■ ORIGINAL ARTICLE

Expression of GABA system in Mouse Olfactory Mucosa

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ABSTRACT: Our previous study showed the presence of γ-amonobutyrate (GABA) system composed of GABA synthesize enzyme glutamate decarboxylase (GAD), GABA and GABA receptors in lower airway tract epithelial cells of GAD67-GFP knock-in mice. The aim of this study was to investigate the presence of GABA system in upper airway tract. To observe GAD expression, mucous membranes of upper airway tract of adult male GAD67-GFP mice were observed by fluorescent microscopy. To examine GABA receptor expression, reverse transcription-polymerase chain reaction (RT-PCR) analysis of the mucous membranes was made. Strong GFP fluorescence was exclusively observed in the cells of olfactory epithelium. RT-PCR analysis showed that the olfactory epithelium express subunit mRNAs of all GABA receptor types; $GABA_A$, $GABA_C$ and $GABA_B$. From the results of this study, it was suggested that presence of GABA system in the olfactory epithelium of mammals.

Key words: Olfactory epithelium, GABA system, GAD67-GFP knock-in mouse

INTRODUCTION

γ-Aminobutyrate (GABA) is a principal inhibitory neurotransmitter in adult mammalian brain. GABA is also detected in many peripheral tissues.[1] Mammalian species expresses two isoforms of GAD, GAD65 and GAD67, where the numbers refer to the molecular mass in kDa. The two GAD isoforms are products of two distinct genes. In the brain, GAD67 is responsible for synthesis of >90% of GABA and is a soluble cytosolic protein, whereas GAD65 is preferentially localized near neuronal synaptic vesicles.[2] Therefore, to

study the precise distribution and morphology of GABAergic neurons, GAD67-green fluorescent protein (GFP) knock-in mouse (GAD67-GFP mouse) has been successfully employed.[3] We investigated the male reproductive organs of this transgenic mouse by fluorescence microscopy to survey GABAergic cells, and found cells with strong GFP signal in the epithelium of the initial segment and proximal caput of the epididymis.[4] After that, same survey has been made for the male respiratory organs including trachea and lung, and also found cells with intense GFP signal in the airway epithelium.[5] In the present study, we examined the upper respiratory tract of this transgenic mouse by fluorescent microscopy and observed cells with strong GFP signals in olfactory mucosa. Furthermore, results of RT-PCR analysis indicated expression of $GABA_A$, $GABA_B$ and $GABA_C$ receptor subunits.

MATERIALS AND METHODS

Animals

The generation of GAD67-GFP mice has been described by Tamamaki et al.[3], and these mice were maintained at the Department of Anatomy, Osaka Medical College. Male wild-type ICR mice (4 weeks old) were obtained from Clea Japan (Osaka, Japan). All animals were caged in a temperature-controlled room (23˚C), and allowed water and normal food (CE-2, Nihon Clea) ad libitum. We used a standard dark/light schedule of 12/12 hr. All animal experiments were reviewed and approved by the Ethics Review Committee for Animal Experimentation of Osaka Medical College.

GFP Fluorescence Observation

Twenty adult male GAD67-GFP mice aged from 4 weeks to 4 months were used for the analysis of GFP-distribution in their upper respiratory tract including nasal cavity, pharynx and larynx. The mice were anesthetized with pentobarbital (50mg/kg i.p.) and perfused with Ringer's solution via left ventricle followed by the 4% formaldehyde in 0.1 M phosphate-buffered saline (PBS, pH7.4). Their nasal cavity, pharynx and larynx were dissected and post-fixed for 5 hours in the same fixative in a refrigerator (4˚C). In addition to these organs, cerebellum was also dissected as a positive control of GFP-protein expression. Then the specimens were rinsed with PBS and immersed in 30% sucrose for cryoprotection. Specimens were embedded in OCT compound (Miles, Elkhart, USA). The blocks were cut into 20 μm thick sections with a cryostat (Leica CM3050, Nussloch, Germany), air-dried at room temperature and mounted with aqueous/dry mounting medium Crystal/Mount (Biomeda, Foster, CA, USA). Some sections were stained with propidium iodide (PI, Molecular Probes, Eugene, OR) after treatment with RNAase for 60 minutes at 37˚C.

Fluorescence was observed and photographed using a fluorescence microscope (Nikon Eclipse E600, Tokyo, Japan) equipped with a digital camera (VB7000/7010, Keyence Co., Osaka, Japan).

RNA preparation and reverse Transcription polymerase chain reaction (RT-PCR)

The GAD67-GFP mice and ICR mice were anesthetized with pentobarbital (50 mg/kg, i.p.) and perfused transcardially with Ringer's solution via the left ventricle. The olfactory mucosa were dissected from the nasal cavity of the animals (n=6). We amplified $GABA_A$ and $GABA_B$ receptor subunits by RT-PCR as described by Abe et al.[4] and Tamayama et al.[6], except we used new mouse primer 1; (forward 5'-ACAGACCTGCTCTCTGGAAATCG-3' and reverse 5'-GGGTTTCTCTCCGTTCTCAGGC-3' (633 bp product), Genbank accession number NM-008075) and new mouse primer 2; (forward

5'-AGCAGCACTGGCTGGTACAACC-3' and reverse 5'-AGAATGTGGCTTGTTGGGTAGCC-3' (452 bp product), Genbank accession number NM-008076) for $GABA_C$ receptor subunits. The PCR products were separated on 1.5% agarose gels, stained with 0.1 mg/mL ethidium bromide, visualized by UV transillumination, and documented on black and white instant film.

RESULTS

Distribution of GFP-Positive Cells

In 4 weeks- to 4 months-old GAD67-GFP mouse upper respiratory tract, strong GFP-positive cells were found in the olfactory epithelium of nasal cavity (Fig. 1), and no GFP fluorescence was observed in the nasal mucosa except for olfactory mucosa, and pharyngeal and laryngeal mucosae (data not shown). The Purkinje cells in the cerebellum as a positive control of GFP protein expression showed extremely strong GFP fluorescence (Fig. 2).

The distribution pattern and GFP fluorescence intensity were unchanged from 4 weeks-old to 4 months-old mice.

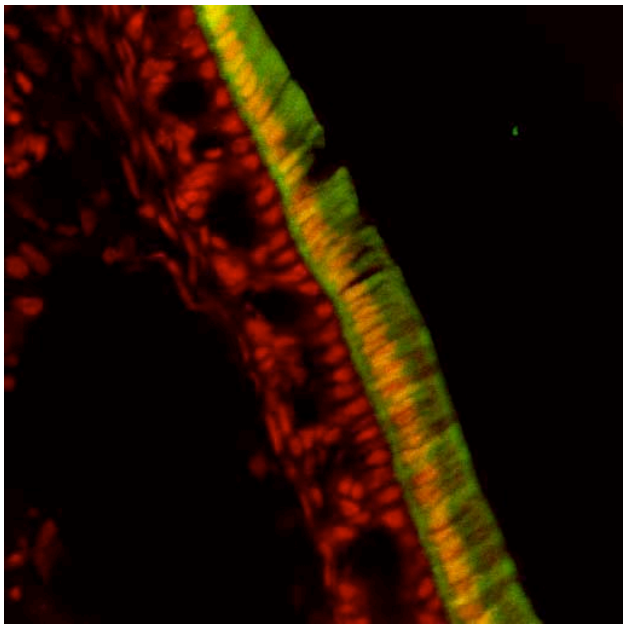

Fig.1. Fluorescent micrograph of green fluorescent protein (GFP)-positive cells in the olfactory mucosa of GAD67-GFP knock-in mouse. GFP fluorescence (green). Propidium iodide staining of nuclei (red).

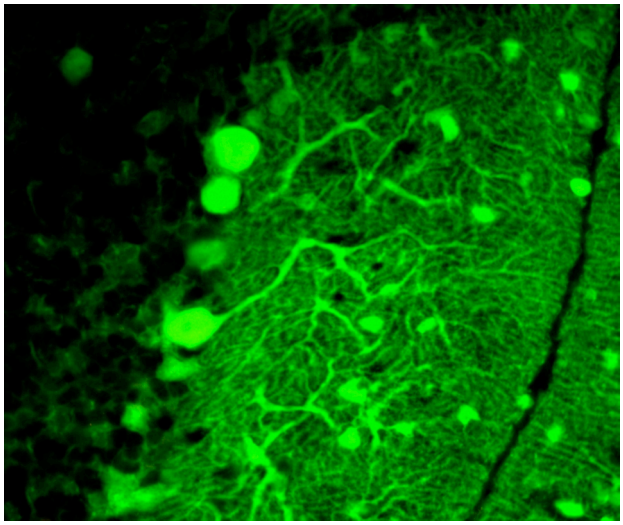

Fig.2. Fluorescent micrograph of green fluorescent protein (GFP)-positive Purkinje cells in the cerebellum of GAD67-GFP knock-in mouse.

Analysis of $GABA_A$, $GABA_B$ and $GABA_C$ receptor subunit mRNAs

Analysis of $GABA_A$, $GABA_B$ and $GABA_C$ receptor subunit mRNAs of olfactory mucosae from GAD67-GFP and ICR mice showed identical results. $GABA_A$ receptor subunit $\alpha1$, $\alpha5$, $\beta2$, $\beta3$, and $\gamma3$ mRNAs, $GABA_B$ receptor subunit R1a, R1b and R2 mRNAs and $GABA_C$ receptor subunit were detected (Fig. 3).

DISCUSSION

The olfactory epithelium of mammals contains four major cell types: the olfactory neurons, sustentacular (supporting) cells, basal cells, and secretory cell of the Bowman's glands. The olfactory epithelium is unique in the mammalian nervous system as it is a site of continual neurogenesis[7) 8)]. The basal cells are

the progenitors which proliferate and differentiate into olfactory neurons, even in adult animals. Therefore, the olfactory neurons are further divided into two types; immature (differentiating) olfactory neurons and mature

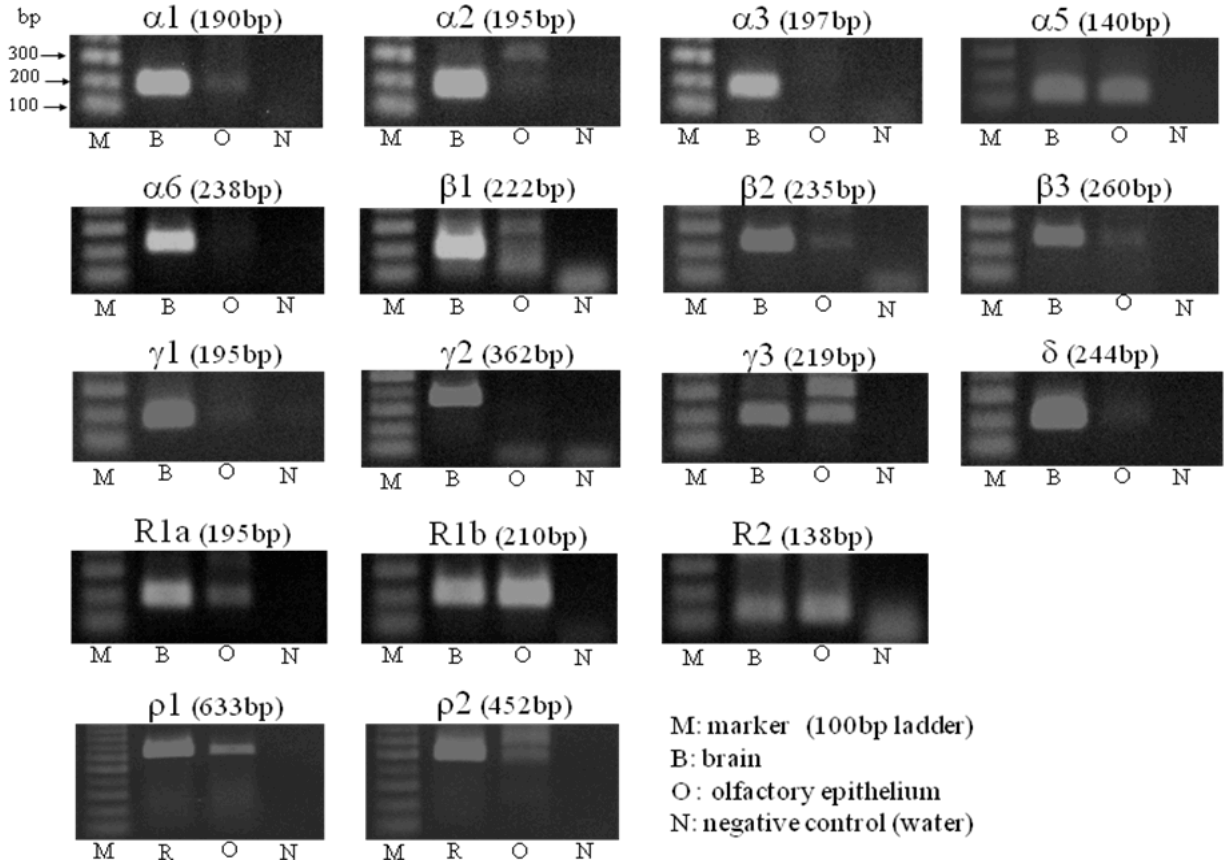

Fig. 3. RT-PCR analysis of $GABA_A$, $GABA_B$ and $GABA_C$ receptor subunit mRNAs from GAD67-GFP knock-in mouse olfactory mucosa and brain as a positive control. In the mouse olfactory mucosa, α1, α5, β2, β3, γ3 $GABA_A$ receptor subunits, R1a, R1b and R2 $GABA_B$ receptor subunits, and ρ1 and ρ2 $GABA_C$ receptor subunits are detected

olfactory neurons. Fluorescence observation of GAD67-GFP mouse nasal mucosa showed that cells with intense GFP fluorescence were localized in the olfactory mucosa, but not in the nasal mucosa. This result indicates that certain cells in mouse olfactory epithelium can synthesize GABA.

The effects of GABA are mediated through ionotropic $GABA_A$ and $GABA_C$ or metabotropic $GABA_B$ receptors[1]. As to the $GABA_A$ receptors, most studies of heterologous expression systems have shown that the functional $GABA_A$ receptor contains at least one α subunit, one β subunit, and one γ or δ subunit. In the present study, RT-PCR analyses showed expression of certain mRNAs of $GABA_A$ receptor subunits to assemble functional $GABA_A$ receptors. As our RT-PCR analysis showed that mRNAs of $GABA_C$ receptor subunits were also detected. $GABA_C$ receptors were first found exclusively in the retina [9], and recent studies showed that these receptors are expressed in many brain regions[10][11][12], while in neuronal and non-neuronal peripheral tissues functional

$GABA_C$ receptors have not been found so far.

In addition to ionotropic GABA receptors, $GABA_A$ and $GABA_C$, we confirmed here the expression of metabotropic $GABA_B$ receptor subunit mRNAs. $GABA_B$ receptors are coupled to G proteins, and active functional $GABA_B$ receptors appear to be heterodimers of the R1 and R2 subunits. In the R1 subunit, two splice variants (R1a and R1b) have been identified[13]. By RT-PCR, mRNAs of the R1a, R1b and R2 subunits of $GABA_B$ receptors were detected. The function of the GABA system composed of the GABA production system and GABA receptors in olfactory epithelial cells remains unclear. However, it is well known that the olfactory epithelium of adult mammals contains a population of basal stem cells capable of dividing and differentiating into olfactory neurons[7)8)], and GABA has been implicated in modulating of important cellular events including proliferation, migration, axonal growth, differentiation in the developing CNS[14]. Furthermore, in the peripheral non-neuronal tissue, rat growth plate chondrocytes, it has been shown that activation of the GABA receptors promotes proliferation of the cells [6]. Taken together, it is possible to estimate that GABA system might contribute physiological functions in the mammalian olfactory epithelium.

Further studies should be directed towards defining which epithelial cell types possess GABA production system and GABA receptors to clarify the functions of GABA in the olfactory epithelial cells.

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