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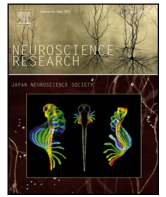

Immunohistochemical study on the neuronal diversity and three-dimensional organization of the mouse entopeduncular nucleus

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ABSTRACT

The entopeduncular nucleus (EPN) is one of the major output nuclei of the basal ganglia in rodents. Previous studies have divided it into rostral and caudal halves, with the former containing somatostatin (SOM)-immunoreactive neurons and the latter dominated by parvalbumin (PV)-containing neurons, respectively. However, it is unclear whether this simple rostrocaudal segmentation is appropriate, and the possibility of the existence of other neuronal populations remains to be investigated. In this study the cytoarchitecture of the mouse EPN was analyzed immunohistochemically. Substance P (SP)-immunoreactivity determined the extent of the EPN, which was 800 μm-long along the rostrocaudal axis. PV-positive neurons were concentrated in the caudal two-thirds of this range. PV-negative neurons were abundant in the rostral half but were further located caudally around the PV neuron-rich core. PV(+)/SOM(−) and PV(−)/SOM(+) neurons constituted 28.6% and 45.7% of EPN neurons, respectively, whereas the remaining population (25.7%) exhibited neither immunoreactivity. Eleven percent of EPN neurons lacked immunoreactivity for glutamic acid decarboxylase, indicating their non-GABAergic nature. Three-dimensional reconstruction revealed that PV-rich/SP-poor core was surrounded by PV-poor/SP-rich shell region. Therefore, presumptive thalamus-targeting PV neurons are outnumbered by other populations, and the regional heterogeneity shown here might be related to functionally distinct pathways through the basal ganglia.

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1. Introduction

The basal ganglia consist of several subcortical nuclei and are important for coordinated movement, cognition, procedural memory and reward-based learning (Pennartz et al., 2009). Dysfunction in the basal ganglia causes not only movement disorders such as Parkinson disease and Huntington's chorea but also some cognitive and mental disturbances (Crittenden and Graybiel, 2011). Although the structure of the basal ganglia has been extensively studied, there still remain many questions to be solved for the precise understanding of the action of the basal ganglia. One such issue concerns the complexity in the internal structure of the individual nuclei of the basal ganglia.

The output nuclei of the basal ganglia include the internal segment of the globus pallidus (GPi) and the substantia nigra pars reticulata (SNr) in primates. In the rodent brain, the entopeduncular nucleus (EPN) corresponds to the GPi. Tracer experiments have revealed that EPN neurons in the rat project to the lateral habenula (LHb), ventral anterior-ventral lateral thalamus, parafasicular-center median complex, and tegmenti pedunculopontis nucleus of the brain stem; the latter three regions are thought to be innervated by collaterals of the same EPN neurons (van der Kooy and Carter, 1981). EPN of the rat has been classified roughly into the rostral and caudal halves: the rostral EPN contains somatostatin (SOM)-immunoreactive neurons, which project to the LHb (Vincent and Brown, 1986), whereas the latter contains parvalbumin (PV)-immunoreactive neurons (Rajakumar et al., 1994; Hontanilla et al., 1997, 1998), which project to the thalamus (Rajakumar et al., 1994). However, the position of the border of these two regions along the rostrocaudal axis is ambiguous and

Abbreviations: BSA, bovine serum albumin; CB, calbindin; CeM, medial part of the central nucleus of amygdala; ChAT, choline acetyltransferase; CLSM, confocal laser scanning light microscopy; CR, calretinin; EPN, entopeduncular nucleus; GAD, glutamic acid decarboxylase; GPi, globus pallidus internal segment; ic, internal capsule; LHb, lateral habenula; NOS, nitric oxide synthase; NPY, neuropeptide Y; PFA, paraformaldehyde; PBS, phosphate-buffered saline; PV, parvalbumin; SD, standard deviation; SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; SOM, somatostatin; SP, substance P; TRN, thalamic reticular nucleus; VIP, vasoactive intestinal peptide; VPL, ventral posterolateral thalamic nucleus.

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differs among studies, ranging from one-third to two-thirds along the axis. The topography of PV neurons was also characterized by their concentration in the central core of the nucleus at the caudal level (Hontanilla et al., 1997). It remains unknown, however, if distributions of other neuronal populations are similar or complementary to that of PV neurons. Moreover, quantitative analysis of both the number and spatial position of EPN neurons belonging to different populations has not been attempted so far.

Chemical heterogeneity of EPN neurons is another issue that is not fully understood. It has been a general idea that output nuclei of the basal ganglia consist of only GABAergic neurons, which also receive GABAergic input from the striatum, thereby regulating the target structures via a disinhibitory mechanism that is triggered by the activation of GABAergic striatal neurons. However, the presence of excitatory connections from the EPN to LHb has been recently demonstrated in the rat (Shabel et al., 2012, 2014). The LHb has gained considerable attention due to its potential contribution to the decision making in behavior of primates by conveying anti-reward or aversive signals (Matsumoto and Hikosaka, 2007, 2009; Hong and Hikosaka, 2008, 2013). Furthermore, the glutamatergic nature has also been shown in some neurons in the substantia nigra in mice (Antal et al., 2014), which is another major output nucleus of the basal ganglia. Thus, the detailed analysis of both the cytoarchitecture and three-dimensional organization of EPN will provide a basis for a broad range of studies on the basal ganglia.

In this study the chemical heterogeneity and spatial distribution of EPN neurons were analyzed immunohistochemically using various molecular markers combined with a quantitative method based upon stereology. The obtained results revealed an unrecognized diversity and spatial arrangement of EPN neurons, both of which will have great importance when stereotaxic experiments including deep brain stimulation are performed.

2. Materials and methods

2.1. Tissue preparation

All of the experiments and animal procedures were performed according to the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publications No. 80-23, revised 1996), and all of the protocols were approved by the Institutional Animal Care and Use Committee at the Kumamoto University. All efforts were made to minimize the number of animals used and their suffering.

Eighteen male C57BL/6J mice (21–26 g, 7–8 weeks old) were deeply anesthetized with sodium pentobarbital (100 mg/kg, i.p.). Fifteen of the animals were perfused via the ascending aorta with phosphate-buffered saline (PBS, pH 7.4) followed by 50 ml of 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4) at room temperature. The remaining three mice were perfused using the same procedure, but PBS and 4% PFA were replaced by 0.9% NaCl and zinc-aldehyde fixative at pH 6.5 (Mugnaini and Dahl, 1983), respectively, in order to enhance the glutamic acid decarboxylase (GAD) immunoreactivity in somata. Brains fixed with PFA were removed from the skull and stored overnight in the same fixative at 4 °C. Next, the fixative was replaced by PBS containing sodium azide. Brains fixed with zinc-aldehyde were processed for sectioning 1 h after the perfusion.

2.2. Immunohistochemistry

Serial 40-μm-thick coronal sections were cut using a vibrating microtome (TTK-3000, Dosaka) from the brain block that contained the entire EPN. After cryo-protection in 25% sucrose in PBS, the sections placed on aluminum foil were rapidly frozen in the vapor of liquid N_2, rapidly thawed in 25% sucrose in PBS, and then processed for triple-fluorescent immunohistochemistry, as previously described (Fukuda and Kosaka, 2000; Tajima and Fukuda, 2013) using slight modifications. Briefly, the sections were incubated with 1% bovine serum albumin (BSA; Sigma) and 0.3% Triton-X in PBS overnight, with a mixture of mouse anti-NeuN (1:250, Millipore), rabbit anti-PV (1:5000, Swant) and rat anti-substance P (SP; 1:400, Millipore) antibodies diluted in 1% BSA–0.3% Triton-X in PBS for 7 days at 20 °C, with biotinylated donkey anti-rat IgG (1:250, Jackson ImmunoResearch) overnight, and with a mixture of Alexa 488-conjugated donkey anti-mouse IgG (1:250, Jackson ImmunoResearch), Cy3-conjugated donkey anti-rabbit IgG (1:400, Jackson ImmunoResearch), and streptavidin-DyLight 649 (1:100, Jackson ImmunoResearch) overnight. The long incubation period with the primary antibodies was essential to improve the permeation of the antibodies into the deep part of the 40-μm-thick sections and thus to obtain confocal images of constant and sufficient quality throughout the depth of the sections (Fukuda et al., 1998; Fukuda and Kosaka, 2000). The second set of triple immunostaining was performed using a mixture of mouse anti-NeuN (1:250, Millipore), rabbit anti-PV (1:5000, Swant) and rat anti-SOM (1:200, Millipore) antibodies, followed by the same procedures as described for the first set of triple immunostaining. Several other sets of triple immunostaining were performed by combining the primary and secondary antibodies listed in Tables 1 and 2, respectively. When the sections were processed for GAD immunostaining, Triton-X was omitted from the incubation medium of all steps because it reduced the immunoreactivity in the soma (Fukuda et al., 1996, 1997). To incubate sections that were fixed by zinc-aldehyde, PBS was replaced by 0.5 M Tris HCl buffer (pH 7.6) in all of the procedures described above. Sections were mounted in Vectashield (Vector Laboratories) and examined using a confocal laser-scanning light microscope (C1 plus, Nikon), which was equipped with three single laser beams, 488, 543, and 633 nm in wavelength, and a filter set of BA 515/30, BA 590/50, and 650 LP. Control sections were prepared by omission of primary antibodies and by mismatching secondary antibodies; both provided only weak non-specific staining.

Table 1
Primary antibodies and dilutions used in the study.

Antibody	Host	Dilution	Source
SP	Rat	1:400	Chemicon (MAB356)
NeuN	Mouse	1:250	Chemicon (MAB377)
NeuN	Guinea – pig	1:1000	Chemicon (ABN90P)
PV	Rabbit	1:5000	Swant (PV25)
PV	Mouse	1:5000	Swant (235)
SOM	Rat	1:200	Chemicon (MAB354)
GAD 65/67	Rabbit	1:5000	Sigma (G5163)
vGluT2	Guinea – pig	1:500	Frontier institute (VGluT2-GP-AF810)
ChAT	Goat	1:1000	Chemicon (AB144P)
CB	Mouse	1:5000	Swant (300)
CR	Goat	1:2500	Chemicon (AB1550)
bNOS	Mouse	1:5000	Sigma (N2280)
NPY	Goat	1:5000	Chemicon (AB1583)
VIP	Rabbit	1:250	Immunostar (20077)

2.3. Confocal laser scanning microscopy

Images for confocal laser scanning light microscopy (CLSM) were obtained using ×4 (Plan Apo, N.A. = 0.2, Nikon), ×10 (Plan Fluor, N.A. = 0.3, Nikon), ×20 (Plan Fluor, N.A. = 0.5, Nikon), ×40 (Plan Fluor, N.A. = 0.75, Nikon), and ×60 (Plan Apo VC, N.A. = 1.4, Nikon) objectives. The ×4 and ×10 objectives were used to visualize the EPN in a single frame in CLSM, whereas the ×20, ×40 and ×60 objectives were used to identify and analyze EPN neurons with sufficient resolution. The size of each frame was 1024 × 1024 pixels, and images of the optical slices were acquired from the

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