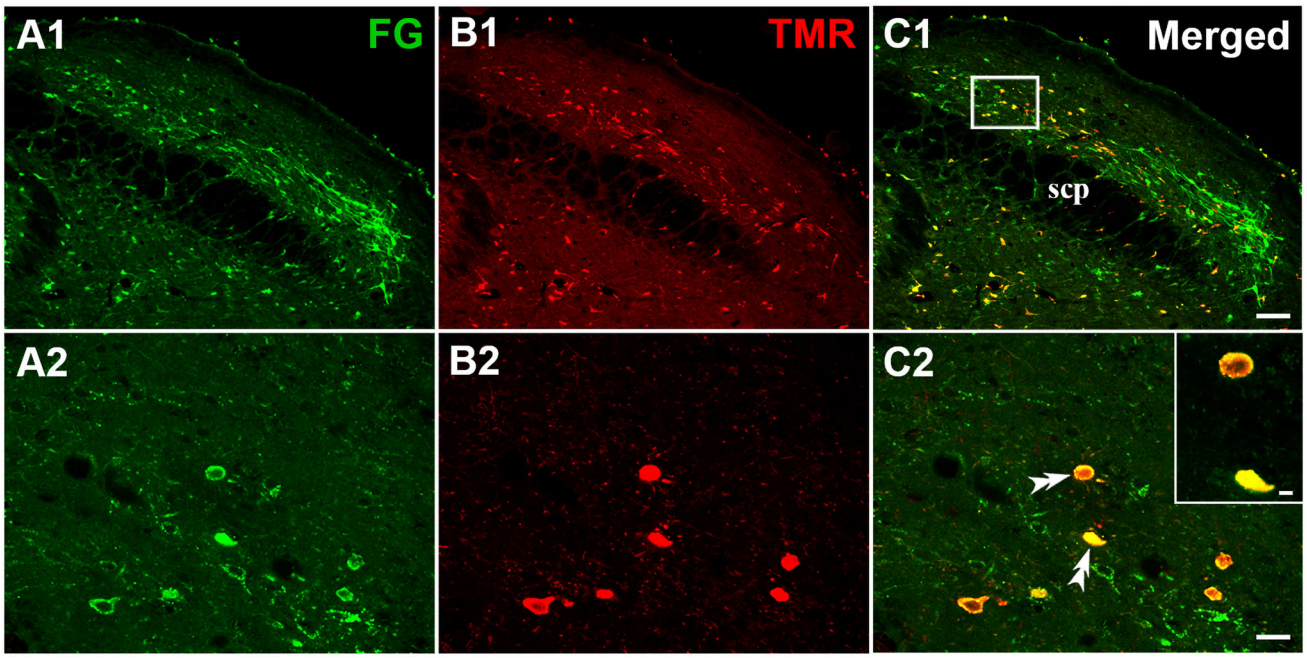

Fig. 2. The FG/TMR double-labeled neurons in the LPB. The immunofluorescent histochemical photomicrographs showed the FG-labeled neurons (A1 and A2, green), TMR-labeled neurons (B1 and B2, red), and FG/TMR double-labeled neurons (C1 and C2, Merged) in the LPB. The area demarcated with a rectangle in C1 was enlarged in A2–C2. The double-arrowheads in C2 indicated FG/TMR double-labeled neurons and were further enlarged (inset). Scale bar = 100 μm (A1–C1), 20 μm (A2–C2), and 5 μm (inset).

neurons, while 26.73% of the TMR-labeled neurons were CGRP positive. Specifically, almost 92.67% of the FG/TMR double-labeled neurons were CGRPergic in the LPB (Table 2).

Neurons in the LPB Sent Collateral Projections to the CeA and VTA were FOS-Positive in Neuropathic Pain

To further examine whether the LPB collateral projection neurons identified above may be involved in the transmission of somatic nociceptive information in the SNI model of rats, FG and TMR retrograde tract tracing combined with FOS immunohistochemistry was performed. The brain slices of five SNI rats (Rat 11, Rat 12, Rat 15, Rat 19, and Rat 21) with successful injections were carried out FG/TMR/FOS triple-labeled immunofluorescence histochemistry. FOS immunoreactive neurons showed extensive distribution in the LPB and mainly labeled in the neuronal nucleus (Fig. 4). By cell counting, the FG/FOS and TMR/FOS double-labeled neurons accounted for 89.49% and 77.87% of the total FG and TMR retrograde-labeled neurons, respectively. While FG/TMR/FOS triple-labeled neurons were 93.33% of the total FG/TMR double-labeled neurons in the LPB (Table 3).

DISCUSSION

In this study, we revealed that part of CGRP immunopositive neurons in the LPB projected to the CeA and VTA by the way of axon collaterals. Furthermore, in the SNI condition, these collateral projection neurons in the LPB can be activated by nociceptive stimuli. Our results indicated that the CGRP immunopositive neurons in the LPB of rat which send the projection fibers to both the CeA and VTA might be related with nociceptive signal transmission.

It is known that the LPB is a critical region in the integration and transmission of peripheral nociceptive information, which delivers various kinds of sensory information to the thalamus, amygdala, VTA, periaqueductal gray, and other nuclei (Fulwiler and Saper, 1984). On the other hand, the CeA is a part of the classical spino-parabrachio-amygdaloid pain-processing pathway in the central nervous system (Hunt and Mantyh, 2001). The LPB–CeA pathway is related with not only nociceptive information transmission but also regulation of pain-related negative emotions (Watabe et al., 2013; Han et al., 2015; Sato et al., 2015). Recently, it has been reported that a part of LPB neurons send projection fibers to VTA, and involved in modulation of salt appetite related functions (Miller et al., 2011). In our present experiment, we observed that the distribution of FG retrograde-labeled neurons from CeA and TMR retrograde-labeled neurons from VTA in LPB were similar to the previous studies (Coizet et al., 2010; Miller et al., 2011; Watabe et al., 2013; Han et al., 2015; Sato et al., 2015). Importantly, there were also neurons in the LPB double-labeled with FG and TMR, indicating these neurons sending their projection fibers to the CeA can also project to the VTA by the way of axon collaterals.

CGRP is a kind of neuropeptides, widely distributed in the nervous system. A lot of studies reported that the neurons in the LPB projected to CeA are mainly CGRPergic, which make CGRP one of functional markers of parabrachial inputs to the CeA (Dobolyi et al., 2005; D'Hanis et al., 2007). Moreover, the exclusive source of CGRP in

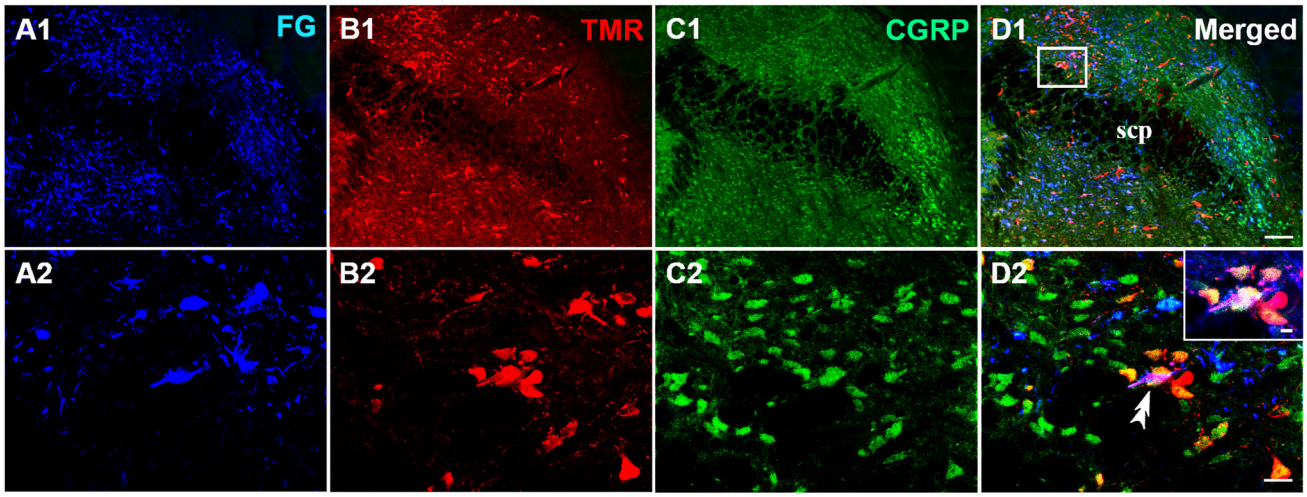

Fig. 3. The FG/TMR/CGRP tripled-labeled neurons in the LPB. The immunofluorescent histochemical photomicrographs showed the FG- (A1 and A2, blue), TMR- (B1 and B2, red), CGRP-labeled neurons (C1 and C2, green) and the FG/TMR/CGRP tripled-labeled neurons (D1 and D2, Merged) in the LPB. The area demarcated with a rectangle in D1 was enlarged in A2–D2. The double-arrowhead in D2 represented an FG/TMR/CGRP tripled-labeled neuron and was further enlarged (inset). Scale bar = 100 μm (A1–D1), 20 μm (A2–D2), and 5 μm (inset).

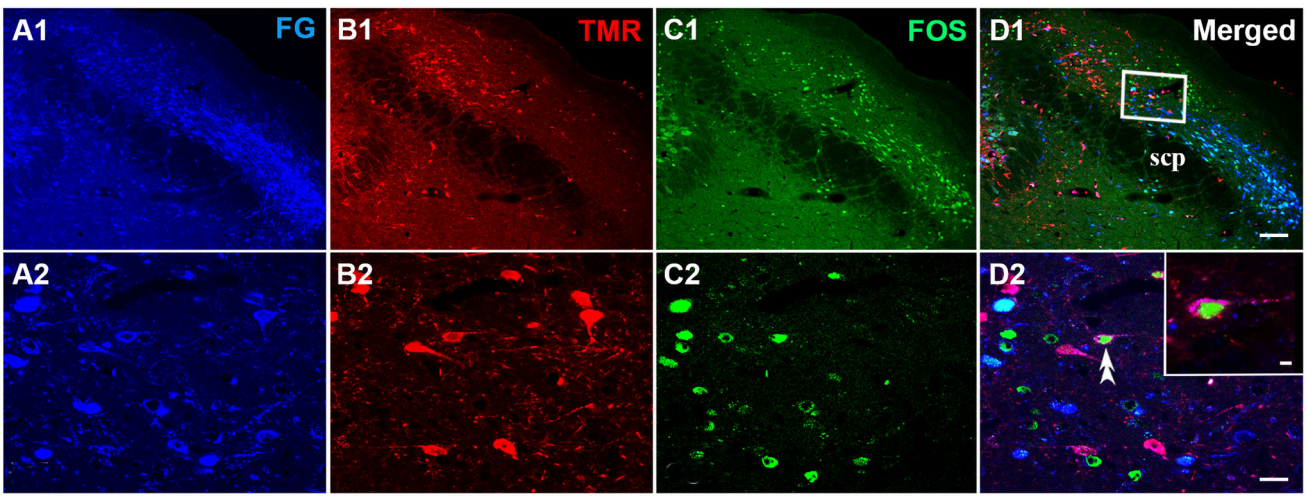

Fig. 4. The FG/TMR/FOS tripled-labeled neurons in the LPB. The immunofluorescent histochemical photomicrographs showed the FG- (A1 and A2, blue), TMR- (B1 and B2, red), FOS-labeled neurons (C1 and C2, green), and the FG/TMR/FOS tripled-labeled neurons (D1 and D2, merged) in the LPB, respectively. The area demarcated with a rectangle in D1 was enlarged in A2–D2. The double-arrowhead in D2 indicated an FG/TMR/FOS tripled-labeled neuron and was further enlarged (inset). Scale bar = 100 μm (A1–D1), 20 μm (A2–D2), and 5 μm (inset).

the amygdala is from the LPB, and studies showed that block the CGRP1 receptor in the amygdala has antinociceptive effect in different pain model. Electrophysiologic study also revealed that CGRP in the amygdala could facilitate excitatory postsynaptic currents through NMDA receptor (Han et al., 2010). Consistent with previous studies, in the present study, we also observed nearly 85.57% neurons from the LPB to CeA are CGRP positive. On the other hand, the previous study showed the distribution of CGRP-like immunoreactive axon terminals in dopaminergic cell group of VTA in rat, and these CGRP-positive terminals may selectively modulate the activity of VTA dopaminergic neurons that innervate the prefrontal cortex (PFC) (Deutch and Roth, 1987). In this study, we also observed some VTA projection neurons in LPB were CGRP positive, although there were also a part of VTA projection neurons in the LPB showing CGRP-negative. Thus, the present results combined with the previous reports imply that the two kinds of VTA projection neurons in LPB may innervate different VTA neurons. Furthermore, our results also showed that the CGRP-like immunoreactive neurons in LPB projecting to CeA sent their axon collaterals to the VTA. These collateral projection neurons may play an important role in the synchronization of salient signals, like pain, transmitted *via* LPB to the CeA and VTA, as both of the two regions play important roles in the modulation of pain sensation and processing of pain related affections.

FOS protein is conventionally used as a marker of functional activation of neurons. The neurons can quickly

express FOS protein when subjected to external stimulation (Dragunow and Faull, 1989; Perrin-Terrin et al., 2016). Previous studies showed that the expression of FOS in variety of regions in the central nervous system increased for animals in multiple kinds of pain models, including acute pain and chronic pain. In this study, we also observed about 89.49% and 77.87% of the projection neurons in the LPB to CeA and VTA were FOS positive, respectively, in the condition of neuropathic pain of SNI model in rats, indicating that the LPB–CeA and LPB–VTA pathways directly participate in the nociceptive information transmission.

On the other hand, CeA and VTA are two important regions that process affective aspects of pain. For instance, the VTA are closely related with the aversion response after pain stimuli. Consistently, with the use of retrograde tract tracing combined with FOS immunohistochemical staining in mice with SNI surgery, we found almost all the projection neurons in the LPB to CeA are FOS positive in the condition of neuropathic pain of SNI model, indicating that the LPB–CeA pathway directly participates in the nociceptive information transmission. On the other hand, few studies have reported neurons in the LPB projected to the VTA, and the retrograde tract tracing experiment showed that the LPB–VTA pathway was involved in the transmission of nociceptive signals as well as most of the VTA projection neurons in the LPB are FOS positive. Meanwhile, it has also been reported that the CGRP-positive neurons in the LPB sent collateral projection neurons to both CeA and PVT (Liang et al., 2016). Thus, it seems that the CGRP neurons in the LPB probably may innervate diverse brain regions simultaneously, and are deduced important function in the synchronization and process of different aspects of pain-related emotions.

CONCLUSION

Taken together, our data provide direct evidence that part of CGRP-like immunoreactive neurons in the LPB of rat send projections to both of the CeA and the VTA by way of axon collaterals, and these collateral projection neurons may be involved in the peripheral nociceptive signal transmission.

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