Selective distribution of GLUT3-expressing nerve fibers in the lamina terminalis among the circumventricular organs of mice

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ABSTRACT
Sensory circumventricular organs contain the subfornical organ, organum vasculosum of the lamina terminalis (OVLT), and area postrema. Here, immunostaining for GLUT3 in the murine brain selectively labeled the subfornical organ and OVLT. The immunoreactive neural tract of the subfornical organ formed into thin bundles and extended ventro-rostrally over the anterior commissure. After turning over the commissure, the neural tract passed through the median preoptic nucleus (MnPO) and reached the OVLT; thus, a continuous neural tract expressing GLUT3 connected the subfornical organ, MnPO, and OVLT in the lamina terminalis. In the OVLT, GLUT3-immunoreactive fibers gathered in both the dorsal cap and lateral periventricular zone. Electron microscopically, the immunoreactive structures in the subfornical organ corresponded to nerve fibers or nerve terminals containing many small clear vesicles. The area postrema, another sensory organ, was immunonegative for GLUT3. This study not only presented a useful marker tracing the neural tract in the sensory sites of the lamina terminalis but also suggested a unique system for sensing and determining the metabolism of circulating glucose in the circumventricular organs.

The circumventricular organs (CVOs), specialized brain structures located on the midline of the third and fourth ventricles, are characterized by a condensed vascularization that lacks a blood-brain barrier but possesses sufficient perivascular spaces. The CVOs contain the lamina terminalis and area postrema as sensory apparatuses, while the CVOs of the secretory type are located in the median eminence, pituitary gland, and pineal gland. The lamina terminalis consists of the subfornical organ (SFO), organum vasculosum of the lamina terminalis (OVLT), and median preoptic nucleus (MnPO). There is an intimate relation between neural connections and functions—especially osmoregulation—among these three sites of the lamina terminalis. Morphologically, the SFO has a ventral stalk which reaches the MnPO, while the OVLT dorsally extends towards the anterior commissure to contact with the MnPO. However, the MnPO is usually excluded from sensory CVOs, due to the conventional vasculature with the blood brain barrier.

The SFO and OVLT hold a rich vasculature composed of fenestrated capillaries to supply a kind of “window” for systemic circulation (7). In fact, they express a variety of receptors and binding sites for peripherally released signals (16). The most intensely studied function is that of serving the predominant site of osmoreception. Specific groups of neurons in the lamina terminalis both detect changes in plasma tonicity and transmit this information to the vasopressin-secreting magnocellular neurons of the supraoptic and paraventricular nuclei. Complete ablation of the lamina terminalis (i.e. OVLT, MnPO, and SFO) is needed to abolish an increased vasopressin release in response to the intravenous infusion of hypertonic saline (17), suggesting that all parts of the lamina terminalis may participate in the osmo-
regulatory control of vasopressin release and thirst (17). The CVOs also play important roles in the initiation of neuroinflammatory responses in the brain, as circulating LPS and cytokines induce the quick production of proinflammatory molecules in the CVOs (31). Although CVOs share some morphological and functional characteristics, no common molecules specific for them are known. The present study reports how GLUT3 represents a reliable marker substance for the lamina terminalis and is useful to trace the direct connection. This finding is in contrast to the widely held notion that GLUT3 is a neuronal glucose transporter broadly expressed in the brain.

MATERIALS AND METHODS

**Tissue samplings.** Ten-week-old adult female and male ddY mice were supplied by Japan SLC (Shizuoka, Japan). For immunohistochemistry, ten deeply anesthetized mice were perfused via the aorta with physiological saline, followed with 4% formaldehyde in a 0.1 M phosphate buffer, pH 7.4. The brain was removed, dissected into blocks containing the circumventricular organs and the area postrema, and immersed in the same fixative for an additional 12 h at room temperature. The formaldehyde-fixed tissues were dipped in a 30% sucrose solution overnight at 4°C, embedded in O.C.T. compound (Sakura Finetek, Tokyo, Japan), and quickly frozen in liquid nitrogen. All experiments using animals were performed under protocols following the Guidelines for Animal Experimentation, Hokkaido University Graduate School of Medicine.

**Immunohistochemistry.** Frozen sections 40 μm in thickness were prepared and collected in small glass bottles. After pretreatment with normal donkey serum, floating sections were incubated with a mixture of two antibodies in the following panel: guinea pig anti-mouse GLUT3 antibody (1 μg/mL, GLUT3-GP-Af720; Frontier Institute, Ishikari, Japan), guinea pig anti-GLUT1 antibody (1 μg/mL, GLUT1-GP-Af610; Frontier Institute), rabbit anti-CCKA receptor (1 μg/mL, CCKAR-Rb-Af260; Frontier Institute) (13), rabbit anti-mouse calbindin (1 μg/mL, calbindin-Rb-Se-1; Frontier Institute), rabbit anti-calretinin (1 μg/mL, calretinin-Rb-Af200; Frontier Institute), and rat anti-mouse CD31 antibody (0.5 μg/mL, Clone MEC 13.3; BD PharMingen) for 48 h, followed by incubation with Cy3-labeled anti-guinea pig IgG (Jackson ImmunoResearch, West Grove, PA) and one Alexa488-labeled anti-rabbit antibody (Invitrogen) or Alexa488-labeled anti-rat IgG (Invitrogen). During staining, 0.01 M phosphate buffered saline (PBS, pH 7.2) containing 0.3% Triton X-100 was used for diluting antibodies and rinsing. The stained sections were mounted with glycerin-PBS and observed under a confocal laser scanning microscope (Fluoview; Olympus, Tokyo, Japan).

**Silver-intensified immunogold method for electron microscopy.** Frozen sections 20 μm in thickness were mounted on poly-L-lysine-coated glass slides, incubated with the normal donkey serum followed by the anti-GLUT3 antibody (1 μg/mL) overnight, and subsequently reacted with goat anti-guinea pig antibody covalently linked with 1-nm gold particles (1: 200, Nanogold; Nanoprobes, Yaphank, NY). After silver enhancement using a kit (HQ silver; Nanoprobes), the sections were osmificated, dehydrated, and directly embedded in Epon (Nisshin EM, Tokyo, Japan). Ultrathin sections were prepared and stained with both uranyl acetate and lead citrate for observation under an electron microscope (H-7100; Hitachi, Tokyo, Japan).

RESULTS

The SFO is usually divided into two major subdivisions: the rostrodorsal “outer shell” and central “ventromedial core”. In the present study using mice, the anti-GLUT3 antibody labeled both subregions of the SFO supplied by a plexus of CD31-immunolabeled blood vessels (Fig. 1a–c). GLUT3-immunoreactive nerve fibers gathered in the SFO without any noticeable accumulation sites or directions throughout this organ. Small neurons expressing two types of calcium-binding proteins—calbindin and calretinin—intermingled in the SFO (Fig. 1d, e): calbindin-containing neurons were reported to occupy exclusively the ventromedial core (16). In the present staining, calbindin-immunoreactive cell bodies dominated in the ventromedial core of the GLUT3-immunoreactive SFO, while calretinin-immunoreactive cell bodies tended to occupy the outer shell. Calretinin-immunoreactive nerve fibers were extremely numerous at the boundary to the hippocampal commissure (Fig. 1e). A strand composed of GLUT3 fibers extended rostro-ventrally towards the anterior commissure, though it gradually became thin and compact, corresponding to the ventral stalk (16) or partially to the dorsal MnPO. The CD31-immunoreactive vessels were fewer in the ventral stalk than in the main body of the SFO.

A very thin strand with a condensed immunoreac-
Fig. 1 GLUT3 expression in the lamina terminalis of mice. On a sagittal section, double immunostaining of GLUT3 and CD31 shows that a long GLUT3-immunoreactive strand connects the SFO with the OVLT (a). Sagittal (b) and coronal sections (c) display an abundance of CD31-immunoreactive blood vessels in the SFO. In double immunostainings of GLUT3 and calbindin (d) or calretinin (e), calbindin cells gather in the ventromedial cores of the SFO, while calretinin cells are more numerous in the outer shell. GLUT3-immunoreactive fibers course over the anterior commissure (AC) facing the third ventricle (III). Another group of fibers is dispersed in the rostral part of the peri-commissure region indicated by an arrow (f). Fig. 1g is a bright-field image of the same section. HC: hippocampal commissure Bars 200 μm (a), 50 μm (b–e), 100 μm (f, g)
tivity of GLUT3 coursed over the caudal surface of the anterior commissure, appearing exposed to the third ventriculus via the ependyma or tanyocytes (Fig. 1f, g). On the rostral side of the anterior commissure, GLUT3 fibers were more or less dispersed into the dorsal and ventricular portions of the pericommisural subdivision of the MnPO, as classified by Johnson et al. (8). After turning over the anterior commissure, the GLUT3-positive neuropil on the caudal side further extended towards the OVLT without any intervals (Fig. 1a). Noteworthy, the GLUT3-immunoreactive neuropils changed into a thick band and passed through the subependymal region of the ventral part of MnPO towards the OVLT. The intra-MnPO band with GLUT3 immunoreactivity often accompanied a large vessel, possibly a venule, but not capillary networks (Fig. 2a, b). Blood vessels in the whole brain were intensely positive to an antibody against GLUT1, which is a marker of the blood-brain barrier. Blood vessels in both the SFO and OVLT were weakly positive to this GLUT1 antibody.

As for the zonation of the OVLT, McKinley et al. (16) and others (28) proposed a classification of three functional subdivisions. First, the rostromedial vascular region accommodates a vascular plexus—which was immunolabeled with CD31 antibody in the present study. The second portion, the arching dorsal cap, covers the vascular region of OVLT and contains calretinin-expressing neurons. The third one is the lateral (and posterior) periventricular zone which surrounds the central vascular region. Fig. 2c and d show a set of three adjacent sections obtained from two mice each. Approaching the OVLT, the GLUT3-immunoreactive strand dispersed into individual nerve fibers to form a dense nerve plexus in the dorsal cap and lateral zone adjacent to the vascular region. In fact, the densest distribution of positive fibers was seen in the dorsal cap.

Under an electron microscope, positive reactions for GLUT3 were detected as aggregations of silver-intensified gold particles (Fig. 3). The gold particles in the SFO were distributed mainly along the cell membranes of nerve fiber and terminals of various sizes. The nerve terminals often contained small clear vesicles as well as small mitochondria and lysosomes.

The area postrema, another sensory CVO, functions as a center of satiety and vomiting in the medulla oblongata. The area postrema was not immunoreactive for GLUT3, while the nucleus tractus solitarius (NTS) adjacent to the area postrema were diffusely immunoreactive (Fig. 4a). The NTS functions as an important feeding control center and receives a variety of peripheral signals, such as cholecystokinin (CCK). Because of the significant inhibition of the CCK-induced satiety by a vagotomy, vagal afferent input is the most important pathway for peripheral CCK to activate neurons in the NTS (3, 10, 24, 29). In addition to the NTS, a CCK1 receptor (CCKAR) antibody we used was able to intensely immunolabel the area postrema, where immunoreactive fibers gathered and intermingled with CD31-positive blood vessels (Fig. 4b). GLUT3 immunoreactivity in the NTS inside the blood-brain barrier was less intense as compared with immunoreactivity of the CCK1 receptor.

DISCUSSION

GLUT3, originally established as a neuronal glucose transporter, actually contributes to glucose uptake for a unique neuronal metabolism (27). GLUT3 mRNA is highly expressed in the brain, exclusively in neurons (23). Immunohistochemically, GLUT3 of the brain is localized predominantly in the neuropils, namely axons and dendrites, with less labeling in the cell body (1, 4, 6, 15), suggesting the active glucose uptake by GLUT3 for synaptic transmission. However, many immunohistochemical studies for GLUT3 have failed to demonstrate its selective cellular and subcellular localization in neurons, instead reporting diffuse and obscure immunolabelings. In fact, the antibodies we used also failed to immunolabel neural elements in the central nervous system or only produced some immunolabeling of background levels, except for the lamina terminalis (SFO and OVLT), while the same antibody did stain selectively cellular elements out of the brain, such as cell membranes of seminiferous tubules and spermatozoa (12).

It is generally believed that sensory CVOs are composed of the SFO, OVLT and area postrema. Marker substances for equally detecting these apparatuses are not available. Here we displayed GLUT3 as a reliable marker for the SFO and OVLT; it is an especially powerful marker to trace the direct neural connection between them. The intensive expression of GLUT3 in the ventromedial core of the SFO coincides with a high sensitivity for blood-borne angiotensin signals in this SFO subregion (16). GLUT3-immunoreactive fibers in the SFO were also found to be rich in the outer shell and ventral stalk, indicating that signals of GLUT3 cover all regions of the SFO. It has been postulated that the SFO and OVLT are independent organs, but functionally...
Fig. 2  GLUT3 expression in the OVLT. Both sections of Fig. 2 a and b are sagittal sections of the OVLT. A GLUT3-expressing thick nerve strand with a CD31-immunoreactive vein approaches the OVLT and is dispersed in association with the vascular plexus of the OVLT immunolabeled with a CD31 antibody (a). Fig. 2b is a double immunostaining of GLUT3 and calbindin. Fig. 2 c and d are two sets of serial coronal sections of the OVLT obtained from two mice. The order of sectioning is not clear, due to the staining of floating sections in one bottle. A dense distribution of GLUT3 nerve fibers surrounds the green colored vascular plexus labeled with the CD31 antibody. OX: optic chiasm Bars 100 μm (a, b), 50 μm (c, d)
blood vessels with any CD31 immunoreactivity. Therefore, the ventral stalk should be excluded from true sensory CVOs. Accordingly, the blood vessels in the ventral stalk of the rat SFO were reported to be non-fenestrated in type and possess no sufficient pericapillary spaces (26).

Characteristically, the thin and compacted strands of GLUT3-immunoreactive fibers passed over the
GLUT3 in the circumventricular organs

The SFO and OVLT send fibers to the MnPO, in addition to projections to the paraventricular and supraoptic nuclei (18). Within the lamina terminalis, MnPO-derived nerve fibers form synapses with neurons in the SFO and OVLT (20, 30, 32). Thus, reciprocal connections exist between the MnPO and SFO/OVLT. Neuroanatomical tracing combined with c-Fos expression suggests that the circulating peptides—angiotensin II and relaxin—which stimulate thirst—act on neurons within the periphery of the SFO and the dorsal cap of the OVLT (9, 17). The MnPO region differed from the SFO and OVLT in the distribution pattern of GLUT3 fibers, in that they extensively intermingled with the vascular system. Although the MnPO lies inside the blood-brain barrier (16), the novel and continuous neural tract expressing GLUT3 intimately interconnected the SFO and VOLT via the MnPO. However, it remains unknown whether the GLUT3-positive neuropils only pass through the MnPO or supply an important neural tract to the MnPO itself.

The dorsal vagal complex containing the area postrema, NTS, and dorsal motor nuclei of the vagus, is the major viscerosensory center of the medulla oblongata. The area postrema and NTS implement a sensory function for blood-borne messengers including angiotensin II (19). The diffuse and weak immunoreactivity of GLUT3 was found in the NTS but not in the area postrema. Under the light microscope, however, we could not identify any individual nerve elements with GLUT3 immunoreactivity in the NTS, unlike in the SFO and OVLT, indicating a possibility that the immunoreactivity in the NTS may be at a background level. Further investigations are required to determine the expression level of GLUT3 in the NTS.

The area postrema intensely expressed the CCK1 receptor which detects the satiety-related gut hormone released from the upper small intestine. The CCK1 receptor-mediated induction of c-Fos protein was found in the area postrema and NTS after the peripheral administration of CCK (2, 5, 25, 33). Based on the results of a vagotomy, it is considered that exogenous and endogenous CCK induces satiety to reduce food intake via the vagus. Besides this vagally afferent-mediated NTS activation, peripherally released circulating CCK may act directly on the CCK1 receptor in the area postrema, which has a porous blood-brain barrier and an intimate neural connection to the NTS. This immunohistochemical finding of the CCK1 receptor is consistent with the many binding studies showing a restricted distribution of CCK binding sites in the area postrema and NTS of the rat brain (14, 21, 22). Recently, an in vitro patch-clamp study demonstrated a possibility that GLP-1 released from intestinal endocrine cells acts directly on the area postrema in mice (11). The area postrema may be a sensory center of circulating signals derived from the visceral organs, especially the gut.

Several questions remain unresolved: (1) do GLUT3-immunoreactive fibers correspond to axons or dendrites; (2) where do the cell bodies reside; (3) what kind of neurons are they? While the present study may be just one step in studies of CVOs, this finding may prove useful to study the ontogeny and developmental changes of CVOs and their differentiation.

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