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THALAMIC NUCLEUS SUBMEDIUS RECEIVES GABAergic PROJECTION FROM THALAMIC RETICULAR NUCLEUS IN THE RAT

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Abstract—GABAergic projection from thalamic reticular nucleus to thalamic nucleus submedius in the medial thalamus of the rat was studied by using immunohistochemistry for GABA, retrograde labeling with Fluoro-Gold combined with immunohistochemistry for GABA, and anterograde labeling with biotinylated dextranamine. Immunohistochemistry displayed that only GABA immunoreactive terminals were observed in the thalamic nucleus submedius, while GABA immunoreactive neuronal cell bodies were located in the thalamic reticular nucleus and lateral geniculate nucleus. Injection of Fluoro-Gold into the thalamic nucleus submedius resulted in massive retrogradely labeled neuronal cell bodies in the rostroventral portion of the ipsilateral thalamic reticular nucleus and a few in the contralateral thalamic reticular nucleus, and most of these cell bodies showed GABA immunopositive staining. Many biotinylated dextranamine anterogradely labeled fibers and terminals in the thalamic nucleus submedius were observed after injection of biotinylated dextranamine into the thalamic reticular nucleus. The present results provide a morphological evidence for a hypothesis that a disinhibitory effect on output neurons elicited by opioid or 5-hydroxytryptamine inhibiting a GABAergic terminal in the thalamic nucleus submedius may lead to activation of the descending inhibitory system and depression of the nociceptive inputs at the spinal cord level.

Key words: thalamic nucleus submedius, thalamic reticular nucleus, anterograde and retrograde transport, GABA, immunohistochemistry, rat.

Anatomic studies in the rat and the cat have established that the thalamic nucleus submedius (Sm) receives a major projection from the neurons in lamina I of the trigeminal subnucleus caudalis and spinal dorsal horn (Craig and Burton, 1981; Yoshida et al., 1991), some of which express substance P receptor, indicating that they are nociceptive in nature (Li, 1999). The neurons in the Sm project primarily to the ventrolateral orbital cortex (VLO) (Yoshida et al., 1992; Coffield et al., 1992). The VLO contains neurons that project to the midbrain periaqueductal gray (PAG) (Hardy and Leichnetz, 1981; Craig et al., 1982), a region intensively involved in descending modulation of nociception (Fields and Basbaum, 1999). Behavioral and electrophysiological studies (Zhang et al., 1995, 1996, 1998, 1999) have indicated that electrically or chemically evoked activation of the Sm depresses the tail flick reflex, jaw-opening reflex and the nociceptive response of spinal dorsal horn neurons, and these depressive effects could be attenuated or eliminated by a lesion or depression of the VLO or PAG, suggesting that the antinociception produced by activation of the Sm is produced by activation of the Sm–VLO–PAG–brainstem descending inhibitory system.

Microinjection of morphine or serotonin (5-HT) into the Sm produces a naloxone or 5-hydroxytryptamine 1A (5-HT_{1A}) receptor antagonist reversible antinociception in the tail flick test or in the formalin test (Dong et al., 1999; Yang et al., 2002; Xiao et al., 2005). It has been indicated that the GABAergic modulation is implicated in the Sm morphine- and 5-HT-evoked antinociception (Jia et al., 2004; Xiao et al., 2002). In fact, in the rat medial thalamus there are no GABAergic interneurons at all (Oertel et al., 1983; Ottersen and Storm-Mathisen, 1984). GABAergic terminals in the Sm are most likely to originate from the projection of the thalamic reticular nucleus (Rt) which contains rich GABAergic neurons (Oertel et al., 1983; Ottersen and Storm-Mathisen, 1984), but this assumed GABAergic projection still lacks of neurochemical and morphological evidences. The present study was designed to examine whether the Sm received a GABAergic projection from the Rt by using Fluoro-Gold (FG) retrograde tracing combined with immunohistochemical staining for GABA, and using biotinylated dextranamine (BDA) anterograde tracing technique to further confirm the retrograde tracing results.

EXPERIMENTAL PROCEDURES

The experiment was performed on 30 male Sprague–Dawley rats weighing 200–260 g. All procedures of present experiment have been approved by the Committee of Animal Use for Research and Education of the Fourth Military Medical University (Xi'an, China). All surgical procedures were carried out by anesthesia induced by

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Abbreviations: ABC, avidin–biotin–peroxidase complex; BDA, biotinylated dextranamine; DAB, 3,3-diaminobenzidine tetrahydrochloride; FG, Fluoro-Gold; LG, lateral geniculate nucleus; NGS, normal goat serum; PAG, midbrain periaqueductal gray; PB, phosphate buffer; PBS, phosphate-buffered saline; Rt, reticular thalamic nucleus; Sm, nucleus submedius; VB, ventrobasal complex; VPL, ventral posterolateral thalamic nucleus; VPM, ventral posteriomedial thalamic nucleus; VLO, ventrolateral orbital cortex; 5-HT, 5-hydroxytryptamine (serotonin); 5-HT_{1A}, 5-hydroxytryptamine 1A.

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sodium pentobarbital (50 mg/kg body weight, i.p.). According to the guidelines of the International Association for the Study of Pain (Zimmermann, 1983), all efforts were made to minimize the number of animals used and their suffering.

Immunohistochemical staining for GABA

Ten rats were perfused transcardially with 100 ml of 0.01 M phosphate-buffered saline (PBS, pH 7.4), followed by 500 ml of 4% (weight/weight, w/w) paraformaldehyde, 0.05% (volume/volume, v/v) glutaraldehyde and 75% (v/v) saturated picric acid in 0.1 M phosphate buffer (PB, pH 7.4). The brains were removed immediately and placed into the same fresh fixative to be fixed for 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) as a cryprotectant overnight at 4 °C and then cut serially into 20 μm-thick coronal sections on a freezing microtome (Kryostat 1720; Leitz, Mannheim, Germany). The sections were collected serially into three dishes containing 0.01 M PBS. Each set of the sections in a dish contained a complete serial of sections of every third serial sections. All sections were washed carefully with 0.01 M PBS.

The first set of the sections in the first dish was used for immunohistochemistry for GABA with avidin–biotin–peroxidase complex (ABC) method (Hsu et al., 1981). Briefly, the sections were incubated successively with: (1) rabbit antiserum against GABA (A2052, 1:2000 dilution; Sigma, St. Louis, MO, USA) in 0.01 M PBS containing 5% (v/v) normal goat serum (NGS), 0.3% (v/v) Triton X-100, 0.05% (w/v) NaN_3 and 0.25% (w/v) carrageenan (PBS-NGS, pH 7.4) for 48–72 h at 4 °C; (2) biotinylated goat anti-rabbit IgG (1:200 dilution; Vector, Burlingame, CA, USA) in PBS-NGS overnight at 4 °C; (3) ABC Elite complex (Vector: 1:50) in 0.01 M PBS (pH 7.4) containing 0.3% (v/v) Triton X-100 for 2 h at room temperature. The bound peroxidase was visualized by incubation with 0.05% 3,3-diaminobenzidine tetrahydrochloride (DAB; Dojin, Kumamoto, Japan) and 0.003% H_2O_2 and 0.04% (w/v) $Ni(NH_4)_2SO_4$ in 0.05 M Tris–HCl buffer (pH 7.6) for 20–30 min. The sections were rinsed at least three times in 0.01 M PBS after every incubation and each time lasted over 10 min. The sections were mounted onto gelatin-coated glass slides, air dried, dehydrated and cleaned, coverslipped with DPX and observed under light microscope (BX-60; Olympus, Tokyo, Japan). The microphotographs were taken with digital camera (DP-70, Olympus) attached to the microscope.

The second set of the sections in the second dish was mounted onto gelatin-coated glass slides and processed for Nissl staining. The sections in the third dish were used for control tests. The primary antibody was omitted or replaced with NGS; no positive staining was detected in control slides.

Immunofluorescence histochemical staining of GABA for FG-labeled neurons

After anesthesia, a 4% (w/v) saline solution of retrograde fluorescent tracer FG (Fluorochrome, Denver, CO, USA) was stereotaxically (Paxinos and Watson, 1986) and iontophoretically injected into one side of the Sm (2.5 mm posterior to Bregma, 0.5 mm lateral to the midline, 6.5 mm from cerebral surface) (n=10) through a glass micropipette (tip diameter of 10–20 μm) using a 2 μA positive-pulsed direct current (7 s on/off for 10–15 min). Three days later, the rats were treated according to above mentioned schedule. The brains were cut into coronal sections 20 μm thick. All sections were collected serially into four dishes containing 0.01 M PBS (pH 7.4) and washed carefully with 0.01 M PBS. The sections in the first dish were mounted onto clean glass slides, air dried, coverslipped with a mixture of 50% (v/v) glycerin and 2.5% (w/v) triethylene diamine (anti-fading reagent) in 0.01 M PBS. Then they were examined with an epifluorescence microscope (BX-60; Olympus) under appropriate ultraviolet filter (excitation 360 nm; emission 450 nm) to view the distribution of gold-emitting FG retrogradely labeled neurons and FG injection sites. In order to determine the neurochemical nature of the retrogradely labeled neurons, only the sections from the rats (n=5) whose injections sites were confined to the Sm were used in the following procedures.

The sections in the second dish were incubated at 4 °C with: (1) rabbit antiserum against GABA (1:2000 dilution; Sigma) in PBS-NGS (pH 7.4) for 48–72 h; (2) biotinylated goat anti-rabbit IgG (1:200 dilution; Vector) in PBS-NGS overnight, and (3) Texas Red-labeled avidin D (1:200 dilution; Vector) for 4 h. The immunofluorescence histochemically stained sections were treated as mentioned above, and then observed with epifluorescence microscope under appropriate ultraviolet filter for gold-emitting FG (excitation 360 nm; emission 450 nm), or for red-emitting Texas Red (excitation 550 nm, emission 615 nm). The microphotographs were taken with digital camera (DP-70, Olympus) attached to the microscope. FG-labeled neurons, GABA immunopositive neurons and GABA/FG double-labeled neurons were counted on all of the second serial sections.

The sections in the third dish were mounted onto gelatin-coated glass slides and processed for Nissl staining. The location of FG retrogradely labeled neuronal cell bodies in the nuclei of the diencephalon was plotted on projection drawings of the Nissl-stained sections.

In the control experiment, the primary antibody was omitted or replaced with a mixture of NGS and normal mouse serum to incubate the sections in the fourth dish. The other incubation steps were the same as that used for the sections in the second dish. No immunofluorescence histochemistry staining for the omitted or replaced antibodies was detected.

Anterograde labeling experiment

The rats (n=10) received a unilateral injection of 10% biotinylated dextranamine (BDA, molecular weight 3000; Molecular Probes, Eugene, OR, USA) in 0.9% saline at Rt (Rajakumar et al., 1993). BDA was stereotaxically and iontophoretically injected unilaterally into the Rt (1.4 mm anterior to Bregma, 1.6 mm lateral to the midline, 6.2 mm from cerebral surface) through a glass micropipette (tip diameter of 10–20 μm) using a 2 μA positive-pulsed direct current (7 s on/off for 10–15 min) (Paxinos and Watson, 1986). Three days later, the rats were anesthetized and perfused transcardially with 100 ml of 0.01 M PBS (pH 7.4), followed by 500 ml of 4% (w/v) paraformaldehyde containing 75% (v/v) saturated picric acid in 0.1 M PB (pH 7.4). The brains were removed immediately, fixed for additional 4 h at 4 °C, 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. The sections were collected serially in three dishes containing 0.01 M PBS. The BDA injection sites and BDA-labeled fibers and axon terminals on the sections in the first dish were revealed by incubating the sections 4 h at room temperature in 0.01 M PBS solution containing ABC Elite complex (1:200 dilution; Vector) and 0.3% (v/v) Triton X-100 (pH 7.4). Finally, the peroxidase-bound to ABC complex was visualized by incubating the sections with 0.05% DAB, 0.003% H_2O_2 and 0.04% $Ni(NH_4)_2SO_4$ in 0.05 M Tris–HCl buffer (pH 7.6) for 20–30 min. The sections were mounted onto gelatin-coated glass slides, air dried, coverslipped with DPX, examined and photographed with BX-60 microscope (Olympus). The microphotographs were taken with digital camera (DP-70, Olympus) attached to the microscope. The distribution of BDA anterogradely labeled fibers and terminals in the nuclei of the diencephalon was plotted onto a two-dimensional plane by using a camera lucida with the aid of a drawing tube attached to a BX-60 light microscope (Olympus).

The sections in the second dish were mounted onto clean glass slides, air dried and used for Nissl staining. In the control experiment, the primary antibodies were omitted or replaced with