Acta Histochemica 117 (2015) 642–648

Contents lists available at ScienceDirect

Acta Histochemica

journal homepage: www.elsevier.de/acthis

Immunohistochemical localization of GABAergic key molecules in the main olfactory bulb of the Korean roe deer, *Capreolus pygargus*

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ARTICLE INFO

Article history:
Received 29 October 2014
Received in revised form 14 May 2015
Accepted 10 June 2015

Keywords:
Roe deer
Main olfactory bulb
Gamma-amino butyric acid
Immunohistochemistry

ABSTRACT

Gamma-amino butyric acid (GABA) negatively regulates the excitatory activity of neurons and is a predominant neurotransmitter in the nervous system. The olfactory bulb, the main center in the olfactory system, is modulated by inhibitory interneurons that use GABA as their main neurotransmitter. The present study aimed to evaluate GABAergic transmission in the main olfactory bulb (MOB) of the Korean roe deer (*Capreolus pygargus*) by examining the immunohistochemical localization of GABAergic key molecules, including glutamic acid decarboxylase (GAD), vesicular GABA transporter (VGAT), GABA transporters (GATs; GAT-1 and GAT-3), and potassium sodium chloride co-transporter 2 (KCC2). GAD, VGAT, and KCC2 were expressed in the glomerular layer (GL), external plexiform layer (ePL), mitral cell layer (ML), and granule cell layer (GrL). Intense GAT-1 expression was observed in the GL; GAT-1 expression was discernible in the ePL, ML, and GrL. However, intense GAT-3 expression was extensively observed in all layers of the MOB. These results suggest that substantial GABAergic synapses are present in the GL, ePL, ML, and GrL. Furthermore, the released GABA may be removed by GAT-1 and GAT-3 in the GL, and the majority of GABA, which is present in the ePL to GrL, may undergo reuptake by GAT-3. This is the first morphological and descriptive study of GABAergic transmission in the MOB of Korean roe deer.

1. Introduction

Gamma-amino butyric acid (GABA), a predominant inhibitory neurotransmitter in the mature mammalian central nervous system, negatively regulates the excitatory activity of neurons (Macdonald and Olsen, 1994; Olsen and Avoli, 1997). GABA is synthesized from glutamate by glutamic acid decarboxylase (GAD), and packaged by vesicular GABA transporter (VGAT), which transports GABA into the synaptic cleft. Once released, GABA is taken up by GABA transporters (GATs) for its removal into presynapses and astrocytes. Potassium sodium chloride co-transporter 2 (KCC2), which reduces the intracellular chloride ion concentration, is abundantly expressed in the GABAergic postsynapse. The expression of KCC2 is necessary for hyperpolarizing GABAergic inhibition (Rivera et al., 1999). GABA is the most common inhibitory transmitter and is detected in parts of the nervous system, including the cerebrum (Takayama and Inoue, 2010), cerebellum (Takayama and Inoue, 2004, 2006), spinal cord (Kosaka et al., 2012; Schaffner et al., 1993), and some cranial nerves, such as the olfactory (Yokosuka, 2012), trigeminal (Kin et al., 2014) and hypoglossal nerves (Kosaka et al., 2012).

The olfactory bulb, the main center in the olfactory system, is located in the rostral portion of the forebrain and is oval-shaped in structure. The olfactory system is composed of the main olfactory bulb (MOB) and accessory olfactory bulb systems (Caba et al., 2014).

Abbreviations: CNS, central nervous system; CR, calretinin; ePL, external plexiform layer; GABA, γ-amino butyric acid; GAD, glutamate acid decarboxylase; GAT, GABA transporter; GFAP, glial fibrillary acidic protein; GL, glomerular layer; GrL, granule cell layer; iPL, internal plexiform layer; ML, mitral cell layer; MOB, main olfactory bulb; KCC2, potassium chloride co-transporter 2; ONL, olfactory nerve cell layer; PG cell, periglomerular cell; TH, tyrosine hydroxylase; VGAT, vesicular GABA transporter.

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http://dx.doi.org/10.1016/j.acthis.2015.06.006
0065-1281/

MOB, the primary brain center for olfactory sensation, accepts the odorous information detected in the olfactory epithelium and sends the olfactory signals to the primary olfactory cortex (de Olmos et al., 1978). According to microscopic analyses of rat and mouse brain, the MOB is divided into six layers (Caba et al., 2014; Kosaka and Kosaka, 2013); beginning with the most superficial layer, these include the (1) olfactory nerve cell layer (ONL), (2) glomerular layer (GL), (3) external plexiform layer (ePL), (4) mitral cell layer (ML), (5) internal plexiform layer (iPL), and (6) granular cell layer (GrL). Morphological characteristics of GABAergic signaling surrounding the olfactory bulb have been studied over the past few decades in various animals, including the frog (Puopolo and Belluzzi, 1998), dog (Nadi et al., 1980), mouse (Kosaka and Kosaka, 2013; Pallotto et al., 2012; Panzanelli et al., 2007; Yokosuka, 2012), rabbit (Nolasco et al., 2012), and rat (Caba et al., 2014; Gracia-Llanes et al., 2010; Kosaka et al., 1997; Liu and Shipley, 1994; Panzanelli et al., 2007; Puopolo and Belluzzi, 1998). However, there are few reports regarding GABAergic transmission in the olfactory system of small ruminants, such as goat (Mogi et al., 2007), sheep (Kendrick et al., 1992, 1997; Levy, 2002), including ewes (Keverne et al., 1993; Levy et al., 1995).

The Korean roe deer, *Capreolus pygargus* (*C. pygargus*), is a species of Siberian roe deer in the family Cervidae, order Artiodactyla. *C. pygargus* is the most abundant wild animal on Jeju Island, South Korea, and it inhabits forests, meadows, the fields of Halla Mountain, and parasitic cones. However, little is known regarding the biology of the Korean roe deer, although a few studies have reported histological studies about olfactory mucosae, vomeronasal organ, and accessory olfactory bulb (Park et al., 2014a, 2014b), and have performed genetic analysis (Han et al., 2007).

Various wild animals, including deer, are highly dependent on odorous information for territory disputes, reproduction and sexual behavior (Asher et al., 1990; Jaczewski, 1989; Villagran and Ungerfeld, 2013; Wood, 2003). The olfactory bulb is filled with neuroactive substances, including acetylcholine, glutamate, glycine, taurin, and GABA. Among them, GABA is the most predominant neurotransmitter derived from amino acids in the olfactory bulb (Halasz and Shepherd, 1983). However, little is known with respect to GABAergic transmission in the olfactory system of Cervidae. In the present study, key molecules participating in GABAergic transmission, including GAD, VGAT, GATs, and KCC2, were analyzed by immunohistochemistry, as the first step toward understanding GABAergic transmission in the MOB of *C. pygargus*.

2. Materials and methods

2.1. Animals

Olfactory bulb samples (male, $n = 3$) from 2- and 3-year-old Korean roe deer (*C. pygargus*) were obtained from the Jeju Wildlife Rescue Center. The ages of the deer were estimated by the number of horn branches (spikes). Olfactory bulb was removed from the skull immediately after death and fixed in 10% buffered formalin for 48 h. The Institutional Animal Care and Use Committee of Jeju National University approved all protocols used in this study. The protocols for the care and handling of animals conformed to current international laws and policies (NIH Guide for the Care and Use of Laboratory Animals, NIH Publication No. 85-23, 1985, revised 1996).

2.2. Tissue preparation and histological examination

The olfactory bulb containing the MOB was dehydrated in a graded ethanol series (70%, 80%, 90%, 95%, and 100%), cleared in xylene, embedded in paraffin, and sectioned at a 6-μm thickness. Sagittal serial sections of the MOB were mounted onto glass slides coated with silane (Sigma–Aldrich, St. Louis, MO, USA). After deparaffinization, sections were stained with hematoxylin and eosin (H&E) solution. To visualize the MOB nerve fiber tract, Klüver-Barrera staining (0.1% Luxol-fast blue and 0.1% cresyl violet) was applied to paraffin sections (Kluver and Barrera, 1953).

2.3. Immunohistochemistry

Sections (6 μm) of paraffin-embedded tissues were deparaffinized and heated in a microwave oven (700 W) in citrate buffer (0.01 M, pH 6.0) for 3 min. After cooling the slides to room temperature, sections were exposed to aqueous 0.3% hydrogen peroxide for 20 min to block endogenous peroxidase activity. Nonspecific binding was blocked using 3% normal goat serum in phosphate buffer saline (PBS, pH 7.4) for 1 h and then allowed to react with GAD65/67 (Millipore, Billerica, MA, USA), GAT-1 (Sigma–Aldrich, St. Louis, MO, USA), GAT-3 (Sigma–Aldrich, St. Louis, MO, USA), VGAT (Takayama and Inoue, 2004), and KCC2 (Takayama and Inoue, 2006) primary antibodies overnight at room temperature. After rinsing three times with PBS for 15 min, sections were visualized using the avidin–biotin–peroxidase complex method (Histofine kit, Nichirei, Tokyo, Japan) (Hsu et al., 1981). Sections were then counterstained with hematoxylin for 15 s, washed in running tap water for 10 min, dehydrated through a graded ethanol series, cleared with xylene, and mounted with Canada balsam (Sigma–Aldrich, St. Louis, MO, USA).

For double immunofluorescence staining, sections were incubated with rabbit anti-tyrosine hydroxylase (TH) (Millipore, Billerica, MA, USA), calretinin (CR) (Swant, Bellinzona, Switzerland), GAT-1, and GAT-3 antibodies, and were visualized using anti-rabbit IgG conjugated to Alexa Fluor® 488 (Life Technologies, Carlsbad, CA, USA). After rinsing three times in PBS, sections were incubated with guinea pig anti-GAD (original antibody) or mouse anti-glial fibrillary acidic protein (GFAP) (Millipore, Billerica, MA, USA), and visualized using anti-guinea pig IgG conjugated to Alexa Fluor® 568 (Life Technologies, Carlsbad, CA, USA) or anti-mouse IgG conjugated to Alexa Fluor® 568 (Life Technologies, Carlsbad, CA, USA), respectively. The immunofluorescence images were acquired using a laser scanning confocal microscope (Olympus FV1000).

2.4. Antibody characterization

Characterization of all antibodies used in the present study is shown in Table 1. We determined the proper dilution factor for each antibody by testing serial dilutions of the antibody concentrations. The specificities of GAD, VGAT, and KCC2 antibodies were examined in a previous study (Kobayashi et al., 2013; Takayama and Inoue, 2004). To evaluate the immunohistochemical reactivity specificities of rabbit polyclonal GAD, VGAT, and KCC2 antibodies, the antibodies were pre-incubated at room temperature for 1 h with the antigen peptides for GAD (rat GAD control peptide AB1511, catalog number AG252; Millipore, Billerica, MA, USA), VGAT (synthetic peptide from the N-terminus of mouse VGAT, aa 1022–1042), and KCC2 (synthetic peptide from the N-terminus of mouse KCC2, aa 44–64) (10 μg/mL of each diluted antibody). Negative controls were included by omitting GAT-1 and GAT-3 primary antibodies from a few sections in each immunoreaction. No obvious staining was detected in the olfactory bulb (Figs. 2A, 2D, 2G, 3E, and 3F).

3. Results

3.1. Histological architecture of the MOB

Using a light microscope, the MOB of the Korean roe deer was found to be comprised of five layers, including the ONL, GL, ePL, ML, and GrL (Fig. 1A and B). We observed three or four rows of glomeruli