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Lateralization, maturation, and anteroposterior topography in the lateral habenula revealed by ZIF268/EGR1 immunoreactivity and labeling history of neuronal activity

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ABSTRACT

We report habenular lateralization in a simple transgenic mouse model used for labeling a facet of neuronal activity history. A transgenic construct comprised of a *zif268/egr1* immediate-early gene promoter and a gene for normal Venus fluorescent protein with a membrane tag converted promoter activity into long-life fluorescent proteins, which was thought to describe a facet of neuronal activity history by summing neuronal activity. In addition to mapping the immediate-early gene-immunopositive cells, this method helped demonstrate the functionality of the lateral habenular nucleus (LHb). During postnatal development, the LHb was activated between postnatal days 10 and 16. The water-immersion restraint stress also activated the LHb over a similar period. LHb activation was functionally lateralized, but had no directional bias at the population level. Moreover, the posterior LHb was activated in the early stage after the stress, while the anterior LHb was activated in the later stage. Our results indicate lateralization, maturation, and anteroposterior topography of the LHb during postnatal development and the stress response.

1. Introduction

Identifying the neuronal circuits responsible for specific functions is important for understanding the neural basis of animal behaviors. The habenula (Hb) consists of medial and lateral nuclei (MHb and LHb), receives inputs from the basal ganglia and limbic system, extends axons to the fasciculus retroflexus (FR), and sends outputs to forebrain and midbrain nuclei including monoaminergic neurons. The Hb is involved in the regulation of emotional behaviors. In zebrafishes, silencing the MHb activity results in freezing, suggesting its involvement in expression of fear responses ranging from the active coping response of flighting to the passive coping response of freezing (Agetsuma et al., 2010; Okamoto et al., 2012). Another hypothesis suggests that the MHb is involved in controlling levels of anxiety. Because silencing the MHb activity causes elevated levels of anxiety, it may induce greater fear responses, resulting in the transition from active to passive coping (Lee et al., 2010; Mathuru and Jesuthasan, 2013). Further research in mice shows that two parallel pathways through the ventral and dorsal MHb are involved in anxiety- and fear-related behaviors, respectively (Yamaguchi et al., 2013). Neurons in the Hb also respond to stress and aversive stimuli. For instance, neuronal activity as assessed by immediate-early gene (IEG) expression is elevated in the LHb after stress (Wirtshafter et al., 1994; Shumake et al., 2003). Dysfunction of the LHb has been implicated in depression, schizophrenia, and drug addiction (Hikosaka, 2010). Fiber connection from the MHb to the LHb (Kim and Chang, 2005) suggests that the habenular complex integrates information about potentially harmful environments against survival and regulates emotional behaviors. In addition, the Hb shows structural asymmetry in lower vertebrates, thus attracting considerable interest as a model of brain lateralization (Concha et al., 2012). In zebrafish, asymmetry in the dorsal Hb (the fish homolog of the mammalian MHb) is well documented in terms of its development,

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gross wiring, and single axon morphology (Aizawa et al., 2005; Gamse et al., 2005; Aizawa et al., 2007; Bianco et al., 2008). It is still unclear whether there is asymmetry in the ventral Hb (the fish homolog of the mammalian LHb) (Amo et al., 2010). Hb asymmetry in mammals is controversial; further investigation is required to understand the links between the structural symmetry and functional lateralization of the Hb (Bianco and Wilson, 2009).

Various methods have been employed for evaluating circuit functionality. IEG expression level is a reliable marker of increased neuronal activity (Saffen et al., 1988; Worley et al., 1993; Zangenehpour and Chaudhuri, 2002; Guzowski et al., 2005); thus, transgenic reporters of neuronal activity that utilize destabilized fluorescent proteins under the control of IEG promoters are useful (Wilson et al., 2002; Barth et al., 2004; Wang et al., 2006; Man et al., 2007; Eguchi and Yamaguchi, 2009; Kawashima et al., 2013). However, fluorescent proteins persist significantly longer than neuronal activity even if they are destabilized. The long-term functions of neuronal circuits can therefore be examined using the slow kinetics of normal fluorescent proteins. Theoretically, neuronal activity history can be described by summing neuronal activity; however, this has not been thoroughly investigated. It is hypothesized that normal long-life fluorescent proteins controlled by IEG promoters are accurate for summing neuronal activity, with the resulting signal indicating a facet of neuronal activity history (Supplementary Fig. 1a–h). To test our hypothesis, we generated transgenic mice having the *zif268/egr1* IEG promoter linked to the normal yellow fluorescent protein gene (*Venus*) with a membrane tag (Man et al., 2007; Arni et al., 1998; Nagai et al., 2002; Slade et al., 2002; Knapska and Kaczmarek, 2004; Barth, 2007). This method was useful in connecting experiences to their corresponding neuronal circuits. By examining a facet of neuronal activity history in addition to mapping the IEG-immunopositive cells, we report the functional lateralization, maturation, and anteroposterior topography of the LHb during postnatal development and water-immersion restraint stress response in mice.

2. Materials and methods

Animal experiments were carried out in accordance with the National Institute of Health Guide for the care and use of laboratory animals. All experimental protocols were approved by the Committees for Animal Care and Use of the University of Tsukuba and the University of Toyama. All efforts were made to minimize the number of animals used and their suffering.

2.1. Transgenic mice

The gene construct consisted of five elements: a promoter derived from *zif268/egr1*, the QBI SP163 translational enhancer element, a membrane localization sequence, a gene for the brighter variant of the yellow fluorescent protein with normal long-life (*Venus*), and an SV40 poly(*A*) sequence. The human *egr1* (also called *krox-24*, *zif268*, *TIS8*, or *NGFI-A*) gene was purchased from American Type Culture Collection (ATCC, Manassas, VA; TIS8H7, No. 65849) (Sakamoto et al., 1991). TIS8H7 was amplified using *Pfu* DNA polymerase (Promega, Madison, WI) with the following primers to obtain the *zif268/egr1* promoter (1004-bp length of the 5′-untranslated region of the gene): 5′-end with an AseI site, GGGATTAATAATCAGCTTCCCCACTTCGG, and 3′-end with an XhoI site, GGGCTCGAGAGTTCTGCGGCTGGATCTCT (Hokkaido System Sciences, Sapporo, Japan). The QBI SP163 element derived from *pcDNA4/HisMaxB* (Invitrogen, Carlsbad, CA) was inserted between the promoter and *Venus*, which functions as a strong translational enhancer through ribosome recruitment and a cap-independent translation mechanism (Stein et al., 1998). The sequence of the 20-amino acids N-terminal fragment of GAP43 was used for membrane localization, which was fused in frame to *Venus* (Supplementary Fig. 1i).

To generate the transgenic mice, the linearized construct (2274 bp) was excised from the vector, electrophoresed on a 2% agarose gels, purified from the gel, and diluted in 50 ng/μL with 100 mM Tris–HCl and 10 mM EDTA (pH 7.4). DNA solution was injected into fertilized oocytes derived from BDF1 mice (Laboratory Animal Resource Center, University of Tsukuba, Tsukuba, Japan). Tail genomic DNA was prepared and germline transmission of the transgenes in each line was verified using polymerase chain reaction; the primers were directed against regions in *Venus* (CTGGTCGAGCTGGACGGCGACG and CACGAACTCCAGCAGGACCATG). Nine founder lines were obtained. Five of the nine showed no fluorescence for Venus, and one line exhibited uniform fluorescence for Venus in the brain; the other six lines were not used for further investigations. Three transgenic lines were maintained as heterozygotes and crossed for multiple generations into C57BL/6J mice. To ascertain transgenic integration into the genome, Southern blotting was performed with the probe against *Venus* on the three lines with three restriction enzymes. One of the three lines exhibited a single integration site and this line (*zsgv-a*) was used for experiments in this report. The other two lines were designated as *zsgv-b* and *zsgv-c*. Animals were maintained in single-sex cages with up to five littermates per cage.

2.2. Immunohistochemistry and in situ hybridization

Mice were deeply anesthetized with pentobarbital (50 μg/g body weight) (Dai-nippon Sumitomo Pharma., Osaka, Japan), and transcardially perfused with PBS and 3.7% formaldehyde in PBS. Their brains were immersed in the same fixative at 4 °C overnight, embedded in gelatin (16.7% gelatin, 16.7% glycerol in PBS), and post-fixed in the same fixative for 4 d at 4 °C. The gelatin block was cut into 70-μm-thick sections using the Vibratome (VT1500, St. Louis, MO) and rinsed with PBS. Sections were divided into two sets containing every other section. One set was washed in PBS with 0.5% Triton-X100 (PBT), blocked with PBT with 10% normal goat serum, and subsequently stained with a 200-fold dilution of anti-ZIF268/EGR1 antibody (C-19, Santa Cruz Biotechnology, Santa Cruz, CA) with an appropriate secondary antibody coupled with Alexa594 (Invitrogen). The other set was washed in PBS with 0.5% Triton-X100 (PBT) and stained with a 50-fold dilution of NeuroTrace 530/615 red fluorescent Nissl stain (Invitrogen) in PBS for 20 min. The sections were mounted on glass slides in Mowiol 4-88 (Merck Millipore) and observed under a fluorescence microscope (DMRXAA2, Leica, Wetzlar, Germany). IEG-immunopositive cells in the LHb and labeling in the FR were examined during postnatal development from P9 to P70. The images were obtained with a digital camera (VB-7000, Keyence, Osaka, Japan). They were processed with the same parameters in ImageJ software (National Institutes of Health, Bethesda, MD). The number of immunopositive cells was counted in the LHb.

Brains of the *zsgv-a* transgenic mice were dissected on P13. They were divided into the anterior (including the Hb) and posterior regions (including the FR) by using a stainless brain slicer matrix (Neuroscience Inc., Tokyo, Japan). From the anterior parts, fresh-frozen coronal sections 20 μm in thickness were prepared and processed for *in situ* hybridization. *Venus* (nucleotides 1–720) was used as template. Digoxigenin-11-UTP (Roche)-labeled cRNA probe was synthesized according to the manufacture instructions. *In situ* hybridization was carried out as described by Esumi et al. (2005). The posterior parts were fixed in 3.7% formaldehyde in PBS, embedded in gelatin and cut into 70-μm-thick sections using the Vibratome. The sections were stained with NeuroTrace 530/615 red fluorescent Nissl stain.