CALL FOR PAPERS | Central Control of Fluid and Electrolyte Homeostasis

Anatomical organization of the rat organum vasculosum laminae terminalis

Masha Prager-Khoutorsky and Charles W. Bourque

Center for Research in Neuroscience, Research Institute of the McGill University Health Centre, Montreal, Quebec, Canada

Submitted 7 April 2015; accepted in final form 26 May 2015

Prager-Khoutorsky M, Bourque CW. Anatomical organization of the rat organum vasculosum laminae terminalis. Am J Physiol Regul Integr Comp Physiol 309: R324–R337, 2015. First published May 27, 2015; doi:10.1152/ajpregu.00134.2015.—The organum vasculosum of the laminae terminalis (OVLT) is a circumventricular organ located along the ventral part of the anterior wall of the third ventricle. Because it lacks a complete blood-brain barrier (BBB), blood-borne signals detected in the OVLT provide the brain with information from the periphery and contribute to the generation of centrally mediated responses to humoral feedback and physiological stressors. Experimental studies on the rat OVLT are hindered by a poor understanding of its precise anatomical dimensions and cellular organization. In this study, we use histological techniques to characterize the spatial outline of the rat OVLT and to examine the location of neurons, astrocytes, tanycytes, and ependymocytes within its confines. Our data reveal that OVLT neurons are embedded in a dense network of tanycyte processes. Immunostaining against the neuronal marker NeuN revealed that neurons are distributed throughout the OVLT, except for a thick midline septum, which comprises densely packed cells of unknown function or lineage. Moreover, the most ventral aspect of the OVLT is devoid of neurons and is occupied by a dense network of glial cell processes that form a thick layer between the neurons and the pial surface on the ventral aspect of the nucleus. Lastly, combined detection of NeuN and c-Fos protein following systemic injection of hypertonic NaCl revealed that neurons responsive to this stimulus are located along the entire midline core of the OVLT, extending from its most anterior ventral aspect to the more caudally located “dorsal cap” region.

OVLT; osmoregulation; thirst; vasopressin; tanycytes; blood-brain barrier

Together with the subfornical organ and the area postrema, the organum vasculosum of the lamina terminalis (OVLT) is one of the brain’s sensory circumventricular organs. These are highly vascularized midline structures that lack a complete blood-brain barrier (BBB) and that serve as sites where peripheral circulating factors can penetrate the brain to influence neuronal activity (34). Basic features of the OVLT have been documented in several species of mammals, including sheep (32), rabbit (12, 71), mouse (27), rat (71), and human (61). As in other species, the rat OVLT is located between the anterior-dorsal aspect of the preoptic recess of the third ventricle, and the prechiasmatic cistern that encloses the preoptic vascular plexus (13, 26, 35).

Previous work has shown that the OVLT is a heterogeneous nucleus that contains several types of neurons that differ in their pattern of gene expression. For example, subsets of neurons in this area express cytokine receptors (44), epithelial Na+ channels (36), estrogen receptor (57), gonadotropin-releasing hormone (14, 18, 40), leptin receptor (39), nitric oxide synthase (3), prolactin (39, 62), transient receptor potential vanilloid type ion channels type 1 and 4 (11, 19, 28), and ANG II (angiotensin II) receptors (33, 36, 59, 70). Although it is not yet known whether the expression of these molecules specifically defines distinct subsets of neurons within the OVLT, these findings are consistent with the involvement of this nucleus in a wide diversity of centrally regulated processes, including food anticipatory activity (37), fever (5), sickness behavior (16, 20), gonadal function (47), and systemic osmoregulation (7).

One of the well-characterized functions of the OVLT is its role as a central osmoreceptor and sodium detector (21, 31, 49, 63). Indeed, many of the neurons in this nucleus are intrinsically sensitive to hypertonic NaCl or mannitol, and these cells display changes in action potential firing rate that are proportional to extracellular fluid osmolality (8, 11, 69). Sodium or osmosensitive neurons in the OVLT contribute to body fluid homeostasis via axonal projections to many central homeostatic structures, including the hypothalamic paraventricular (56) and supraoptic nuclei (42, 51), the median preoptic nucleus (9, 31), and thalamic neurons projecting to frontal thirst areas (19, 53). Previous studies examining the expression of c-Fos protein (a product of the activity-dependent early gene c-fos) have suggested that sodium- or osmo-responsive neurons are found predominantly in a region of the OVLT termed the “dorsal cap” (42). However, it is unclear whether, and to what extent, these sensory neurons might be located in other parts of the OVLT, because the precise boundaries of this nucleus have not been defined. In this study, we used a combination of serial sectioning and labeling techniques to provide a high-resolution description of the anatomical boundaries of the rat OVLT, together with information regarding the location of non-neuronal cells (glia, ependymocytes, and tanycytes) and neurons responsive to hypertonic NaCl.

MATERIALS AND METHODS

Animals. Adult male Long-Evans rats (80–150 g; Charles River Laboratories, Saint-Constant, QC, Canada) were used throughout this study. The animals were treated in strict accordance with the guidelines outlined by the Canadian Council on Animal Care (http://www.ccac.ca/), and experiments adhered to protocols approved by the Facility Animal Care Committee of McGill University (protocol no. 1190).

Histology. Rats were anesthetized with isoflurane and perfused via the heart with 10 ml of PBS followed by 300 ml of PBS containing 4% paraformaldehyde. The brains were extracted and postfixed by immersion for 48 h in 4% paraformaldehyde in PBS. A vibratome was used to obtain serial tissue sections (50 μm thick) in the coronal, sagittal, and horizontal planes. Horizontal sections were made parallel...
to the ventral surface of the brain (an angle of ~30° relative to the surface of the cortex). For the immunolabeling of vimentin protein, heat-mediated antigen retrieval in citrate buffer was performed. Sections were blocked with 10% normal goat serum (in PBS containing 0.3% Triton-X) and incubated overnight at 4°C with primary antibodies. Following wash, sections were incubated for 1 h with fluorescently labeled secondary antibodies. DAPI (4',6-diamidino-2-phenylindole, 1:2,000; Life Technologies, Burlington, ON, Canada) and/or fluorescently labeled phalloidin (1:500; Life Technologies) were added to the secondary antibody solution to visualize cell nuclei or actin, respectively. Sections were then washed and mounted on coverslips using SlowFade Gold Antifade reagent (Life Technologies). All images were collected using a confocal microscope (FV1000, Olympus Canada, Richmond Hill, ON, Canada). Stereotaxic coordinates mentioned in the text were derived from the 6th edition of the rat brain atlas published by Paxinos and Watson (45).

Evans blue injection. Evans blue is a dye with high affinity for serum albumin that is commonly used to assess the permeability of the...
BBB (17, 58, 68), and thus, it was used to evaluate the boundaries of OVLT. Rats were anesthetized with isoflurane and injected intravenously with 0.5 ml of 1% Evans blue dissolved in PBS. After 30 min, the animals were transcardially perfused with 20 ml PBS, and then the brain was extracted and fixed by immersion for at least 48 h in 4% paraformaldehyde dissolved in PBS. Serial sections (50 μm thick) were cut and mounted onto slides, and Evans blue fluorescence was visualized.

Antibodies. The following primary antibodies were used: vimentin mouse monoclonal antibody developed by Alvarez-Buylla et al. (1) and obtained from the Developmental Studies HybriGoma Bank, created by the National Institute of Child Health and Human Development of the National Institutes of Health and maintained at The University of Iowa, Department of Biology (Iowa City, IA; 1:100), GFAP (glial fibrillary acidic protein) rabbit polyclonal antibody (Sigma-Aldrich Canada, Oakville, ON, Canada; 1:500) (15), rabbit polyclonal c-Fos antibody (Millipore, Billerica MA; 1:10,000) (55), chicken anti-NeuN (Hexaribonucleotide Binding Protein-3a), polyclonal antibody (ABN91, 1:500; EMD Millipore). The anti-NeuN antibody obtained from Millipore is a polyclonal antibody that has been raised against the mouse protein and shown to detect this protein with high specificity (23). Although its ability to specifically detect rat NeuN has not been formally demonstrated, the rat NeuN sequence displays over 98.9% homology to the mouse ortholog, so we assume that the protein was efficiently detected in sections of the rat brain. Secondary antibodies were fluorescently labeled Alexa Fluor-conjugated [488 nm, 568 nm, and 647 nm; (Life Technologies; 1:500)].

Hypertonic stimulation. The localization of osmosensitive neurons was performed by immunohistochemical detection of c-Fos protein, as previously described (46). Briefly, animals were anesthetized with isoflurane (<1 min) and injected subcutaneously with lidocaine (0.25 ml) followed by 2 M NaCl or isotonic (295 mosmol/kg H2O) NaCl saline (2 ml/100 g body wt). Rats were perfused with 4% paraformaldehyde 100 min following the injection. The blood was collected from the left atrium just before the perfusion line was inserted into the right ventricle. The blood samples were placed on ice for 1 h, centrifuged at 1,600 × g, and then serum samples were assayed for osmolality (in triplicate). Serum osmolality in saline-treated animals was 297.2 ± 1.6, and 340 ± 9.7 in rats injected with 2 M NaCl.

RESULTS

Anatomical boundaries of the OVLT. To define the borders of the OVLT, six healthy rats were injected intravenously with 1% Evans blue (0.5 ml/100 g), a compound that binds to albumin and, thus, selectively permeates the brain through
fenestrated capillaries in areas lacking a functional BBB (17, 58, 68). After 30 min, the rats were perfused with PBS to wash out the dye from the vasculature, and the brains were fixed with paraformaldehyde. Blocks of tissue containing the anterior ventral hypothalamus were sectioned into consecutive sections in the coronal, sagittal, or horizontal planes. Sections were mounted, and Evans blue fluorescence was imaged at 647 nm using confocal microscopy. As illustrated in Fig. 1, bright fluorescence was detected along the ventral midline of coronal sections taken between anterior-posterior bregma (APB) coordinates +0.30 and +0.65. To delineate the perimeter of this zone of fluorescence (defined as the OVLT), we performed image thresholding at an intensity level 250% greater than the average background signal intensity. The apparent dimension of objects detected using this approach can be affected by the threshold value if fluorescence intensity declines gradually over a significant distance. However, in this case, lowering the threshold from 250% to 100% above the background caused the apparent dimension of the OVLT to increase by less than 10%. Thus, there was a clear demarcation between the region where the dye had penetrated and the surrounding tissue. The dotted lines superimposed on the line drawings shown in Fig. 1 illustrate the outline of the OVLT determined from average findings based on coronal sections from three rats and are consistent with an equivalent analysis of sections taken in the sagittal (Fig. 2, A−C) and horizontal (Fig. 2, D−F) planes.

These data indicate that the OVLT spans about 400 μm in the rostrocaudal direction. Its rostral pole is located about 350 μm in front of the most anterior portion of the optic recess of the third ventricle (Fig. 2C). When looking at serial sections taken in the coronal (Fig. 1) or horizontal planes (Fig. 2), the width of the OVLT is observed to increase from ~400 μm to ~700 μm within 100 μm of the rostral pole. This value is maintained up to the middle of the nucleus (i.e., near APB +0.40), from which point, it becomes progressively narrower toward the caudal pole (Fig. 2F). The height of the OVLT (relative to the ventral surface of the brain) increases from ~150 μm at the rostral pole to ~700 μm at APB +0.4 (Fig. 2C).

Despite its small size, the OVLT is a complex three-dimensional structure. In coronal view, the rostral OVLT is shaped like an inverted heart bordered dorsally by the vertical limbs of the nucleus of the diagonal band of Broca (DBB; Figs. 1 and 3, A and B). The ventral surface of the rostral OVLT lies directly adjacent to the third ventricle (3V).

Fig. 3. Distribution of neurons in the OVLT analyzed in coronal plane. Panels show the distribution of NeuN staining (magenta) and glial fibrillary acidic protein (GFAP; yellow) in coronal sections (left) and corresponding schematics taken at several APBs (values indicated above each of six positions in A−F). Dotted lines superimposed onto the panels delineate the boundaries of the OVLT at corresponding positions, as determined in Fig. 1. Note the absence of NeuN staining in the midline septum (open white arrows). Axes at the bottom of each column show lateral dimensions in millimeters. Abbreviations: 3V, third ventricle; DBB diagonal band of Broca, dc, dorsal cap; MNPO, median preoptic nucleus; och, optic chiasma; pcc, prechiasmatic cistern; or, optic recess of the third ventricle, sept, midline septum.
above the prechiasmatic cistern that houses the preoptic vascular plexus (Fig. 3, A and B). The caudal portion of the OVLT is vertically elongated and bordered ventrally by the optic chiasma and dorsally by the median preoptic nucleus (Fig. 3, C–F). The caudal pole of the OVLT interfaces with the ventral part of the third ventricle, including the small optic recess located directly above the optic chiasma. Histological features of the OVLT are described below and illustrated in Figs. 3–10.

**Distribution of neurons and the midline septum.** Analysis of staining for the neuronal marker NeuN revealed that the rostral part of the OVLT comprises loosely packed neurons bordered dorsally by the vertical limbs of the DBB (e.g., Fig. 3A), which features a higher density of NeuN-positive cells. A prominent layer (100–200 µm thick) of weak NeuN staining was found to separate the area populated by neurons from the ventral glia limitans superficialis (GLS) throughout the rostral OVLT (Fig. 3, A–C). The caudal part of the ventral OVLT also displayed a much lower density of neurons than the core of the nucleus. The caudal part of the OVLT was also traversed by a sagittal septum comprising small (∼5 µm) and densely packed cells oriented vertically (e.g., Fig. 4, A–C). These DAPI-labeled cells featured an intense actin rim (Fig. 4C) but were not stained by any of the other markers tested (e.g., Figs. 5, A–D and 6, A–G). This septum extended dorsally from the midline GLS (Figs. 3, C–E and 5B). The anterior part of the septum was quite narrow (∼15 µm; e.g., Fig. 4, A and B), but clearly divided the left and right sides of the OVLT (e.g., Figs. 5, A–D and 6, C–E). The height of the septum increased progressively from the rostral to caudal direction (Fig. 1 and 5, A–D) and contributed to the formation of the most medial aspect of the anterior wall of the third ventricle in the lower half of the OVLT (Fig. 7).

**Ependymocytes, tanycytes, and glial cells.** Although ependymocytes and tanycytes express a high density of the intermediate filament vimentin (27), this protein is also commonly found in glial cells (10, 54). Conversely, while glial cells can be recognized by the presence of GFAP (glial fibrillary acidic protein), this protein can also be detected in tanycytes (50). The identification of these cell types, therefore, requires a consideration of their appearance and position; notably, ependymocytes and tanycytes are normally restricted to the lining of the cerebral ventricles (27). Vimentin-positive cells lining the third ventricle in the most dorsal region of the OVLT and in the lateral walls of the ventricle caudal to the OVLT displayed a typical ependymocyte morphology, namely, a cuboidal shape organized as a single layer of cells displaying intense vimentin staining surrounding a prominent round and centrally located nucleus (Figs. 5, E and F, 6, E–G, 7, G–I, and 8, A and B) (24, 29). In contrast, vimentin-positive cells lining the ventricle in the ventral part of the OVLT exhibited the typical features of tanycytes, namely, an elongated oval soma (Fig. 8, A, C, D) and vimentin-positive apical processes that extended into the parenchyma (Figs. 5G, 6, C–F, 7, C–I, and 8, A, C–E). The fine processes of tanycytes created an interwove network that surrounded neuronal somata located within the ventral core of the OVLT (e.g., Figs. 5G, 7, H and 9, A, D, E). These processes were also observed to project toward capillaries within the OVLT (Fig. 7, H and I and 9A). Interestingly, while capillaries located within the OVLT were contacted by the processes of tanycytes, these vessels were not wrapped by the GFAP-positive astrocyte end-feet, characteristic of capillaries located outside the OVLT (Fig. 9B).

In addition to ependymocytes and tanycytes, the OVLT comprised two distinct types of GFAP-positive cells: stellate astrocytes, and marginal astrocytes (Figs. 9 and 10). Stellate astrocytes were scattered throughout the OVLT (e.g., Figs. 7H and 9C) and resembled stellate astrocytes located outside the OVLT (e.g., Fig. 8B). A layer of tightly packed marginal astrocytes formed the GLS along the ventral surface of the brain. These cells were strongly labeled by both GFAP and vimentin (Figs. 5, 7, and 10). Interestingly, marginal astrocytes underlying the anterior surface of the OVLT displayed a high...
density of elongated processes that projected into the parenchyma of the nucleus (Figs. 5G, 7C–I, 9, D and E, and 10). In addition to the cell types described above, we observed two additional patterns of GFAP/vimentin staining. As illustrated in Fig. 10, the thickness of GFAP/vimentin staining increased significantly in the vicinity of the interface between the dorsal surface of the optic chiasma and the ventral surface of the OVLT (i.e., between APB 0.55 mm (A and G), at APB 0.45 mm (B and I), at APB 0.40 mm (C), at APB 0.30 mm (D), and APB 0.25 mm (E and F). H shows higher magnification of another section taken around APB 0.45 mm (GFAP is not labeled in this section). Note the prominent neuron-free layer in the ventral part of the rostral OVLT (A, B, and G). This layer is occupied by a network of GFAP- and vimentin-positive glia-like cells (G). In the medial part of OVLT (B–D), a dense network of fibers appears to contact the midline septum and penetrate into the neuron-rich parenchyma. This interwoven network of tanyctye-like cells appears as two distinct populations: a dorsally located population (white open arrowheads), showing immunofluorescence for vimentin but not for GFAP, and ventrally located population (red open arrowheads), is positive for both GFAP and vimentin (I). Note that the midline septum (white open arrows in B–D and I) is devoid of fluorescence. E and F: sections taken caudal to the OVLT, which feature a complete opening of the third ventricle. Abbreviations: 3V, third ventricle; bv, blood vessel; pcc, prechiasmatic cistern.

Another notable enhancement of GFAP staining was observed along the anterior ventral edges of the septum, where densely packed GFAP-positive processes were observed to course in a lateral orientation (Figs. 5I and 6C). Interestingly, processes contacting the anterior-dorsal and caudal parts of the midline septum contained vimentin, but not GFAP (Fig. 5B–D, I).

Distribution of neurons responsive to hypertonic NaCl. Previous electrophysiological studies in vitro have reported that a large fraction of OVLT neurons are osmosensitive or sodium-sensitive (11, 69), and in vivo studies using the expression of the immediate early gene c-fos have shown that neurons responsive to hypertonic NaCl are consistently found in the region of the dorsal cap (4, 19, 42, 43, 55). However, studies using the c-Fos approach are commonly illustrated using a single coronal tissue section.
taken at the level of the optic recess. To examine whether neurons responsive to hypertonic NaCl are also located in other regions of the OVLT, adult rats received an intraperitoneal injection of either hypertonic NaCl (2 M; n = 100) or isotonic saline (n = 5), and the distribution of c-Fos immunoreactivity was examined in serial sections taken throughout the OVLT. As illustrated in Fig. 11, c-Fos-positive neurons were found along the entire rostro-caudal extent of the nucleus. The expression of c-Fos was specifically related to the hypertonic NaCl rather than other stimuli because animals injected with isotonic saline did not show any c-Fos-positive staining. In the rostral part of the OVLT, these cells were mainly located in the ventral and midline core of the nucleus (within ~100 µm of midline). In the caudal part of the OVLT, c-Fos-positive cells were observed both in the region of the dorsal cap and, to a lesser extent, in the lateral margins bordering the midline septum.

**DISCUSSION**

As one of the brain’s sensory circumventricular organs the OVLT is a privileged site where brain cells can monitor blood-borne signals that affect physiological and pathological states due to the absence of a BBB (34). Notably, the OVLT is a component of the anteroventral third ventricle [AV3V; e.g., (21)], a region identified as being important for the regulation of cardiovascular status (22), hydromineral balance (31), reproduction (18, 60), sleep (67), and thermoregulation (41, 67). The osmolality and sodium concentration of the extracellular fluid are known to influence most of these systems, and previous studies have shown that neurons in the OVLT can monitor and relay osmotic or natremic information to other sites both within the AV3V (19, 51, 56, 65) and outside the AV3V (9, 30, 64). Although many studies have reported neuronal c-Fos expression in this area in response to systemic hypertonicity, such studies have not defined the full outline of this nucleus or the presence and distribution of nonneuronal cells within its confines. Our study provides a more complete understanding of the anatomical boundaries of the rat OVLT than previously documented. It also provides a characterization of local non-neuronal cells and reveals that neurons responsive to hypertonic NaCl are located along the entire rostro-caudal axis of the nucleus.

**Boundaries of the OVLT.** Our data reveal that the OVLT is a spheroid midline structure that extends ~400 µm in the rostro-caudal axis, and measures a maximum of 600 µm wide and 700 µm high (Fig. 12). While the OVLT is bordered dorsally by the DBB at its anterior end and by the median...
preoptic nucleus at the caudal end, none of the markers tested in our study (other than Evans blue) could clearly define the lateral and dorsal edges of the nucleus. Indeed, the perimeter of the OVLT defined by the leakage of Evans blue did not correspond to any visible change in neuronal density (e.g., Fig. 3).

The absence of a gross morphological demarcation between the OVLT and more dorsal structures, such as the median preoptic nucleus, has been reported by others (4, 72). Yet differences in local function have been highlighted by a previous study that showed that the dorsal border of the caudal OVLT, defined by the accumulation of HRP, marks a sharp transition between osmoreponsive neurons in the OVLT, and nonosmoreponsive neurons in the median preoptic nucleus (4, 33). Although the perimeter of the OVLT defined by Evans blue may delineate distinct functional zones, a recent study has shown that local gonadotropin-releasing hormone neurons positioned outside the borders of the OVLT send long dendritic processes within the BBB-free area (18). The possibility that other types of neurons can sample circulating factors via dendrites extending into the OVLT remains to be determined.

Ependymocytes, tanycytes, and glial cells. Previous work has shown that the anterior part of the OVLT harbors a dense superficial plexus of capillaries that arises from branches of the anterior preoptic arteries located in the prechiasmatic cistern (38, 71). Consistent with the absence of a BBB, the capillaries that emerge from this network and

Fig. 7. Distribution of OVLT cell types in the sagittal plane. A: transmitted light micrograph of the sagittal plane. B: schematic diagram of the area shown in A. Abbreviations: 3V, third ventricle; MNPO, median preoptic nucleus; och, optic chiasma; or, optic recess of the third ventricle; PA, preoptic arteries; DBB, diagonal band of Broca; pcc, prechiasmatic cistern. C: immunofluorescence image of the section shown in A. C-G: fluorescence immunolabeling showing the distribution of NeuN (magenta), vimentin (green), GFAP (red), and DAPI (blue) in a series of consecutive 50-µm-thick sections taken near the midline. H and I are higher magnification fluorescence images in the same region. Note that the midline septum (open arrows) in midsagittal view (C, E, I), appears as a layer of cells lacking NeuN, GFAP, and vimentin. The midline septum forms the most ventral part of the anterior wall of the third ventricle (red open arrows) surrounding the optic recess (yellow open arrows). Note that blood vessels arising from the preoptic arteries (PA) create the preoptic capillary plexus, including longitudinally oriented vessels (red arrows) and vessels coursing upward into the neuron-rich parenchyma (white arrows). Notice that blood vessels within the OVLT are contacted by vimentin-positive tanycyte processes, but not by GFAP-positive glial processes (red and white arrows). In contrast, the blood vessels outside the OVLT boundaries (yellow arrows) appear to be embedded in GFAP-positive glia. Note an example of a classical stellate astrocyte (H) (white open arrowhead).
Fig. 8. Anatomical organization of ependymocytes, tanycytes, and neurons in the OVLT. High-magnification immunofluorescence images show the distribution of different cell types in the OVLT. A and D are labeled for NeuN (magenta), vimentin (green), GFAP (red), and DAPI (blue). B and C are labeled for vimentin (green) and GFAP (red). E is labeled for NeuN (magenta), vimentin (green), and GFAP (red). A: an image of the horizontal OVLT section showing the organization of tanycytes, neurons, and glia cells. Note the spatial separation of tanycytes (t) and ependymocytes (e) in the OVLT. Vimentin-positive tanycytes occupy the anterior wall of the third ventricle and project their processes anteriorly into the neighboring parenchyma, while vimentin-positive ependymocytes are lining the posterior wall of the ventricle outside the OVLT boundaries. B and C: representative examples of ependymocytes and tanycytes respectively. C and D: high-magnification sagittal image of OVLT, a portion of the anterior ventricular wall harboring a few layers of tanycyte somata that project a high density of processes in the OVLT. High-magnification images of NeuN-positive neurons embedded within a network of interwoven tanycyte processes in sagittal (C) and coronal planes (D).

penetrate the OVLT feature a fenestrated endothelium surrounded by perivascular spaces of varying dimension (71). The pial surface through which these capillaries enter the OVLT is associated with a thickened GLS comprising marginal glia that harbor a high density of elongated GFAP-positive processes (e.g., Figs. 9, D and E, 10, and 12). Although the function of these processes remains to be determined, it is possible that they serve to prevent the diffusion of substances between the parenchyma of the OVLT and the cisternal CSF. In the caudal and ventral region of the OVLT, the GLS is replaced by a thick network of GFAP-positive cells and processes, which span the ventral border of the OVLT and the dorsal surface of the optic chiasma.

In contrast to the rostral pole of the OVLT, which interfaces the cisternal CSF via extensively arborized marginal glia, the caudal pole of the OVLT interfaces the ventricular CSF through a thick layer of tanycytes. Indeed, a multicellular layer of tanycytes formed the anterior wall of the third ventricle along the full extent of the BBB-free zone (Figs. 7, 8, C and D, and 12). It has been proposed that tanycytes may serve as a physical barrier between the parenchyma of the OVLT and the third ventricle (27), an arrangement that would prevent blood-borne molecules diffusing into the OVLT from accessing the ventricular CSF. The possibility that tanycytes can regulate the local diffusion of substances released by neurons and glia, or their access to the general circulation, remains to be investigated.

Unlike capillaries outside the OVLT, which are wrapped by astrocytic end-feet that contribute to the BBB (2), capillaries coursing within the OVLT were devoid of GFAP staining. In contrast, these vessels appeared to be contacted by vimentin-positive processes arising from the tanycytes forming the rostral wall of the third ventricle (Figs. 5, H and I, 9, A and B, and 12). Indeed, a previous electron microscopy study has indicated that fenestrated capillaries located within the rat OVLT are wrapped by several layers of tanycyte processes (26). Interestingly, a similar arrangement has been documented in the median eminence, where tanycytes lining the floor of the third ventricle send long processes that form end-feet structures that wrap the capillaries of the pituitary portal system (48) and may play a role in the local transport and diffusion of blood-borne molecules (6, 52).

Location of neurons responsive to hypertonic NaCl. Reports on the OVLT are commonly illustrated using coronal sections taken at the level of the optic recess (~APB +0.3). Sections taken in this plane encompass regions that have been referred to as the lateral margins and the dorsal cap. Although these regions were not demarcated by changes in neuronal density or cytoarchitectonic features (Fig. 3), they have been reported to contain neurons that differ in their responsiveness to osmotic and humoral stimuli and in patterns of connectivity to other brain areas. For example, experiments involving c-Fos detection have shown that sodium depletion, or increases in circulating ANG II, preferentially activate neurons in the lateral margins (33, 36, 59, 70), whereas increases in the circulating levels of the hormone relaxin and systemic hypertonicity activate neurons in the dorsal cap (25, 42, 43, 60). Moreover, retrograde labeling studies have shown that OVLT neurons projecting to the supraoptic nucleus, ventrolateral periaqueductal gray, and ventrolateral preoptic area are concentrated within the dorsal cap (4, 33, 42, 60, 66, 67), whereas those projecting
to the bed nucleus of the stria terminalis are located in the lateral margins (59).

Our study reveals that a large number of neurons responsive to hypertonic NaCl are also located in the anterior part of the OVLT; i.e., in the ventral midline region that extends rostrally from the septum. Our analysis of c-Fos expression in serial sections obtained from rats injected with hypertonic saline indicates that responsive neurons form a continuous cluster that runs along the entire rostro-caudal extent of the OVLT, beginning in the anterior ventral midline, then rising along the dorsal aspect of the septum, and terminating within the dorsal cap, where a high density of responsive cells can be observed. The projection sites of neurons in the anterior part of the OVLT remain to be determined.

Although, our study reveals that the majority of cells expressing c-Fos after the stimulation with hypertonic saline are NeuN-positive neurons (Fig. 11), it is also possible that a small number of nonneuronal cells (e.g., astrocytes) may also express c-Fos in response to this stimulus. Moreover, the use of hypertonic NaCl injection as an experimental stimulus during in vivo experiments causes an increase in both serum sodium concentration and serum osmolality. As such, the induction of c-Fos expression observed in our experiments could have been mediated by the involvement of either sodium-sensitive or osmosensitive mechanisms. Further work is required to establish the location and involvement of distinct subsets of osmo-sensitive and sodium-sensitive neurons, as well as astrocytes in vivo.

**Perspectives and Significance**

The OVLT lacks a conventional BBB and, thus, allows local neurons to detect circulating molecules and direct adaptive central responses to peripheral signals. Our study provides a comprehensive description of the dimensions and spatial organization of the rat OVLT, which should facilitate future studies in this area. In addition, our study provides a detailed description of the location and interrelationships between different cell types within the borders of OVLT. Notably, we highlight the presence of a population of tanyocytes whose densely interwoven processes occupy a central part of the OVLT and embed local neurons. Tanyocytes may play an important role in the structural and metabolic support of local neurons, and also in the regulation of their electrical activity. Lastly, our data revealed the presence of a midline septum, formed from densely packed cells of unknown lineage. Further characterization will be required to understand the nature of these cells as well as their function.

**ACKNOWLEDGMENTS**

This work was supported by operating grants FRN82818 and MOP9939 from the Canadian Institutes of Health Research to C. W. Bourque, and by a James McGill Chair awarded to C. W. Bourque by McGill University. M. Prager-Khoutorsky was supported in part by a Postdoctoral Fellowship Award from the Heart and Stroke Foundation of Canada. The Research Institute of the McGill University Health Center receives generous support from Le Fonds de Recherche du Québec-Santé.
DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: M.P.-K. and C.W.B. conception and design of research; M.P.-K. performed experiments; M.P.-K. analyzed data; M.P.-K. and C.W.B. interpreted results of experiments; M.P.-K. prepared figures; M.P.-K. drafted manuscript; M.P.-K. and C.W.B. edited and revised manuscript; C.W.B. approved final version of manuscript.

REFERENCES

Fig. 11. Distribution of neurons responsive to hypertonic NaCl analyzed in serial coronal planes. Abbreviations: 3V, third ventricle; dc, dorsal cap; MNPO, median preoptic nucleus; och, optic chiasma; OVLT, organum vasculosum of the lateral terminalis; pcc, prechiasmatic cistern; sept, midline septum. A series of sequential 50-µm-thick coronal sections immunolabeled for NeuN (red) and c-Fos (green) (APB values are indicated in the bottom part of each image). Four schematic diagrams in the left upper corner show the location of the OVLT in the sections with the corresponding APB coordinates. First and third columns show maximal projection from eight sections derived from rats injected with isotonic saline (iso). Second and fourth columns show maximal projection from eight equivalent sections derived from rats injected with 2 M NaCl saline (hyper). The increase in the c-Fos immunoreactivity in the OVLT was seen only in hypertonic-stimulated OVLT.


