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Neurochemical features of enkephalinergic neurons in the mouse trigeminal subnucleus caudalis

Jing Huang [a], Wei Wang [a], Jing Chen [a], Shun-Nan Ge [b], Yan-Yan Wei [a], Ya-Yun Wang [a], Takeshi Kaneko [c], Yun-Qing Li [a,*], Sheng-Xi Wu [a,**]

[a] Department of Anatomy, Histology and Embryology, K.K. Leung Brain Research Centre, The Fourth Military Medical University, Xi'an 710032, PR China
[b] Department of Neurosurgery, Tangdu Hospital, The Fourth Military Medical University, Xi'an 710038, PR China
[c] Department of Morphological Brain Science, Graduate School of Medicine, Kyoto University, Kyoto 606-8501, Japan

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ABSTRACT

Enkephalinergic (ENKergic) neurons have been proposed to play crucial roles in pain modulation in the trigeminal subnucleus caudalis (Vc). To assist an advance in the research of ENKergic neurons, here we used preproenkephalin-green fluorescent protein (PPE-GFP) transgenic mice, in which all ENKergic neurons were fluorescent. We first performed fluorescent *in situ* hybridization combined with immunofluorescent histochemistry to confirm the specificity of this transgenic mouse and its advantages in showing ENKergic neurons in the Vc. Then based on this useful transgenic mouse, we examined the phenotypic diversity of PPE-GFP neurons by immunostaining for several markers that characterize ENKergic neuron subtypes. About 25.9 ± 1.9% of GFP-positive neurons were regarded as immunoreactive for glutamic acid decarboxylase $(GAD)_{67}$ mRNA and 14.7 ± 1.4% of GFP-positive neurons were positive for γ-aminobutyric acid. The proportions of calbindin-, calretinin-positive cells among the ENKergic neurons were 8.4 ± 1.2% and 7.3 ± 1.7%, respectively. Only 1.1 ± 0.1% of GFP-positive neurons colocalized with parvalbumin and no GFP-positive neurons were found to co-express neuronal nitric oxide synthase. We then injected retrograde tracer into the thalamic regions and observed that a small number of ENKergic neurons in the Vc were retrogradely labeled with the tracer. The present results provide a detailed morphological evidence of the neurochemical features of ENKergic neurons. These results have broad implications for understanding the functional roles of ENKergic neurotransmission in the Vc.

1. Introduction

The trigeminal subnucleus caudalis (Vc) is the first-order relay nucleus to the higher centers for the trigeminal nociceptive information. Therefore, Vc plays an important role as a critical relay nucleus for the processing of nociceptive signals from the orofacial area (Sessle, 1996, 2000; Hu et al., 1992; Dubner and Bennett, 1983; J.L. Li et al., 1999; Li et al., 2000; Ni et al., 2002). Vc has been known to contain various types of neurons, which can be classified according to their morphological and functional properties (Li et al., 1999a,b). Understanding neuronal mechanisms mediating nociceptive information processing in the Vc will require knowledge of the neurochemical features of its main cell types.

Endogenous opioid peptides play important parts in inhibition on the nociceptive information transmission in the superficial laminae (laminae I and II) of the Vc (Basbaum and Fields, 1984). Previous studies showed that endogenous enkephalinergic system in the Vc could be activated presumably by the nociception caused by experimental tooth movement (Balam et al., 2005) or electrical stimulation of the whole trigeminal ganglion (Nishimori et al., 1989). Like other neuropeptides, enkephalin (ENK) is coexpressed with classical neurotransmitters in the central nervous system and could modulate the neurotransmitter action via activation of presynaptic or postsynaptic receptors (Hokfelt et al., 2000; Merighi, 2002). ENK binds principally to δ-opioid receptor (DOR) and μ-opioid receptors (MOR) and these receptors may affect neurotransmitter release via their numerous effectors (Law et al., 2000; Liao et al., 2005). Therefore, the neurotransmitter coexpressed with ENK is crucial for understanding the functional role of ENK neurotransmission.

It has been reported that calcium-binding proteins (CaBPs) are useful markers for specific neuronal populations in various brain regions (Parent et al., 1996; Frassoni et al., 1998; Miettinen et al.,

* Corresponding author. Tel.: +86 29 84774501; fax: +86 29 83283229.
** Corresponding author. Tel.: +86 29 84773954; fax: +86 29 83283229.
E-mail addresses: deptanat@fmmu.edu.cn (Y.-Q. Li), devneuro@fmmu.edu.cn (S.-X. Wu).

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1997; Morys et al., 1999). The immunoreactivities for the three well-known CaBPs, calbindin (CB), calretinin (CR), parvalbumin (PV), have also been observed in the rat Vc in our previous study (J.L. Li et al., 1999; Li et al., 1999a,b, 2000). The results indicated that the CaBPs, were expressed in both projection and intrinsic Vc neurons, and that some projection neurons expressing CB, CR or PV might be nociceptive neurons (J.L. Li et al., 1999; Li et al., 1999a,b, 2000). However, the coexpression of ENK with CaBPs was not extensively clarified and it is still unclear whether ENK is localized in interneurons or projection neurons.

In addition, the identification of ENKergic cell bodies following immunohistochemical labeling is difficult since ENK is concentrated in the axon terminals (Du and Dubois, 1988). To aid in the identification of ENKergic neurons, we used PPE-enhanced green fluorescent protein (GFP) transgenic mouse in which all ENKergic neurons were identified by the expression of GFP. The specificity of GFP-positive ENKergic neurons has been confirmed (Koshimizu et al., 2008). So the PPE-GFP transgenic mouse provides a convenient tool to study the morphological pattern and functional significance of ENKergic neurons.

This study aimed at characterizing the neurochemical features of ENKergic neurons in the Vc. By using PPE-GFP transgenic mouse, we assessed the co-expression of ENK with glutamic acid decarboxylase $(GAD)_{67}$ mRNA, GABA, CB, CR, PV, neuronal nitric oxide synthase (nNOS) and estimated their proportions among GFP-positive cells. The goal is to provide essential information on the phenotypic properties of these ENKergic neurons in the Vc.

2. Experimental procedures

2.1. Animals

The generation and characterization of the PPE-GFP transgenic mice have been described previously (Koshimizu et al., 2008). Fifteen adult mice were used for *in situ* hybridization (ISH) and immunofluorescence labeling. The experiments were conducted in accordance with the Animal Use and Care Committee for Research and Education of the Fourth Military Medical University (Xi'an, PR China). All efforts were made to minimize the number of animals used, as well as to minimize distress to the animals.

2.2. Immunohistochemistry (IHC) and double immunofluorescent (IF) histochemistry

Adult PPE-GFP transgenic mice were perfused transcardially with 0.1 M phosphate buffer (PB; pH 7.4) containing 4% paraformaldehyde. The brainstem was obtained and postfixed with the same fixative for 4 h, placed in 30% (w/v) sucrose solution in 0.05 M PB solution (pH 7.4) overnight at 4 °C, and cut into 25 μm thick sections. The sections were incubated at room temperature (RT) overnight with rabbit anti-GFP antibody (1:1000, Sigma, USA) and further incubated at RT for 3 h with biotinylated donkey anti-rabbit IgG antibody (1:200, Chemicon, Temecula, CA); the incubation medium was prepared by using 0.05 M PBS (pH 7.4) containing 0.3% (v/v) Triton X-100, 0.25% (w/v) λ-carrageenan, 5% (v/v) normal donkey serum and 0.05% (w/v) NaN_3 (PBS-XCD). The sections were then incubated at RT for 3 h with avidin-biotinylated peroxidase complex (ABC-Elite kit; Vector, USA) which was diluted at 1:50 with 0.05 M PBS (pH 7.4) containing 0.3% (v/v) Triton X-100 (PBS-X). Finally, the bound peroxidase was developed by reaction with 0.02% (w/v) diaminobenzidine-4HCl (DAB) and 0.001% (v/v) H_2O_2 in 50 mM Tris–HCl (pH 7.6). The sections were mounted onto gelatin-coated glass slides, washed in water, dried, cleared in xylene, and cover slipped.

For double IF, the sections were incubated overnight at 4 °C with a mixture of 1 μg/mL guinea pig (GP) anti-GFP antibody and one of the following antibodies: mouse anti-neuronal nuclei protein (NeuN; 1:5000; Chemicon); mouse anti-glial fibrillary acidic protein (GFAP; 1:5000; Chemicon); mouse anti-CB (1:1000; Sigma); rabbit anti-GABA serum (1:1000; Sigma), mouse anti-CR (1:3000; Chemicon), mouse anti-PV (1:8000; Sigma) or mouse anti-nNOS (1:1000; Chemicon). Secondary antibodies included Alexa Fluor 488-conjugated donkey anti-GP IgG (1:500; Molecular Probes, Eugene, OR, USA), Alexa Fluor 594-conjugated donkey anti-mouse IgG (1:500; Molecular Probes) or Alexa Fluor 594-conjugated donkey anti-rabbit IgG (1:500; Molecular Probes). Sections were air dried and cover slipped with a mixture of 50% (v/v) glycerin and 2.5% (w/v) triethylenediamine (anti fading agent) in 0.05 M PBS (pH 7.4). In the double immunofluorescence experiments, some control sections were processed with one of the two primary antibodies omitted. In these cases, only the color of the corresponding secondary fluorescent antibody was observed, indicating that the secondary antibodies were specific for rabbit, mouse or guinea pig IgGs.

2.3. Combined labeling for immunofluorescent (IF) histochemistry and fluorescent in situ hybridization (FISH)

The procedure for non-radioactive ISH has been described before (Koshimizu et al., 2008). Briefly, free-floating sections were acetylated at RT for 10 min by gentle shaking in freshly prepared 0.25% (v/v) acetic anhydride in 0.1 M triethanolamine. After a rinse, the sections were preincubated for 1 h at 60 °C with a hybridization buffer, which was composed of 5× SSC, 2% (w/v) blocking reagent (Roche Diagnostics), 50% (v/v) formamide, 0.1% (w/v) N-lauroylsarcosine (NLS) and 0.1% (w/v) sodium lauryl sulfate. The sections were then hybridized for 18 h at 60 °C with 1 μg/mL sense or antisense digoxigenin-labeled PPE riboprobe (nucleotides 672–1021; GenBank accession number BC049766) or GAD_{67} riboprobe (nucleotides 43–661; GenBank accession number Y12257) in the hybridization buffer. After two washes at 60 °C in 50% (v/v) formamide, 2× SSC and 0.1% (w/v) NLS for 20 min, hybridized sections were treated for 30 min at 37 °C with 10 units/μL RNase H (Toyobo, Osaka, Japan) in 10 mM Tris–HCl (pH 8.0), 1 mM EDTA and 0.5 M NaCl. The sections were washed at RT twice for 10 min in 2× SSC and 0.1% (w/v) NLS, and then twice for 10 min in 0.2× SSC and 0.1% (w/v) NLS. The following incubations were performed at RT with 1% (w/v) blocking reagent (Roche Diagnostics) in 0.1 M Tris–HCl (pH 7.5) and 0.9% (w/v) NaCl. After a wash, the hybridized sections were incubated overnight with a mixture of 1:3000-diluted POD-conjugated anti-DIG sheep IgG Fab fragment (Roche Diagnostics), 1 μg/mL affinity-purified guinea pig antibody to heat-denatured GFP (Nakamura et al., 2008). The sections were then incubated for 2 h with 5 μg/mL AF488-conjugated anti-GP IgG goat antibody (Invitrogen) and 10 μg/mL AF700-conjugated streptavidin (Invitrogen). Finally, the sections were reacted for 20 min at RT with an HNPP Fluorescence Detection kit (HNPP/FastRed TR; Roche Diagnostics). Sections hybridized with the sense probe showed no mRNA signals in the Vc.

2.4. Retrograde tract-tracing with fluoro-gold (FG) combined with triple-immunofluorescence labeling

Three mice were anesthetized with sodium pentobarbital (35 mg/kg body weight) and fixed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA) in the prone position. About 0.2 μl of 4% (w/v) FG (80014; Biotum, Hayward, CA) dissolved in distilled water was injected into the thalamus (1.34 mm posterior to the bregma, 1.5 mm right to the midline, and 3.5 mm deep from the brain surface). Each injection was made by pressure through a glass micropipette attached to a 1-ll Hamilton microsyringe over a period of 10 min. After the injection, the micropipette was left in the place for additional 20 min. Seven days later, the mice were perfused transcardially under deep anesthesia, and 25 μm thick sections of the brainstem and the brain containing the injection sites were prepared as described above. The sections containing the injection sites were counterstained with cresyl violet. The sections of Vc were processed for triple labeling for GFP, CB or CR and FG. Briefly, the sections were incubated at 4 °C sequentially with (1) a mixture of 1 μg/mL guinea pig (GP) anti-GFP antibody, rabbit anti-FG and mouse anti-CB (1:1000; Sigma) or mouse anti-CR (1:3000; Chemicon) overnight; (2) Alexa Fluor 488-conjugated donkey anti-GP IgG (1:500; Molecular Probes), Alexa Fluor 594-conjugated donkey anti-rabbit IgG (1:500; Molecular Probes) and Alexa647-labeled donkey antibody to mouse IgG (1:500; Molecular Probes) for 4 h. The incubation medium was prepared as described above.

2.5. Photomicrographs and analysis

Tissue sections were examined and photographed under a bright-field microscope (AH-3, Olympus, Japan) or a confocal laser scanning microscope (FV-1000, Olympus). To evaluate double and triple immunofluorescence labeling, the total number of positive neurons in the Vc was counted manually from six sections randomly selected from three different animals. The relative quantitative analysis of double-labeled and triple-labeled neurons was performed by counting the number of somata on each section. Profile counts were corrected by using Abercrombie's formula. Abercrombie's formula for the ratio of the "real" number to the observed number is $T/T + h$, where T = section thickness and h = mean diameter of the GFP-positive somata along the axis perpendicular to the plane of the section (Abercrombie, 1946; Guillery, 2002). Neuronal counts were converted into percentages. The values were expressed as mean ± SEM.

3. Results

3.1. The distribution and specificity of GFP-positive cells in the Vc of the PPE-GFP transgenic mouse

GFP-positive cells were present in all laminae, including marginal zone (lamina I), substantia gelatinosa (lamina II) and magnocellular part (lamina III) of the Vc, with a higher concentration in lamina II (Fig. 1A–C). GFP fluorescence existed in GFP-positive cell bodies including their nuclei, dendrites and axons. Some GFP-positive cells had primary dendrites emerging