MICROGLIA AND PUTATIVE MACROPHAGES OF THE SUBFORNICAL ORGAN: STRUCTURAL AND FUNCTIONAL FEATURES

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The subfornical organ is an important regulator of water-salt metabolism and energy balance of the body, involved in the control of the cardiovascular system and immune regulation. The organ comprises several cell populations, among which microglia and macrophages remain uncharacterized. The study aimed at structural, cytochemical, and functional characterization of microglia and macrophages of the subfornical organ in rats. Brain specimens were collected from mature male Wistar rats (n = 8). Microglia and macrophages were revealed by immunostaining with poly- and monoclonal antibodies against calcium-binding protein Iba1 and lysosomal protein CD68; the slides were examined by light and confocal laser microscopy. The study provides a complex morphological characterization of microglial cells and macrophages of the subfornical organ. We demonstrate that the majority of Iba1-expressing cells in this area of the brain are microglial cells, not macrophages. Microglia of the subfornical organ reveals preactivated state, which may reflect structural and functional features of this organ and specific functions of local microglia. Subependymal microglial cells, the processes of which penetrate into the cavity of the third ventricle of the brain, constitute a distinct subpopulation among the Iba1-expressing cells of the subfornical organ. Apart from microglial elements, the subfornical organ contains sparse tissue macrophages with characteristic strong expression of CD68 accompanied by undetectable or weak expression of Iba1.

Keywords: subfornical organ, microglia, macrophages, circumventricular organs

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МИКРОГЛИЯ И ПРЕДПОЛАГАЕМЫЕ МАКРОФАГИ СУБФОРНИКАЛЬНОГО ОРГАНА: СТРУКТУРНО-ФУНКЦИОНАЛЬНЫЕ ОСОБЕННОСТИ

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Субфорникальный орган является важным регулятором водно-солевого обмена и энергетического баланса организма, участвует в контроле работы сердечно-сосудистой системы и иммунной регуляции. В состав субфорникального органа входят разные клеточные популяции, среди которых неохарактеризованными остаются микроглия и макрофаги. Целью работы было изучить структурные, цитохимические и функциональные характеристики микроглии и макрофагов субфорникального органа головного мозга крысы. Исследовали образцы головного мозга половозрелых крыс-самцов породы Вистар (n = 8). Для выявления микроглии и макрофагов применяли поли- и моноклональные антитела против кальций-связывающего белка Iba1 и лизосомного белка CD68 и анализировали препараты методами световой и конфокальной лазерной микроскопии. В рамках исследования дана комплексная морфологическая характеристика клеток микроглии и макрофагов субфорникального органа. Показано, что большинство Iba1-содержащих клеток этой области головного мозга являются микроглиоцитами, а не макрофагами. Микроглия субфорникального органа находится в предактивированном состоянии, что может быть обусловлено структурно-функциональными особенностями этого органа и специфическими функциями местной микроглии. Среди Iba1-содержащих клеток в субфорникальном органе выявлена особая популяция субэпендимных микроглиоцитов, отростки которых проникают в полость третьего желудочка головного мозга. Помимо микроглии в субфорникальном органе обнаружены единичные тканевые макрофаги, для которых характерно высокое содержанием CD68, но незначительное количество или отсутствие Iba1.

Ключевые слова: субфорникальный орган, микроглия, макрофаги, циркумвентрикулярные органы

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The subfornical organ (*organum subfornicum*) is localized near the arch of the telencephalon (*fornix*) along the anterior wall of the third ventricle, where it occupies the dorsal end of the terminal plate, protruding slightly into the lumen of the third ventricle. An important regulator of water-salt metabolism and energy balance of the body, it is involved in the control of the cardiovascular system and immune regulation [1, 2]; hence the considerable interest in overall anatomy and cellular composition of the subfornical organ, which still remains one of the most mystifying brain structures.

Microglia and macrophages are resident cells of the subfornical organ. These two cell types implement similar functions, but differ structurally (by expression profiles of certain genes and immunophenotype) and ontogenetically (by source of origin). The importance of studying these cell types within the context of subfornical organ functionalities is determined by its being one of the circumventricular organs characterized by the lack of the blood-brain barrier. Accordingly, its local macrophages and microglia, by contrast with their counterparts in other brain regions, are in continuous contact with various agents circulating in the blood [3], which implies that these cells have certain structural and functional features. An extra focus on microglia and macrophages of the subfornical organ is due to their possible involvement in the course of coronavirus infection. The strong neurotropism of SARS-CoV-2 is a well-established fact [4]. The chronic activation status of microglia, which is normal for circumventricular organs, is thought to render it hyper-sensitive and hyper-reactive to pathological stimuli (e.g. SARS-CoV-2 infection) [5] and prone to transition into pro-inflammatory phenotypes accompanied by active synthesis of pro-inflammatory mediators and increased rates of phagocytosis. The SARS-CoV-2-associated neuroinflammatory response, which develops in circumventricular organs, confers significant risks of neurodegeneration. In addition, the enhanced migration capacity of the hyper-activated ameboid microglial cells facilitates the spread of neuroinflammation to other brain regions [5], which may be one of the underlying causes of neurological symptoms in patients with coronavirus infections.

This study aimed at structural, cytochemical, and functional characterization of microglia and macrophages of the subfornical organ in rats.

METHODS

The study was carried out on brain specimens of mature (3–5 months old) male Wistar rats (n = 8). The animals were purchased at the “Rappolovo” breeding facilities (Leningrad region, Russia) and housed at standard conditions with ambient temperature, 12-h light cycle, and *ad libitum* access to pellets and water. The brain specimens were fixed in zinc-ethanol-formaldehyde [6] and embedded in paraffin (Paraffin Type 6, ThermoScientific Richard-Allan Scientific; USA) under standard protocol. The blocks were sectioned on a Microm HM 325 rotary microtome (ThermoScientific; USA); the 5 μm thick frontal sections comprising the subfornical organ region were mounted on adhesion slides (Menzel; Germany). After standard deparaffinization and rehydration, the sections were demasked by heating in 10% aqueous solution of sodium thiosulfate for 23 min [7].

The light microscopy immunohistochemistry assay of microglia and/or macrophages involved anti-Iba1 rabbit polyclonal antibodies (Biocare Medical; USA) diluted 1:1500 and anti-CD68 mouse monoclonal (ED1) antibody (Abcam; UK) diluted 1:4000, with REVEAL Rabbit Specific HRP-DAB Detection System in manufaturer’s dilution (Spring Bioscience; USA) as secondary antibody and 3,3′-diaminobenzidine (DAB+, Agilent; USA) as chromogen. After the reaction, some of the sections were treated with alum hematoxylin as a nuclear counterstain.

For immunofluorescent detection of Iba1, the sections were covered with the rabbit polyclonal antibodies diluted 1:1000 (Biocare Medical; USA). The antigen-antibody complexes were visualized with REVEAL Rabbit Specific HRP-DAB Detection System in manufaturer’s dilution (Spring Bioscience; USA) followed by Cy3-conjugated AffiniPure Goat Anti-Horseradish Peroxidase polyclonal antibodies (Jackson ImmunoResearch; USA). The nuclei were counterstained with SYTOX Green fluorescent dye (Invitrogen; USA).

For double-fluorescent immunostaining of Iba1/CD68 the sections were covered with a 1:1 mixture of rabbit polyclonal antibodies to Iba1 diluted 1:500 (Biocare Medical; USA) and mouse monoclonal antibodies to CD68 diluted 1:1000 (Agilent; USA), followed by a mixture of (anti-)Rabbit IgG Biotinylated Antibody (R&D Systems; USA) and EnVision+/HRP-Anti-Mouse reagent (Agilent; USA) as secondary antibodies. After incubation in the mixture of secondary antibodies, the sections were sequentially treated with solutions of Cy2-Streptavidin (Jackson ImmunoResearch; USA) and Cy3-conjugated AffiniPure Goat Anti-Horseradish Peroxidase polyclonal antibodies (Jackson ImmunoResearch; USA).

Examination of the slides and image acquisition were carried out with Leica DM750 light microscope (Leica; Germany) equipped with Leica ICC_{50} camera (Leica; Germany) and Zeiss LSM800 confocal laser microscope (Zeiss; Germany). The fluorescence was excited using a 488 nm laser for Cy2 and SYTOX Green and a 561 nm laser for Cy3. The images were analyzed in ZEN2012 and LSM Image Browser software packages (Zeiss; Germany).

RESULTS

Immunohistochemical detection of Iba1 calcium-binding protein

Immunohistochemical staining for calcium-binding protein Iba1 specifically expressed in microglia and macrophages revealed immunopositive cells of the subfornical region in all studied rat brain specimens (Fig. 1). The subfornical organ is clearly visualized at low magnification (×10) as a compact cellular aggregation protruding into the cavity of the third ventricle (Fig. 1A, SFO). The organ shows high cellularity revealed by counterstaining of the nuclei with hematoxylin (Fig. 1A, SFO, *blue*), as well as the high intensity of the Iba1 signal (Fig. 1A, SFO, brown). Thus, already at a low magnification of the microscope, the subfornical organ presents with abundant Iba1-immunopositive elements distributed rather evenly within the organ.

Examination of the subfornical organ region and its boundary with adjacent white matter at higher magnifications (×40, ×100) revealed specific localization of the Iba1 immunostaining in cells with ramified processes, of diverse morphology (Fig. 1B–E, *brown*). In white matter (Fig. 1B, WM), the Iba1-positive cells are mostly fusiform, with two long non-ramified or poorly ramified processes located at the poles; cell bodies and processes of these cells are oriented along the nerve fibers (Fig. 1B, WM, *brown*). In subfornical organ (Fig. 1B–E, SFO), the Iba1-positive cells are visually larger compared with the corresponding cells in white matter. These cells have more complex architecture of the processes and show considerable morphological heterogeneity: one morphotype has fusiform shape with small

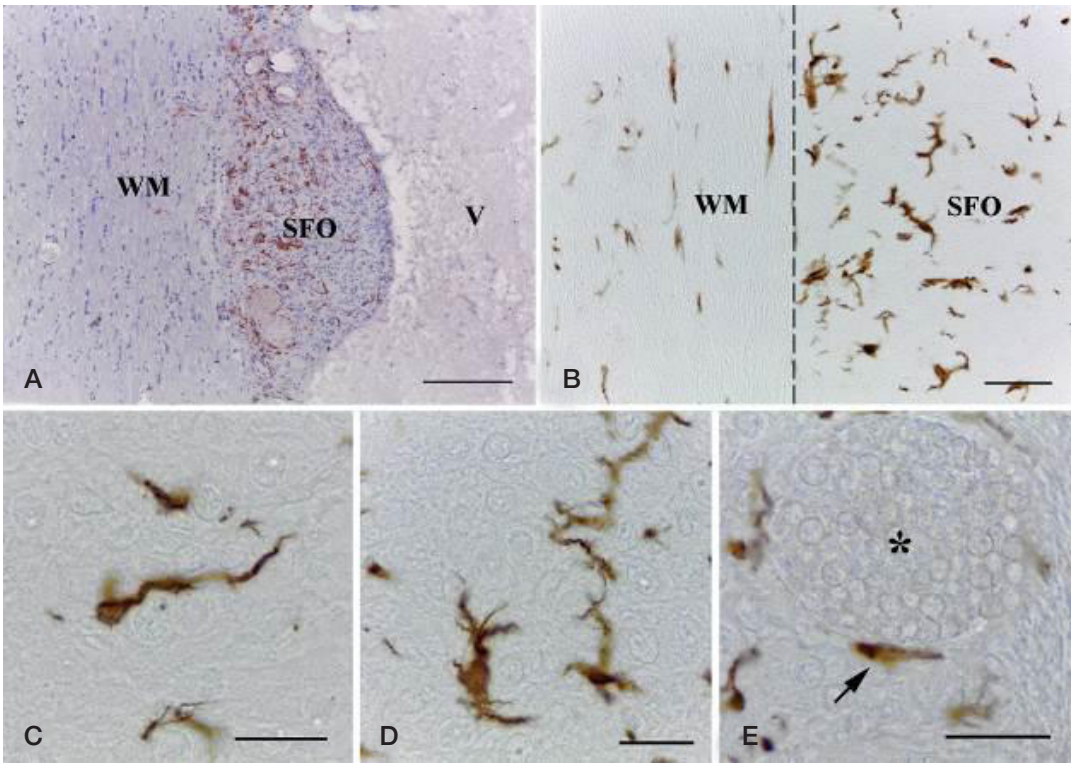

Fig. 1. Iba1-immunopositive cells in rat subfornical organ and adjacent white matter. **A**. Overall view. **B**. The boundary between subfornical organ and the underlying white matter. **C**–**E**. Different morphotypes of Iba1-immunopositive cells within subfornical organ. SFO — subfornical organ, WM — white matter, V — third ventricle of the brain (cavity); the *dashed line* indicates the boundary between subfornical organ and white matter; the *asterisk* indicates blood vessel (lumen); the *arrow* indicates a small perivascular cell with few processes, containing Iba1. Scale bars, 200 μm (**A**), 50 μm (**B**), and 20 μm (**C**–**E**)

body and a long non-ramified process (Fig. 1C, *brown*), another morphotype exhibits relatively thick processes moderately branching in multiple directions (Fig. 1D, *brown*), whereas the sparse perivascular Iba1-positive cells with few processes are spread over the surface of dilated thin-walled vessels of the subfornical organ (Fig. 1E, *arrow*).

The fluorescent immunostaining for Iba1 produced similar results (Fig. 2). The subfornical organ region presents with high cellularity revealed by counterstaining of the nuclei with SYTOX Green fluorescent dye (Fig. 2, *green fluorescence*). Consistenly with the corresponding light microscopy assay, the fluorescent immunostaining for Iba1 revealed high density of Iba1-containing cells within subfornical organ (Fig. 2, *red fluorescence*). Examination of these cells at higher magnifications revealed their considerable morphological heterogeneity. The immunofluorescence assay produced a more contrasted visualization of the thin processes of the Iba1-containing cells compared to light microscopy, which allowed us to describe a specific subpopulation of these cells confined to the ependymal lining of the third ventricle at the level of the subfornical organ. The bodies of these Iba1-immunopositive cells were immediately adjacent to the ependymal layer and often spread over it, and their thin processes permeated the ependymal layer and reached the cavity of the third ventricle (Fig. 2, *arrow*).

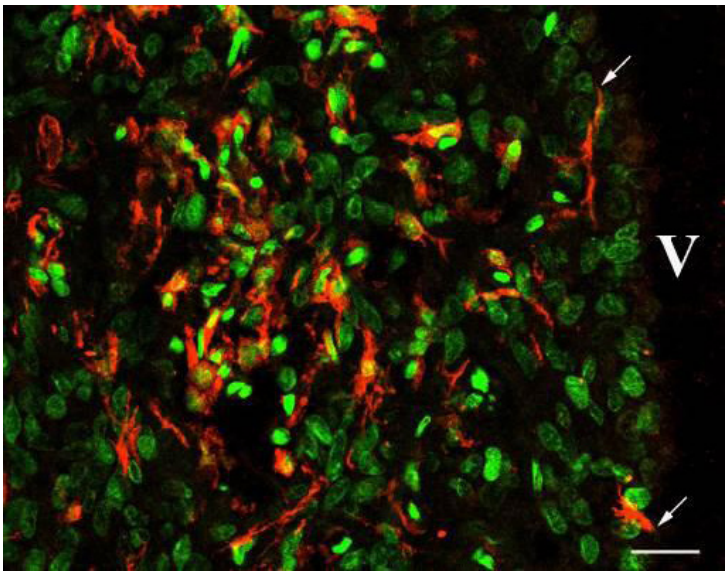

Fig. 2. Immunofluorescent detection of Iba1-positive cells in rat subfornical organ. The Iba1 specific signal is *red* (Cy3 fluorochrome) and the nuclei are *green* (SYTOX Green). V — third ventricle of the brain (cavity); the arrow indicates processes of the Iba1-containing cells reaching the ventricular cavity. Scale bar, 20 μm

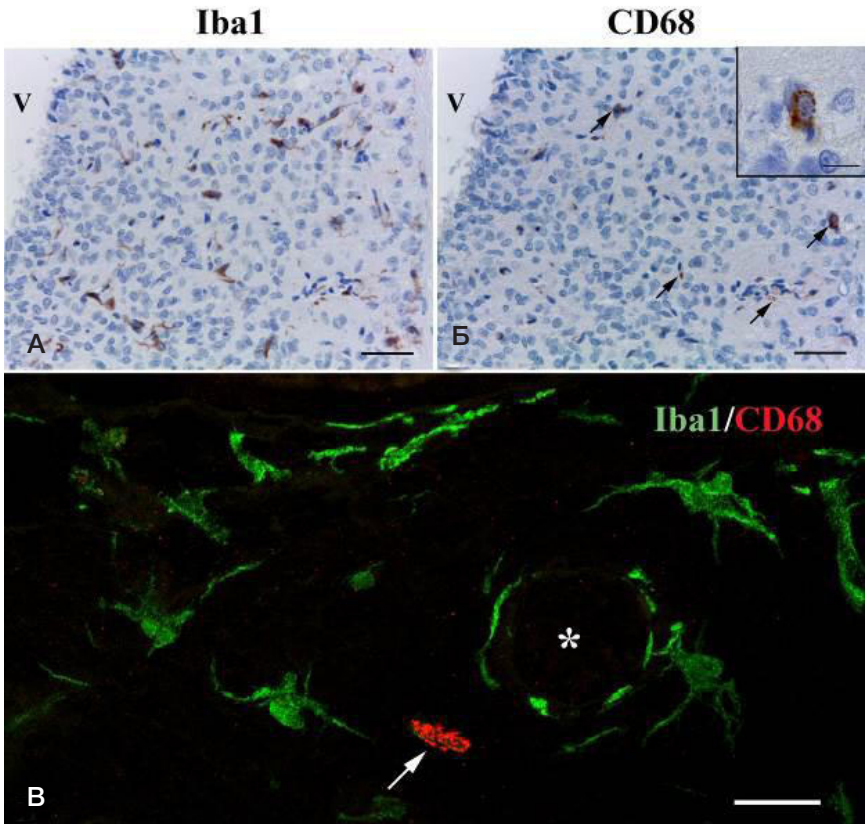

Fig. 3. Characteristic patterns of Iba1 and CD68 immunostaining in rat subfornical organ. **A**. Immunohistochemical reaction for Iba1 with alum hematoxylin nuclear counterstaining. **B**. Immunohistochemical reaction for CD68 with alum hematoxylin nuclear counterstaining; the *arrows* indicate CD68-immunopositive structures within the subfornical organ; the insert shows a magnified area comprising CD68-immunopositive cell. Images **A** and **B** represent histologically identical serial sections of the same specimen (light microscopy). **C**. Double-fluorescent immunostaining of Iba1/CD68 (confocal laser microscopy). The Iba1 signal is *green* (Cy2 fluorochrome) and the CD68 signal is red (Cy3 fluorochrome); the *arrow* indicates a CD68-containing cell; the asterisk indicates blood vessel (lumen). Scale bars, 50 μm (**A**, **B**) and 10 μm (**B** insert, **C**)

Immunohistochemical detection of CD68

Comparative examination of light microscopy immunostaining images for Iba1 (Fig. 3A) and CD68 (Fig. 3B), representing two histologically identical serial sections of the same brain tissue specimen, revealed much lower density of CD68-positive elements and higher density of Iba1-positive elements in the subfornical organ. Only sparse CD68-positive elements were visualized in the subfornical organ, found in parenchyma or perivascular spaces. Most of the signal appeared as small CD68-immunopositive granules scattered in the nervous tissue (Fig. 3B, *arrow*). Only a minority of CD68-immunopositive elements had cellular outlines. These few cells were oval or elongated and showed distinct cytoplasmic granularity (Fig. 3B, insert).

As shown by the double-fluorescent Iba1/CD68 immunostaining, the majority of positively stained cells are $Iba1^{+}/CD68^{-}$ (Fig. 3C, *green fluorescence*). These cells have ramified appearance and heterogeneous morphology, similarly with the Iba1-immunopositive cells revealed by the light microscopy assay. A very minor fraction of cells (solitary cells) in the subfornical organ are $Iba1^{-}/CD68^{+}$ (i.e. contain CD68, but no Iba1). These cells are oval or elongated, with characteristic cytoplasmic granularity, and no observable processes (Fig. 3C; *arrow*, *red fluorescence*). In certain CD68-immunopositive cells, Iba1 was present in small amounts, but it never colocalized with CD68.

DISCUSSION

The subfornical organ remains one of the least studied brain structures. The main scientific findings on its structure and function date back to the 1960–80s. The organ receives synaptic inputs from solitary tract nuclei [8], lateral hypothalamus and medial hypothalamic nuclei [9], while sending projections to diverse brain centers including paraventricular nucleus and lateral hypothalamus [10], arcuate nucleus [11], and median preoptic nucleus [9]. Seminal research on the role of this organ in osmoregulation [12] and control of cardiovascular functionalities [13] also dates to the mid-20th century. The findings obviously need verification and refinement with the use of modern immunomorphological methods, along with the available data on cellular composition of the organ. A comprehensive morphological study on structural and functional features of different subpopulations of neurons, astrocytes, and vascular cells in rat subfornical organ was carried out in 2021 [2]; however, it completely disregarded such important cell types as macrophages and microglia.

Microglia and tissue macrophages of the brain make important contribution to immunity by forming the first-line defense of the central nervous system from various infectious agents capable of crossing the endothelial barrier. Despite their similar functions, these cell populations originate from different embryonic sources [14]. The microglial progenitor cells are formed within the embryonic yolk sac wall during the first wave of hemopoiesis and migrate to the developing brain before the onset of the blood-brain barrier. In the brain, these progenitors differentiate into microglial cells which constitute a self-perpetuating population. Other macrophage lineages found in the brain (meningeal macrophages, perivascular macrophages, choroid plexus macrophages) descend from the erythro-myeloid progenitor cells and hemopoietic stem cells of the embryonic liver and the bone marrow during the second and

the third waves of hemopoiesis. In connection with their yolk sac origin and by contrast with macrophages, the microglial differon comprises no equivalent of the monocyte stage [15–17]. Apart from the origin, microglial cells differ from the "true" brain macrophages both structurally and phenotypically. For instance, microglial cells express unique molecular markers P2RY12, Sall1, and Tmem119 along with very moderate levels of CD45 transmembrane tyrosine phosphatase. By contrast, brain macrophages express CD45 and the major histocompatibility complex class II molecules at much higher levels than microglia, which reflects the important antigen-presenting role of these cells. Also by contrast with microglia, perivascular and meningeal macrophages abundantly express CD206 protein known as macrophage mannose receptor [18].

It is believed that under normal physiological conditions (in the absence of pathogenic processes) microglial cells have "ramified" morphologies with numerous thin branching processes that constantly monitor the microenvironment for potential hazards (the so-called sentinel or resting microglia). Upon the exposure to pathological stimuli, microglia is converted into active (activated) state with characteristic ameboid morphologies. The conversion involves substantial enlargement of the cell body (through increase in the perinuclear cytoplasm volume) accompanied by reduction of the processes. These morphological metamorphoses correspond to a functional shift towards increased phagocytic activity and/or cytokine production [19, 20]. In other words, morphological features of microglial cells reflect their functional status.

In this study, we used calcium-binding protein Iba1 (ionized calcium-binding adaptor molecule 1) as a marker to assess the morphological and functional state of microglia in the subfornical organ. It should be noted that, despite its common use as immunohistochemical marker for microglia [21], Iba1 is not uniquely expressed in microglial cells but also detected in typical tissue macrophages, e.g. in Kupffer cells [22]. Immunostaining with anti-Iba1 antibody reveals both resting and activated microglia and all intermediate states as well [20, 23]. The homogeneous distribution of Iba1 protein in the cytoplasm of microglial cells enables the use of anti-Iba1 immunohistochemistry as a valuable tool for detailed morphological characterization of these cells [24]. Our use of anti-Iba1 immunostaining on paraffin sections of rat subfornical organ revealed numerous positive cells with ramified morphology corresponding to microglia. The examination revealed high density of microglial cells in the subfornical organ and their substantial morphological heterogeneity. Although the identified cells had thicker and shorter processes with reduced degree of branching compared with the classical images of resting microglia, we encountered no amoeboid microglial cell morphologies in the studied histological specimens. Apparently, all microglial cells observed by us in the subfornical organ can be assigned with intermediate status loosely defined as "preactivated".

It is important to emphasize that the subfornical organ a circumventricular organ which lacks the blood-brain barrier. The signs of microglial activation under normal physiological conditions have been previously described for other circumventricular organs. The physiologically activated microglia in circumventricular organs of mice [3] shows overall reduction in the length and number of microglial cell processes compared with other brain regions, accompanied by elevated expression levels of certain molecular markers. The high degree of microglial activation under normal physiological conditions was also observed in the median eminence area of rat brain [25].

Exact causes of the chronic microglial activation observed in circumventricular organs are disputable. Obviously, the condition reflects specific physiological features of these organs. One of such features is the presence of fenestrated capillaries, resulting in the constant exposure of microglial cells to antigens circulating with the blood (by contrast with microglia in other brain regions protected by the blood-brain barrier). A likely responsibility of microglia under these conditions is phagocytosis of neurotoxic molecules that arrive from circulation in order to ensure the maintenance of tissue homeostasis [3]. Another possible function of the activated microglia is its participation in tissue remodeling. Circumventricular organs have been previously characterized as the sites of intensive physiological angiogenesis accompanied by constant proliferation and apoptosis of endothelial cells of the local microcirculatory bed. The activated microglia has been shown to regulate the proliferative activity of endothelium, as well as scavenge the apoptotic leftovers of the dead endothelial cells [26, 27]. Ultimately, microglia can be involved in neurogenesis with concomitant acquisition of an activated morphotype. The presence of neuronal stem cells has been recently demonstrated for certain circumventricular organs including the subfornical organ [28, 29]. This finding suggests a possible contribution of the activated microglia to formation of neurogenic niches in this organ, similar to the well-described involvement of the subventricular zone as well as the subgranular zone of the dentate gyrus in the hippocampus [30].

Another interesting observation made by us in this study reveals a special population of microglial cells of the subfornical organ, which reside beneath the ependymal lining of the third ventricle and reach its cavity with their processes. Similar cell populations termed "subependymal microglial cells" have been described in the subventricular zone of the lateral ventricles [31]. The close contact of subependymal microglia with cerebrospinal fluid may indicate participation of these cells in the control of cerebrospinal fluid composition.

One of the problems that arise in fundamental research on microglia concerns the morphological and cytochemical similarity of these cells with tissue macrophages of the brain. Microglia and macrophages originate from different sources but share a variety of common marker proteins, Iba1 being one of them [32]. It would be virtually impossible to distinguish microglia from tissue macrophages of the brain on a sole basis of immunostaining for Iba1. To specify the identity of the Iba1-immunopositive cells observed by us in the subfornical organ, we additionally performed immunohistochemical staining for CD68 (a transmembrane glycoprotein with a molecular weight of 110 kDa implicated in lysosomal transport). A prominent marker of phagocytic activity, CD68 is highly expressed in monocyte-macrophage lineages and widely used for immunohistochemical detection of Kupffer cells, alveolar macrophages, osteoclasts, dermal Langerhans cells, etc. [33, 34].

Immunostaining with CD68-specific antibody revealed sparse signal and very few CD68-containing cells morphologically similar to macrophages within the subfornical organ. The vast majority of Iba1-immunopositive cells in the subfornical organ do not express CD68, which identifies them as microglia. The absence of CD68 molecules in these cells reveals their rudimentary lysosomal capacity despite the distinct morphological signs of activation. Apparently, the chronic preactivated state of microglia in the subfornical organ has little to do with active phagocytosis and its biological meaning has yet to be discovered. Unexpectedly, the identified CD68-immunopositive macrophages of the subfornical organ contained negligible amounts of the Iba1 protein. According

to the literature, tissue macrophages of the brain express Iba1 in high amounts, which is consistent with our own data for other brain regions in rodents and also in humans [35, 36]. Its low expression in tissue macrophages of subfornical organ may represent a unique cytochemical feature of this local macrophage population.

CONCLUSIONS

The majority of Iba1-containing cells in the subfornical organ are microglial cells, not macrophages. Microglia of the subfornical organ reveals preactivated state, which may reflect physiological features of this organ and specific functions of local microglia. The subfornical organ contains specific population of subependymal microglial cells, which project into the third brain ventricle and contact cerebrospinal fluid with their processes. Apart from microglia, the organ contains solitary tissue macrophages with high content of CD68 and low or negligible expression of Iba1. Continued research on microglia and macrophages is important, considering their regulatory role in normal functioning of the nervous system and notably their involvement in neuroinflammatory and neurodegenerative processes, particularly in the context of targeted pharmacotherapy for neurodegenerative diseases.

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