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GABAergic neurons express μ-opioid receptors in the ventrolateral orbital cortex of the rat

Fu-Quan Huo [a], Jing Wang [a], Yun-Qing Li [b], Tao Chen [b], Feng Han [b], Jing-Shi Tang [a,*]

[a] *Key Laboratory of Environment and Genes Related to Diseases, Ministry of Education, Department of Physiology, School of Medicine, Xi'an Jiaotong University, Xi'an 710061, China*
[b] *Department of Anatomy and K.K. Leung Brain Research Centre, The Fourth Military Medical University, Xi'an 710032, China*

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Abstract

Behavioral studies have indicated that GABAergic modulation is involved in the opioid-induced antinociception in the ventrolateral orbital cortex (VLO). The aim of the current study was to examine whether the GABAergic neurons in the rat VLO expressed μ-opioid receptor subtype 1 (MOR1). This study employed immunofluorescence histochemical double-staining technique and showed that a considerable amount of GABA- and MOR1-like immunoreactive neurons existed in layers II–VI in the VLO. Of these GABA-like immunoreactive neurons, 92.0% of them showed MOR1-like immunoreactivities. Similarly, 80.2% of MOR1-like immuoreactive neurons also exhibited GABA-like immunoreactivities. These results provide morphological evidence that opioid-induced antinociception in the VLO might be due to an inhibitory effect by opioid via MOR1 on GABAergic neurons, resulting in disinhibition of VLO projection neurons and leading to activation of the VLO-PAG brainstem descending pain control system to depress the nociceptive inputs at the spinal cord level.

Keywords: Ventrolateral orbital cortex; μ-Opioid receptor; GABAergic neuron; Immunofluorescence histochemistry; Antinociception; Rat

Previous studies have indicated that the ventrolateral orbital cortex (VLO) is a part of the endogenous analgesic system consisting of the spinal cord-nucleus submedius (Sm)-VLO-periaqueductal gray (PAG)-spinal cord loop [16–18]. Microinjection of morphine into the VLO produces a naloxone reversible antinociception [6] and this effect is enhanced by γ-aminobutyric acid (GABA) A receptor antagonist bicuculline and attenuated by its agonist muscimol, respectively [13]. These results suggest that GABAergic modulation may be involved in the opioid-induced antinociception in the VLO. It has been assumed that microinjection of morphine or stimulation-evoked endogenous opioid peptide release within VLO may directly depress the inhibitory effect of GABAergic interneuron on VLO projection neurons (disinhibition). This leads to activation of the VLO-PAG brainstem descending inhibitory system and depression of the nociceptive inputs at the spinal cord level [13]. However, this proposed mechanism of disinhibition still lacks morphological evidence. Although the prefrontal cortex including VLO contains enkephalinergic neurons, μ-opioid receptors [1,5,9,10] and GABAergic interneurons [4], it is unclear whether GABAergic neurons in VLO expressed μ-opioid receptors. For this reason, the current study was undertaken to examine the expression of μ-opioid receptor (MOR) on the GABAergic neurons in the VLO of the rat by using immunofluorescence histochemical double-staining for MOR and GABA.

The experiments were performed on 16 male Sprague-Dawley rats, weighting 200–260 g. All experimental procedures have been approved by the Committee of Animal Use for Research and Education of the Fourth Military Medical University (Xi'an, PR China). Rats were deeply anesthetized with an overdose of sodium pentobarbital (100 mg/kg body weight, i.p.) and perfused transcardially with 100 ml of 0.01 M phosphate-buffered saline (PBS, pH 7.4), followed by 500 ml of 4% (w/v) paraformaldehyde and 75% (v/v) saturated picric acid in 0.1 M phosphate buffer (PB, pH 7.4). The brains were then removed immediately and placed into the

* Corresponding author. Tel.: +86 29 82655172; fax: +86 29 82656364.
E-mail address: jstang@mail.xjtu.edu.cn (J.-S. Tang).

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same fresh fixative for an additional 4 h at 4 °C. Subsequently the brains were placed into 25% (w/v) sucrose solution in 0.1 M PB (pH 7.4) overnight at 4 °C, and then cut serially into 20 μm-thick coronal sections by the use of a freezing microtome (Kryostat 1720; Leitz, Mannheim, Germany). The sections were placed into three different dishes according to their numerical order while cutting (e.g., sections 1, 4, 7 to dish 1, sections 2, 5, 8 to dish 2 and sections 3, 6, 9 to dish 3, respectively). Each dish usually contained 28–32 sections. All sections were washed carefully with 0.01 M PBS.

The sections in the first dish were used for immunofluorescence histochemical double-staining for GABA and MOR. Briefly, the sections were incubated at 4 °C sequentially with: (1) a mixture of rabbit anti-GABA serum (A2052, 1:2000 dilution; Sigma, St. Louis, MO) and guinea pig anti-μ-opioid receptor subtype-1 (MOR1) polyclonal antibody (AB1774, 1:1000 dilution; Chemicon, Temecula, CA) for 48–72 h; (2) biotinylated goat anti-giunea pig IgG (1:200 dilution; Vector, Burlingame, CA) overnight; (3) Texas red-labeled avidin D (1:1000 dilution; Vector) and donkey anti-rabbit IgG conjugated with fluorescein isothiocyanate (FITC; 1:200 dilution; Jackson Immuno Research, West Groove, PA) for 4–6 h. The diluent used for all antibodies was 0.01 M PBS containing 5% (v/v) normal goat serum, 0.3% (v/v) Triton X-100, 0.05% (w/v) sodium azide (NaN_3) and 0.25% (w/v) carrageenan (pH 7.4). The sections were rinsed at least three times in 0.01 M PBS (pH 7.4) after each incubation and each time lasted over 10 min. Then, the sections were mounted onto clean glass slides, air dried and cover-slipped with a mixture of 50% (v/v) glycerin and 2.5% (w/v) triethylene diamine (anti-fading agent) in 0.01 M PBS. Finally, the sections were observed with an epifluorescence microscope (BX-60; Olympus, Tokyo, Japan) under appropriate filters for green-emitting FITC (excitation 490 nm; emission 520 nm) and for red-emitting Texas Red (excitation 550 nm; emission 615 nm), respectively. The sections were further observed with a confocal laser-scanning microscope (LSM, TCS SP2; Leica, Mannheim, Germany) by using laser beams (488 nm for FITC, 543 nm for Texas Red) and appropriate emission filters (500–535 nm for FITC, 570–654 nm for Texa Red).

The sections in the second dish were mounted on the gelatin coated glass slides and processed for Nissl staining to identify the VLO and the adjacent cortical regions according to Paxinos and Watson [12]. The sections in the third dish were used for control tests. In the control experiments, the primary antibodies were omitted or replaced with normal rabbit and guinea pig sera; no immunofluorescence histochemical staining for the omitted or replaced antibodies was detected. For each rat ($n = 8$), the number of GABA-like, MOR1-like immunopositive neurons and GABA/MOR1 double-labeled neurons were counted on the fifteenth coronal section in the first dish at the same VLO region according to the Nissl stained sections and the rat brain atlas [12]. These data are shown in Table 1.

GABA-like immunoreactive labeling was usually observed in the perikaryon of non-pyramidal neurons and their processes, some of them had bead-shaped varicosities. GABA-like immunoreactive neuronal cell bodies were located scattered throughout all layers of the VLO, but their distribution patterns varied. More GABA-like immunoreactive neuronal cell bodies were found in layers II and VI (Fig. 1). GABA-like immunoreactive neuronal cell bodies were round, bipolar, triangular or multipolar in shape. The diameters of GABA-like immunoreactive neuronal cell bodies were about 10–20 μm (Fig. 2A).

Table 1
Number of GABA- and MOR1-immunoreactive neurons and GABA/MOR1 double-labeled neurons in the VLO

Rat	GABA-ir neurons	MOR1-ir neurons	GABA/MOR1 neurons (%[1], %[2])
R1	90	104	83 (92.2; 79.8)
R2	85	100	80 (94.1; 80.0)
R3	83	95	76 (91.6; 80.0)
R4	87	100	82 (94.3; 82.0)
R5	87	101	79 (90.8; 78.2)
R6	89	102	81 (91.0; 79.4)
R7	93	104	85 (91.4; 81.7)
R8	90	102	82 (91.1; 80.4)
Total	704	808	648 (92.0; 80.2)

%[1], percentage of GABA/MOR1 double-labeled neurons to GABA-ir neurons. %[2], percentage of GABA/MOR1 double-labeled neurons to MOR1-ir neurons.

MOR1-like immunoreactive non-pyramidal neurons were distributed diffusely throughout layers II–VI of the VLO and more frequently encountered in layer II than those in deeper layers. However, MOR1-like immunoreactive neurons were not observed in layer I (Fig. 1). Immunopositive labeling

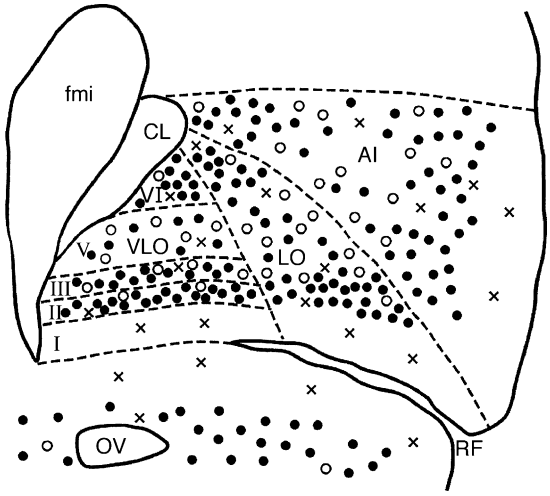

Fig. 1. Schematic drawing showing the distribution of GABA-like immunoreactive neurons (×), MOR1-like immunoreactive neurons (◯) and GABA/MOR1 double-labeled neurons (●) in the VLO which are plotted on coronal sections modified from the Paxinos and Watson's atlas (3.20 mm from Bregma) [12]. Each marker represents one immunoreactive neuron. Abbreviations: AI, agranular insular cortex; CL, claustrum; fmi, forceps minor corpus callosum; LO, lateral orbital cortex; OV, olfactory ventricle; RF, rhinal fissure; VLO, ventrolateral orbital cortex; I–VI, layers I, II, III, V and VI.