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Research article

A comparative study of sex difference in calbindin neurons among mice, musk shrews, and Japanese quails

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HIGHLIGHTS

- The MPN and BNST of mice contain SDNs composed of calbindin neurons.
- The MPN of musk shrews contains a SDN composed of calbindin neurons.
- The BNST of musk shrews contains a SDN composed of non-calbindin neurons.
- Calbindin neruons in the MPN and BNST of Japanese quails were small in number.

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ABSTRACT

The medial preoptic nucleus (MPN) and the bed nucleus of the stria terminalis (BNST) of mice contain sexually dimorphic nuclei (SDNs) that are larger and have more neurons expressing calbindin D-28K (CB), a calcium-binding protein, in males than females. However, it is largely unknown whether such SDNs exist in species other than rodents. In this study, we performed an immunohistochemical study of CB in the MPN and BNST of musk shrews and Japanese quails to examine the existence of homologs of SDNs in mice. Like mice, musk shrews had a SDN exhibiting male-biased sex differences in volume and CB-immunoreactive (ir) cell number in the MPN. The BNST of musk shrews also contained a male-biased SDN, but consisted of non-CB neurons. The paratenial thalamic nucleus of musk shrews, but not mice, had more CB-ir cells in males than females. In Japanese quails of both sexes, CB-ir cells in the MPN and BNST were extremely small in number and did not cluster. These results suggest that the distribution of CB neurons differs among these species. Musk shrews may have a homolog of the SDN composed of CB neurons in the MPN of mice.

1. Introduction

Sexually dimorphic nuclei (SDNs) are found in the brain in many vertebrate species. The first SDN was discovered in the medial preoptic nucleus (MPN) of rats and termed the sexually dimorphic nucleus of the preoptic area (SDN-POA), which is larger and has more neurons in males than in females [1,2]. SDNs located in the MPN/anterior hypothalamus have been shown in other species, including guinea pigs [3], ferrets [4], spotted hyena [5], sheep [6], monkeys [7], and humans [8,9]. Like the MPN, the bed nucleus of the stria terminalis (BNST) contains a subnucleus exhibiting male-biased sex differences in rats and mice with respect to the volume and neuron number [10,11]. The MPN is a key component of the neural system controlling male sexual behavior in a variety of species [12,13]. The BNST is also a region that regulates male sexual behavior in mammalian and avian species [14,15]. The male-biased SDNs of the MPN and BNST may be responsible for the control of sexual behavior in males.

In the MPN and BNST of rats and mice, there are clusters of neurons expressing calbindin D-28K (CB), a calcium-binding protein, which are larger and have more CB neurons in males than in females [16–18]. The cluster of CB neurons in the MPN is termed

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the calbindin-sexually dimorphic nucleus (CALB-SDN) [16], which is regarded as the inner part of the SDN-POA. The cluster of CB neurons in the BNST corresponds to the principal nucleus of the BNST (BNSTp). Thus, CB is a useful marker to identify male-biased SDNs in the MPN and BNST of rats and mice. In addition, rabbits show female-biased sex differences in the number and distribution pattern of CB neurons in the preoptic/anterior hypothalamic area [19], suggesting that sex differences in CB neurons differ among species. However, it has not yet been determined whether other animal species exhibit sex differences in CB neurons of the MPN and BNST and contain nuclei regarded as homologous with the CALB-SDN and BNSTp of mice and rats. In this study, we performed histological analysis of the MPN and BNST in musk shrews, a mammalian species belonging to the order Insectivora, and Japanese quail, a commonly used laboratory animal belonging to the class Aves, to examine the existence of homologs of SDNs in mice.

2. Methods

2.1. Animals

Adult male and female ICR mice (10-weeks old; Sankyo Labo Service Corporation, Tokyo, Japan) were housed in a room maintained at 23 ± 2 °C with a 12-h light/12-h dark cycle (lights on from 8:00 to 20:00) with free access to water and standard diet (Labo MR breeder; Nippon Nousan Kougyo, Yokohama, Japan). Musk shrews and Japanese quails used in this study were bred in our animal facility. Adult male and female musk shrews (16-weeks old) of an outbred KAT strain, which have been established from a wild population in Kathmandu, Nepal, were housed individually in plastic cages equipped with an empty can for a nest box at 23 ± 2 °C with a 12-h light/12-h dark cycle (lights on from 8:00 to 20:00) with free access to water and commercial feeding pellets (number 5P; Nippon Formula Feed Manufacturing, Yokohama, Japan). Adult male and female Japanese quails (8-weeks old) were housed in a room maintained at 23 ± 2 °C with a 14-h light/10-h dark cycle (lights on from 7:00 to 21:00) with free access to water and food (QMAX, Marubeni Nisshin Feed, Tokyo, Japan). All animal procedures were approved and performed in accordance with the Guidelines for the Care and Use of Experimental Animals of Saitama University and the National Institute for Environmental Studies.

2.2. Western blotting for CB

To qualify the anti-CB antibody used in this study, Western blotting was carried out using cerebellar tissues (n = 2 for each species), because CB is strongly expressed in the cerebellum in most species [20]. The tissues were homogenized in a lysis buffer and centrifuged. The supernatant was then mixed with 1/4 vol of 0.29 M Tris-HCl (pH 6.8) containing 8.3% sodium dodecyl sulfate, 25% glycerol, 7.75% dithiothreitol, and 0.01% bromphenol blue and heated at 95 °C for 3 min. Proteins were separated by electrophoresis on a polyacrylamide gel and electrophoretically transferred from the gel to a polyvinylidene difluoride membrane. The membranes were treated with an Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, NE, USA) for 30 min at room temperature and then with a mouse monoclonal anti-CB antibody (1:20,000; C9848; Sigma-Aldrich, St. Louis, MO, USA) in the mixture of equal volume of the blocking buffer and TBS (20 mM Tris-HCl [pH 7.6] and 0.9% NaCl) containing 0.4% Tween-20 for 2 h at room temperature. The membranes were then reacted with goat anti-mouse IgG conjugated with IRDye 800CW (1:5000, LI-COR Biosciences) in the mixture of equal volume of Odyssey Blocking Buffer and TBS containing 0.4% Tween-20 and 0.02% sodium dodecyl sulfate for 1.5 h at room temperature. Immunofluoresent signal on the membranes was visualized by Odyssey CLx Imaging System (LI-COR Biosciences).

2.3. Tissue preparation

Mice (n = 4 for each sex) and musk shrews (n = 6 in male; n = 7 in female) were deeply anesthetized by intraperitoneal injection of sodium pentobarbital (64.8 mg/kg body weight). Animals were then perfused transcardially with 0.05 M phosphate-buffered saline (PBS, pH 7.4), followed by perfusion fixation with 4% paraformaldehyde in 0.05 M phosphate buffer (PB, pH 7.4). Brains were post-fixed with the same fixative at 4 °C overnight. After the brains were immersed in 0.05 M PBS at 4 °C overnight, they were immersed in 30% sucrose-containing 0.05 M PB at 4 °C for 2 days and were quickly frozen. Frozen brains including the MPN and BNST were coronally sectioned at a thickness of 30 μm using a cryostat and were collected at 60-μm intervals.

The brains of Japanese quails (n = 5 for each sex) were obtained after animals were decapitated. The brains were fixed by immersion fixation with 4% paraformaldehyde in 0.05 M PBS at 4 °C for 5 days. The fixed brains were then immersed in 0.05 M PBS at 4 °C overnight and in 30% sucrose-containing 0.05 M PB at 4 °C for 2–3 days, and were quickly frozen. Frozen brain tissues including the MPN and BNST were cut at a thickness of 30 μm and collected at intervals of 60 μm.

In addition, 30-μm-thick cerebellar sections from mice, musk shrews, and Japanese quails (2 males and 2 females in each species) were made. These sections were used for immunohistochemistry to qualify the anti-CB antibody.

2.4. Immunohistochemistry for CB

Brain sections were treated with 0.6% H_2O_2 in 0.05 M PBS containing 1% Triton X-100 (PBST) for 30 min at room temperature. The sections were treated with 5% normal goat serum in PBST for 1 h at room temperature and were then reacted with a mouse monoclonal anti-CB antibody (C9848; Sigma-Aldrich) in 5% normal goat serum-containing PBST at 4 °C. The dilution factor and reaction time of the antibody was 1:15,000 and 2 days for mice, 1:10,000 and 1 day for musk shrews, and 1:5000 and 2 days for Japanese quails. Some cerebellar sections were not reacted with the anti-CB antibody to serve as negative controls for qualifying the primary antibody. After rinsing in PBST, sections were reacted with peroxidase-labeled polymer conjugated to goat anti-mouse immunoglobulin (Dako EnVision Plus, Dako, Carpinteria, CA, USA) for 30 min at room temperature. CB-immunoreactivity was visualized using a Dako DAB Plus chromogen System (DAKO). The sections mounted on gelatin-coated glass slides were counterstained with methyl green.

2.5. Nissl staining

Brain sections from musk shrews and Japanese quails were mounted on gelatin-coated glass slides. They were placed in a graded series of ethanol solutions, and stained with 0.1% cresyl fast violet solution. The sections were then placed in distilled water and a graded series of ethanol solutions, dehydrated with absolute ethanol, cleared with xylene, and covered with an embedding medium and coverslipped.

2.6. Microscopy and stereological analysis

Brain sections were observed under a light microscope. We measured the number of CB-immunoreactive (ir) cells and the volume of the CALB-SDN and BNSTp in mice and those of the CALB-SDN and paratenial thalamic nucleus (PT) in musk shrews